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EDITORIAL

IL-1 induces thromboxane-A2 (TxA2) in COVID-19 causing inflammation and micro-thrombi: inhibitory effect of the IL-1 receptor antagonist (IL-1Ra)

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IL-1 induces a significant number of metabolic and hematological changes. In experimental animals, IL-1 treatments cause hypotension due to rapid reduction of systemic blood pressure, reduced vascular resistance, increased heart rate and leukocyte aggregations. IL-1 causes endothelial dysfunction, the triggering factor of which may be of a different nature including pathogen infection. This dysfunction, which includes macrophage intervention and increased protein permeability, can be mediated by several factors including cytokines and arachidonic acid products. These effects are caused by the induction of IL-1 in various pathologies, including those caused by pathogenic viral infections, including SARS-CoV-2 which provokes COVID-19. Activation of macrophages by coronavirus-19 leads to the release of pro-inflammatory cytokines, metalloproteinases and other proteolytic enzymes that can cause thrombi formation and severe respiratory dysfunction. Patients with COVID-19, seriously ill and hospitalized in intensive care, present systemic inflammation, intravascular coagulopathy with high risk of thrombotic complications, and venous thromboembolism, effects mostly mediated by IL-1. In these patients the lungs are the most critical target organ as it can present an increase in the degradation products of fibrin, fibrinogen and D-dimer, with organ lesions and respiratory failure. It is well known that IL-1 induces itself and another very important pro-inflammatory cytokine, TNF, which also participates in hemodynamic states, including shock syndrome in COVID-19. Both IL-1 and TNF cause pulmonary edema, thrombosis and bleeding. In addition to hypotension and resistance of systemic blood pressure, IL-1 causes leukopenia and thrombocytopenia. The formation of thrombi is the main complication of the circulatory system and functionality of the organ, and represents an important cause of morbidity and mortality. IL-1 causes platelet vascular thrombogenicity also on non-endothelial cells by stimulating the formation of thromboxane A2 which is released into the inflamed environment. IL-1 is the most important immune molecule in inducing fever, since it is involved in the metabolism of arachidonic acid which increases from vascular endothelial organs of the hypothalamus. The pathogenesis of thrombosis,

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vascular inflammation and angiogenesis involves the mediation of the activation of the prostanoid thromboxane A2 receptor. In 1988, in an interesting article we reported for the first time that IL-1 induces thromboxane B2 (TxB2) releases in activated neutrophils and macrophages. An increase in thromboxane can induce leukocyte aggregation and systemic inflammation, which would account for the dramatic thrombi formation and organ dysfunction. Hence, IL-1 stimulates endothelial cell-leukocyte adhesion, and TXB2 production. All these events are supported by the large increase in neutrophils that adhere to the lung and the decrease in lymphocytes. Therefore, eicosanoids such as TxA2 (detected as TxB2) have a powerful action on vascular inflammation and platelet aggregation, mediating the formation of thrombi. The thrombogenesis that occurs in COVID-19 includes platelet and cell aggregation with clotting abnormalities, and anti-clotting inhibitor agents are used in the prevention and therapy of thrombotic diseases. Prevention of or induction of TxA2 avoids thrombi formation induced by IL-1. However, in some serious vascular events where TxA2 increases significantly, it is difficult to inhibit, therefore, it would be much better to prevent its induction and generation by blocking its inducers including IL-1. The inhibition or lack of formation of IL-1 avoids all the above pathological events which can lead to death of the patient. The treatment of innate immune cells producing IL-1 with IL-1 receptor antagonist (IL-1Ra) can avoid hemodynamic changes, septic shock and organ inflammation by carrying out a new therapeutic efficacy on COVID-19 induced by SARS-CoV-2.

Key words: COVID-19; IL-1; SARS-CoV-2; inflammation; thrombosis; thromboxane

It is now known to all that the new coronavirus SARS-CoV-2 infecting humans causes the COVID-19 disease (1). There are thousands of coronaviruses on our planet, some of which are responsible for the common cold and others which are harmless. Virus can be very aggressive and resistant to therapy, as can many bacteria, therefore, today there is substantial interest in viral pathogenesis and how to create new drugs that kill or prevent the spread of the microorganisms into the environment. SARS (severe acute respiratory syndrome) in 2002 caused hundreds of deaths (770) worldwide, but it could have caused many more if precautions had not been taken.

The novel coronavirus SARS-CoV-2 has created a worldwide pandemic with millions of infected and hundreds of thousands of deaths in many nations. Infection with SARS-CoV-2, an RNA virus which is about 29,900 bases long, is transmitted by carriers with symptoms such as fever and dry cough, but can also be transmitted by asymptomatic patients. Coronavirus-19 is constituted by RNA and N-protein inside, while the outside features present a membrane with E and M proteins, lipids and the spike (S) glycoprotein (2). The virus measures approximately 88 nm in diameter plus 50 nm spike

protein, visible under electron microscope. SARS-CoV-2, which uses cellular metabolism to live, after entering the body through the nose and/or mouth, travels in the upper and lower airways by binding to the ACE2 receptor of lung cells; while it binds to the toll like receptor (TLR) on innate immune cells, including macrophages (3-4). The entry of the virus into the host cell stimulates the genes to the cell's ribosomes which cause the translation of proteins that create protective vesicles in the endoplasmic reticulum where, through the viral polymerase RNA, the virus can replicate. An infected cell can release hundreds of copies of the virus as well as interferon, an immune cytokine that alerts and stimulates B cells, T cells, neutrophils and macrophages to protect the host. B cells assisted by T helper cells generate neutralizing antibodies which, when they are efficient and numerous, block the spike protein of the virus (5). The activation of the immune cells that rush to the host's defense causes inflammation.

IL-1 can be generated by innate immune cells but also by many other non-immune cells. IL-1 is mainly a macrophage cytokine emerged as being pivotal, promoting and mediating inflammation. IL-1 is a member of the IL-1 family and its synthesis occurs after a microorganism activates the TLR expressed

on innate immune cells, including macrophages (6). The activation causes pro-IL-1 to accumulate in the cytosol which is secreted in many different ways. The NLRP3 inflammasome plays an important role in the generation of IL-1 causing inflammation, therefore its inhibition could help in thrombosis-mediated diseases which occur in COVID-19. The activation of NLRP3 inflammasome begins with the cleavage of pro-IL-1 by caspase-1 and ends with the release of mature IL-1 which is associated with inflammation. The active metabolites, known as eicosanoids which collectively include PGs, Txs, prostacyclins (PGI₂), LTs and lipoxins, are derived from membrane phospholipids and have biological activities.

In 1986, we showed for the first time that human recombinant IL-1 enhances TxA₂, monitored as TxB₂, in polymorphonuclear leukocytes and macrophages *in vitro*, demonstrating that not only IL-1 is capable of inducing inflammation

by the activation of eicosanoids, but also causes neutrophil, macrophage and platelet aggregation *in vitro*, mediating thrombosis and inflammation (7). IL-1 induces COX-2 which causes an increase of arachidonic acid products, including PGE₂ and thromboxane A₂ (TxA₂), detected as TxB₂ (as TxA₂ is unstable). SARS-CoV-2 infection causes neutrophilia and lymphopenia (4). The numerous neutrophils are activated both by coronavirus-19 and IL-1 secreted by mast cells and macrophages. Activation of neutrophil granulocytes by SARS-CoV-2 and IL-1 leads to the release of prostaglandins (PGs), leukotrienes (LTs) and Txs, which are highly inflammatory substances that contribute to the pathogenesis of COVID-19. These effects alone or in combination with others can be lethal for the patient. In addition, people affected by COVID-19 can have the so-called “cytokine storm” which causes excessive inflammation in the lungs leading to severe respiratory syndrome necessitating life-

Thromboxane A₂ synthesis, aggregation, thrombi generation and lung inflammation

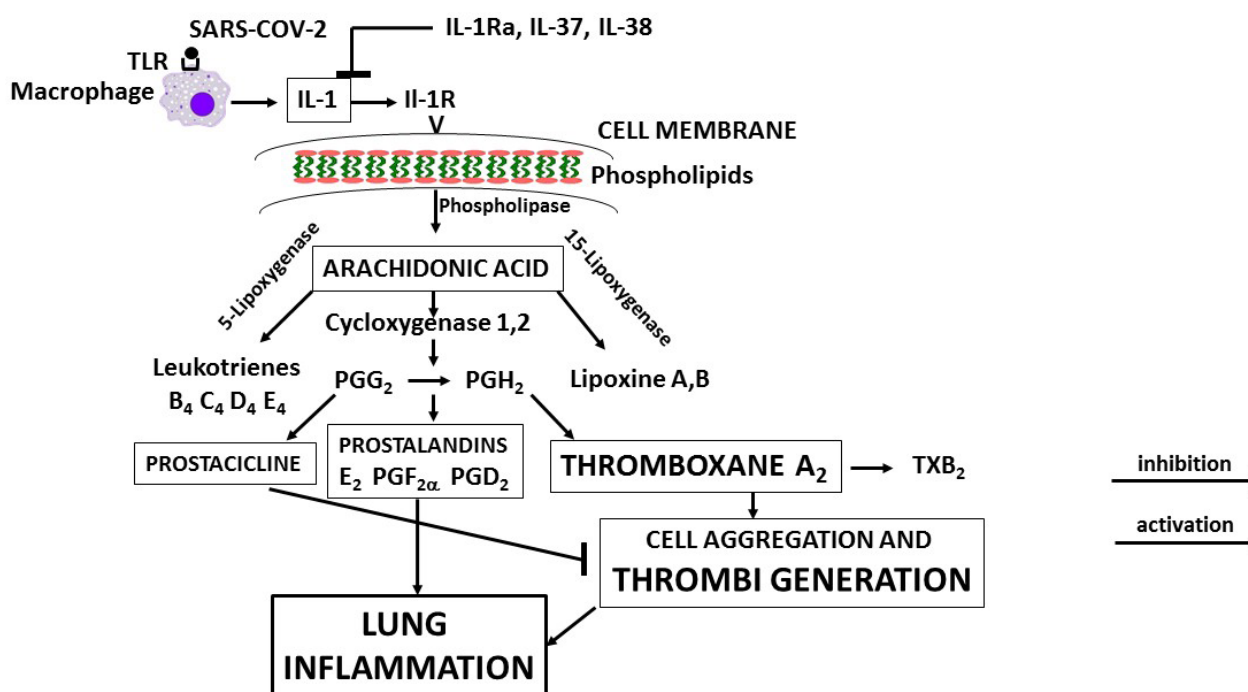


Fig. 1. Generation of thrombi and lung inflammation by macrophage-secreted IL-1, through induction of thromboxane A₂

saving treatment (8). Thus, affected patients have severe disease and lower levels of protecting Treg cells, a phenomenon that occurs less in children who have better-functioning Treg cells.

Endotoxin released by microorganisms activates macrophages and neutrophils to produce inflammatory cytokines and eicosanoids such as TxA₂ (9). TxA₂ belongs to the family of arachidonic acid metabolites produced by the action of enzymes such as phospholipase A₂, cyclooxygenase (COX)-1 and 2, and thromboxane-synthetase. TxA₂ is not only generated by platelets, but also by immune cells such as macrophages, endothelial cells and neutrophils. Platelets can be activated by coronavirus-19 through the TLR, such as TLR4, resulting in the release of neurotransmitters such as substance P, histamine, epinephrine, etc. and they can also produce a series of proinflammatory mediators of both cytokine and chemokine types, such as IL-1, TGFβ1 and CXCL1, CXCL4 CXCL5, CCL3, respectively. In addition, platelets play a role in the regulation of tissue inflammation and may generate platelet-activating factor (PAF) and lipid pro-inflammatory mediators such as PGs and TxA₂. In humans, activation of platelet alpha granules by SARS-CoV-2 induces the synthesis of IL-1β and TGFβ1, promoting pro-inflammatory action.

TxA₂, after being released, is strongly involved in the formation of thrombi by acting on platelet and neutrophil granulocyte aggregation. TxA₂ is a pro-inflammatory eicosanoid acting as a vasoconstrictor involved in various diseases such as myocardial infarction, stroke, bronchial asthma, allergies, kidney damage, angiogenesis, pulmonary hypertension and multi-organ failure (10). An alteration of TxA₂ has been noted in various diseases including diabetes, coronary heart diseases, hypertension, inflammation, auto-immune disorders, atopic eczema, Alzheimer dementia, cancer, and in tissue infections.

SARS-CoV-2-induced COVID-19 disease causes a systemic inflammatory response mediated by IL-1 with severe pathological consequences that can lead to the death of the patient. It has recently been reported that several cytokines are capable of inhibiting IL-1, such as IL-37, IL-38 and IL-1 receptor antagonist (IL-1Ra), an effect that could

aid in microorganism-induced inflammation. In fact, in viral infections, such as COVID-19 where IL-1 is synthesized, there is a high inflammation that is difficult to keep in check (Fig. 1).

In COVID-19, the inflammatory phenomenon can be countered in different ways by: i) preventing the entry of the coronavirus-19; ii) preventing the replication of the virus; iii) reducing the inflammatory reaction; iv) developing a vaccine with different types of viruses; v) weakening or inactivating SARS-CoV-2 with various pieces of the virus.

The action of coronavirus-19 on tissues triggers an inflammatory response mediated by the cytokine storm. The virus activates immune cells such as macrophages which essentially produce two highly inflammatory cytokines, such as IL-1 and IL-6, and cause lymphopenia. IL-1 is the main cytokine of inflammation and after being generated it induces other cytokines and itself in an autocrine-manner. Moreover, the administration in the liver of IL-1 and IL-6 in combination causes a synergistic pro-inflammatory stimulus on the release of serum amyloid A (SAA) (11). IL-1 provokes the activation of other pro-inflammatory compounds such as arachidonic acid compounds including TxA₂. In SARS-CoV-2 infection, in addition to the cytokine storm, there is also a storm of pro-inflammatory eicosanoids which includes the release of PGs, LTs and Txs that can mediate pain, fever, pulmonary fibrosis, thrombosis, and acute respiratory distress syndrome (12). Pro-inflammatory eicosanoids predominantly generated in the endothelium act in the vascular tone and mediate inflammation. Eicosanoid inhibitors could influence the inflammatory cascade and constitute an effective therapeutic approach also in the inflammation that occurs in COVID-19.

Viral infection causes cell death (apoptosis) and necrosis of T helper cells which should aid in the production of antibodies by B cells which should stem the pathogenesis and aid the survival of the host.

This editorial introduces new functions, including thrombus formation, due to TxA₂ released by the neutrophils stimulated by IL-1-derived macrophage induced by SARS-CoV-2 in COVID-19. Furthermore, it proposes inhibition of inflammation through the use of anti-inflammatory cytokines as a new therapy.

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EDITORIAL

Mast cells activated by SARS-CoV-2 release histamine which increases IL-1 levels causing cytokine storm and inflammatory reaction in COVID-19

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SARS-CoV-2 virus is an infectious agent commonly found in certain mammalian animal species and today also in humans. SARS-CoV-2, can cause a pandemic infection with severe acute lung injury respiratory distress syndrome in patients with COVID-19, that can lead to patient death across all ages. The pathology associated with pandemic infection is linked to an over-response of immune cells, including virus-activated macrophages and mast cells (MCs). The local inflammatory response in the lung that occurs after exposure to SARS-CoV-2 is due to a complex network of activated inflammatory innate immune cells and structural lung cells such as bronchial epithelial cells, endothelial cells and fibroblasts. Bronchial epithelial cells and fibroblasts activated by SARS-CoV-2 can result in the up-regulation of pro-inflammatory cytokines and induction of MC differentiation. In addition, endothelial cells which control leukocyte traffic through the expression of adhesion molecules are also able to amplify leukocyte activation by generating interleukin (IL)-1, IL-6 and CXC chemokines. In this pathologic environment, the activation of mast cells (MCs) causes the release of histamine, proteases, cytokines, chemokines and arachidonic acid compounds, such as prostaglandin D2 and leukotrienes, all of which are involved in the inflammatory network. Histamine is stored endogenously within the secretory granules of MCs and is released into the vessels after cell stimulation. Histamine is involved in the expression of chemokine IL-8 and cytokine IL-6, an effect that can be inhibited by histamine receptor antagonists. IL-1 is a pleiotropic cytokine that is mainly active in inflammation and immunity. Alveolar macrophages activated by SARS-CoV-2 through the TLR produce IL-1 which stimulates MCs to produce IL-6. IL-1 in combination with IL-6 leads to excessive inflammation which can be lethal. In an interesting study published several years ago (by E. Vannier et al., 1993), it was found that histamine as well as IL-1 are implicated in the pathogenesis of pulmonary inflammatory reaction, after micorganism immune cell activation. IL-1 in combination with histamine can cause a strong increase of IL-1 levels and,

Key words: histamine; IL-1; SARS-CoV-2; COVID-19; coronavirus; cytokine; inflammation; cytokine storm

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consequently, a higher degree of inflammation. However, it has been reported that histamine alone has no effect on IL-1 production. Furthermore, histamine enhances IL-1-induced IL-6 gene expression and protein synthesis via H2 receptors in peripheral monocytes. Therefore, since MCs are large producers of histamine in inflammatory reactions, this vasoactive amine, by increasing the production of IL-1, can amplify the inflammatory process in the lung infected with SARS-CoV-2. Here, we have proposed for the first time an emerging role for histamine released by MCs which in combination with IL-1 can cause an increase in lung inflammation induced by the viral infection SARS-CoV-2.

Histamine [2-(4-imidazolyl)-ethylamine] is a molecule that has an imidazole ring linked to an ethylamine chain. Histamine is an organic neurotransmitter involved in the immune system and other physiological functions (1). It mediates inflammatory responses and plays a key role in allergic phenomena. Histamine is produced by various cells including mast cells (MCs) and is capable of increasing vascular permeability allowing the passage of microorganisms, fueling infection and inflammation (2). Histamine is stored in MCs which, after activation through the FcεRI receptor, releases (in seconds) the content of the granules into the tissues. Bacteria can also produce histamine through histidine decarboxylase (HDC) an enzyme responsible for catalyzing the decarboxylation of histidine to form histamine and participate in various inflammatory diseases (3-4).

MCs are immune cells that play a key role in allergies and anaphylaxis and possess a high number of affinity receptors (FcεRIs) for IgE antibodies (5). The isolated progenitors of human MCs are c-kit + CD34 + CD13 + FcεRI- capable of differentiating into mature cells (6). MCs are the main source of cytokines in the lungs and they are found in large numbers in the inflamed lung of patients with asthma and respiratory infections due to microorganisms, including the influenza virus (7). The recruitment process of MCs is mediated by innate immunity. In immune responses, MCs bind specifically stimulated IgE and through cross-linking they bind to the FcεRI receptor, inducing receptor aggregation and thus cellular activation, leading to secretion of biologically active mediators that play a key role in allergies and inflammation (2). Histamine can also be produced and released by other cells such as neutrophilic and basophilic granulocytes (8). This vasoactive amine is one of these mediators which is an important mediator of anaphylaxis difficult to

detect in the blood as it has a very low half-life. In MC-mediated experimental models, MC-deficient mice do not produce histamine, proving that these cells are important sources of this biological mediator (9). The activation of H2 receptors and the synthesis of histamine causes an increase in cAMP which improves the production of caspase-1 and therefore of interleukin (IL)-1 in macrophages and also the synthesis of IL-6 induced by IL-1 (10). On the other hand, the activation of histamine

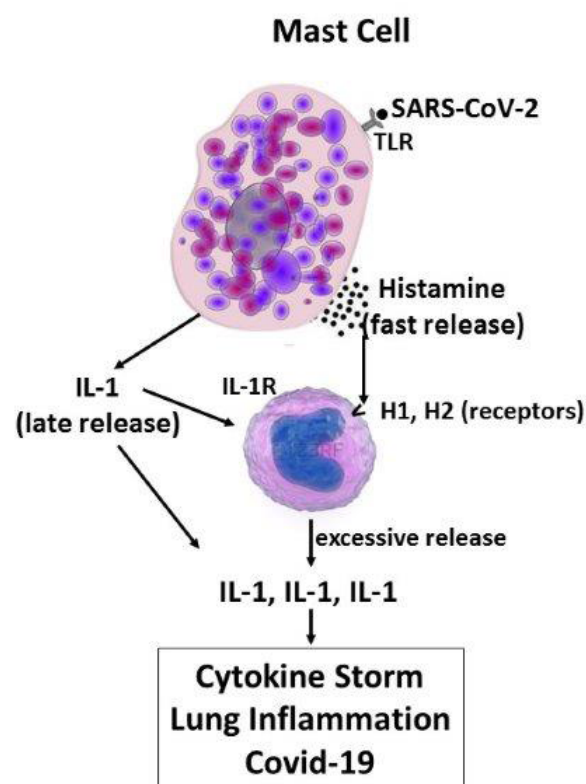


Fig. 1. Activation of mast cell by SARS-CoV-2 causes an early release of histamine and late release of IL-1 which activate macrophage. Histamine potentiates the production of IL-1 which causes cytokine storm and lung inflammation in COVID-19.

H2 receptors does not induce IL-1 synthesis. SARS-CoV-2 is a respiratory pathogen that causes COVID-19, a global pandemic that occurs in young and old with consequences that can be fatal (10). In COVID-19 there is a violent inflammatory response that can affect many vital organs, including the lung where severe tissue damage occurs. COVID-19 has a high morbidity and mortality due mainly to severe lung inflammation. (11). The pathological status of coronavirus-19 infection may be aggravated by subsequent bacterial infections (12). IL-1 is crucial in this inflammatory process and requires cleavage to be activated. Caspase-1 is an inflammasome protease capable of processing IL-1 (13). It is well known that in the lung infected by SARS-CoV-2, there is lymphopenia and a dominant neutrophilia which lead to the production of serine proteases such as cathepsin G, elastase and PR3, which also have the ability to process IL-1 (14). The generation of mature IL-1 causes a violent inflammation which can only be limited in part by the inhibition of neutrophilic granulocytes (15-16). SARS-CoV-2 infection can trigger the recruitment and accumulation of MCs in the lung, a process mediated by innate immunity and therefore by pro-inflammatory cytokines of the IL-1 family (17) (Fig. 1). The accumulation may be mediated by an increase in TLR and other innate receptors provoking the exacerbation of inflammatory symptoms in COVID-19 (18). In this study, we report that there is an important inter-relationship between MCs and IL-1 β -mediated inflammation. In addition, here we emphasize that MCs activated by SARS-CoV-2 release histamine which increases IL-1 levels involved in cytokine storm and, therefore, excessive inflammatory reaction in COVID-19 (19).

In light of the above, we can conclude that the MCs stimulated by SARS-CoV-2 are involved in viral inflammation, contributing to the formation of the cytokine storm by producing histamine and IL-1 which is one of the most potent biological pro-inflammatory molecules with activity that can be lethal.

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EDITORIAL

COVID-19 and multisystem inflammatory syndrome, or is it mast cell activation syndrome?T.C. Theoharides^{1,2,3} and P. Conti⁴

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COVID-19 derives from infection with Coronavirus [severe acute respiratory syndrome (SARS)-CoV-2] and is associated with high morbidity and mortality due to release of a storm of pro-inflammatory cytokines and thrombogenic agents resulting in destruction of the lungs. Many reports indicate that a considerable number of patients who are positive for SARS-CoV-2 are asymptomatic or have mild symptoms. However, increasing evidence suggests that many such patients who either recovered from or had mild symptoms after COVID-19 exhibit diffuse, multiorgan, symptoms months after the infection. These symptoms include malaise, myalgias, chest tightness, brain fog and other neuropsychiatric symptoms that were originally reported in children and named Multisystem Inflammatory Syndrome (MIS-C). Now the US Center for Disease Control (CDC) has announced the recognition of a similar condition in adults, named Multisystem Inflammatory Syndrome (MIS-A). The symptoms characterizing these conditions are very similar to those associated with Mast Cell Activation Syndrome (MCAS, US ICD-110 code D89.42-idiopathic mast cell activation syndrome). Hence, the possibility of MCAS should be evaluated in any patient with MIS and/or multisystem inflammatory symptoms. In either case, these syndromes should be addressed with liposomal formulation (in olive pomace oil) of the flavone luteolin (e.g. PureLut® or FibroProtek®) together with the antihistamine rupatadine, which also has anti-platelet activating factor (PAF) activity and inhibits mast cells that have been implicated in the pathogenesis of cytokine storms in COVID-19.

The recent Coronavirus [severe acute respiratory syndrome (SARS)-CoV-2] is associated with a high morbidity and mortality in adults, known as COVID-19 (1). Infected patients who recover have increased levels of specific antibodies and activated T cells (2, 3), but dysfunctional immune system (4). In particular, the pulmonary pathology results from release of multiple pro-inflammatory cytokines, especially IL-6 (2, 5), but also microthromboses that may involve platelet activating factor (PAF) (6).

Children and adolescents do get sick with COVID-19 (7), but generally present with milder symptoms than adults (8, 9). Nevertheless, a number of papers have reported the presence in children of Multisystem Inflammatory Syndrome (MIS-C) with symptoms resembling toxic shock or Kawasaki syndrome (10, 11). Symptoms in children were mostly nonspecific and included rash (52%), diarrhea (52%), vomiting (45%) and conjunctival injection (45%) with all children having increased indexes of inflammation (12).

Key words: multisystem inflammatory syndrome; MIS-C; MIS-A; COVID-19; SARS-CoV-2; inflammation; mast-cell; immunity; cytokine storm

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There has been increased realization that COVID-19 is associated with many extrapulmonary manifestations, including thrombotic complications, arrhythmias, gastrointestinal symptoms, as well as dermatologic and neurologic complications (12). In fact, the Centers for Disease Control (CDC) recently recognized the existence of a similar syndrome in adults, (<https://www.cdc.gov/mis-c/>) named Multisystem Inflammatory Syndrome (MIS-A). The symptoms characterizing this syndrome are numerous and affect almost all organs, including cardiovascular, gastrointestinal, dermatologic and neurologic symptoms, especially brain fog (Table I) (13, 14). Prominent among the neurologic manifestations is brain fog associated with confusion, inability to focus, loss of memory, and inability to find the right words that has been termed “COVID Brain Fog” (<https://www.nytimes.com/2020/10/11/health/covid-survivors.html>). MIS-A symptoms typically appear weeks to months after infection, giving the term “longhauers” to such patients, and are worsened by psychological stress (15, 16). MIS-A symptoms and their worsening by stress are very similar to those (Table II) experienced by patients with mastocytosis (17) or mast cell activation syndrome (MCAS) (18). A unique aspect of MCAS is that the mast cells in these patients are stimulated by numerous non-allergic triggers, including stress-related peptides (19) without the release of histamine or tryptase (20).

A key source of pro-inflammatory cytokines in COVID-19 (21) is the mast cells (17, 22), which can be triggered by viruses (23), including SARS-CoV-2 (24), and secrete multiple pro-inflammatory mediators (25, 26), including IL-6 (27) and IL-1 β

(28), thus potentially contributing to COVID-19 pathology (24). Mast cells are ubiquitous in the body, located perivascularly, especially in the lungs where they mature under the influence of local micro-environmental factors resulting in different phenotypes. Mast cells are typically stimulated by allergens crosslinking allergen-specific immunoglobulin E (IgE) bound to high affinity Fc epsilon receptor 1 (Fc ϵ RI) (17), but also by non-IgE stimuli (29) such as cationic compounds via activation of the low affinity G-coupled receptor MRGPRX2 (30), as well as neuropeptides, including corticotropin-releasing hormone (CRH), neurotensin (NT), and substance P (SP) via high affinity receptors (31).

Following stimulation, mast cells rapidly secrete the preformed, granule-stored, heparin, histamine, tryptase and TNF, as well as newly synthesized leukotrienes, PAF, prostaglandin D₂ (PGD₂), cytokines (IL-5, IL-6, IL-31, IL-33 and TNF) and chemokines (CCL2, CCL5 and CXCL8) released 6-24 hours later (25). Some of these “late phase” mediators can be released without degranulation (19), as we showed for IL-6 (27) and IL-1 β (28). Mast cell-derived vasoactive mediators, especially cytokines (32), can also increase the permeability of the blood-brain barrier (BBB) (33). Hence, SARS-CoV-2 could lead to “COVID Brain Fog” either directly via activation of mast cells or by permitting cytokines to enter through a disrupted BBB.

Inhibition of mast cell-associated inflammation is necessary (34) and could be accomplished with some naturally occurring flavonoids, especially luteolin (23), which inhibits release of pro-inflammatory molecules

Table I. *Working criteria for MIS-A*

1. Severe illness requiring hospitalization in a person >21 years old
2. Positive test result for SARS-CoV-2 infection (current or within past 12 weeks)
3. Severe dysfunction of one or more extrapulmonary organ systems
4. Laboratory evidence of severe inflammation
5. Absence of severe respiratory illness

Table II. *Common symptoms present in MIS-A and MCAS*

- Angioedema
- Arrhythmias
- Brain fog
- Confusion
- Diarrhea
- Dizziness
- Dysautonomia
- Fatigue
- Gastrointestinal complaints
- Headache
- Hives
- Hypotension
- Lightheadedness (syncope)
- Inability to find the right word
- Memory loss
- Myalgias
- Palpitations
- Shortness of breath
- Skin rashes
- Weakness

from mast cells and also has anti-viral properties (24). Due to the poor solubility and oral absorption of luteolin, preferable formulations would be those using liposomal luteolin (e.g. FibroProtek®, BrainGain®) (35).

The presence of diffuse, multisystem inflammatory symptoms, especially “COVID Brain Fog” may be indicative of MCAS (24). Hence, in addition to the inflammatory markers discussed, it would be important to investigate mast cell-associated mediators and attempt to inhibit their release and/or their biological actions.

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Diagnostic value of the combined detection of CEA, NSE and IL-18 for lung cancer and their relationship with apoptosis gene Bcl-2

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This study aims to investigate the value of the combined detection of carcinoembryonic antigen (CEA), Neuron-specific enolase (NSE) and the level of Interleukin-18 (IL-18) in the serum in the diagnosis of lung cancer. The correlation between these parameters and the expression levels of B-cell lymphoma-2 (Bcl-2) protein were also studied. Eighty patients with lung cancer were included in the lung cancer group. These patients underwent surgery in the Department of Oncology of Huai'an Second People's Hospital between February 2016 and February 2018. During the same period, another 80 patients with benign lung lesions were registered in the benign lesion group and 80 healthy people were enrolled in the control group. Enzyme-linked immunosorbent assay (ELISA) was used to detect the expression levels of CEA, NSE and IL-18. The diagnostic critical value of these factors was used as positive indicator. When CEA, NSE and IL-18 levels were positive at the same time, the combined detection was considered to be positive. WB was used to detect Bcl-2 expression level. We also analyzed the possible correlation between CEA, NSE, IL-18 levels and the Bcl-2 expression levels. The CEA, NSE and IL-18 expression levels in the serum of the lung cancer group were significantly higher than those in the benign lesion and the control groups ($p < 0.05$). The area under ROC curve for CEA, NSE and IL-18 respectively was 0.770 (0.697-0.843), 0.829 (0.767-0.890), 0.721 (0.642-0.800) ($p < 0.001$). IL-18 level was negatively correlated with the level of Bcl-2 mRNA in the tissue ($r = -0.380$, $p < 0.001$). In conclusion, CEA, NSE and IL-18 have a good auxiliary diagnostic value in patients with lung cancer. The combined detection could improve the sensitivity and specificity of lung cancer diagnosis. There was a negative correlation between IL-18 and Bcl-2 levels which suggested a potential inhibitory role of IL-18 on the lung cancer cells apoptosis pathway.

Lung cancer is the worst cancer in terms of morbidity and mortality in the world (1). Due to the relatively latent early symptoms of this disease, most patients usually are in the terminal stage when they are

admitted to hospital (2). Early diagnosis and timely treatment are the two key factors in the prognosis this disease (3). At present, several tumor markers for lung cancer are clinically used. Broad-spectrum

Key words: lung cancer; CEA; NSE; IL-18; Bcl-2

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tumor marker carcinoembryonic antigen (CEA) has been found to be a useful tool for patients with lung cancer (4). Neuron-specific enolase (NSE) is a highly sensitive marker in the diagnosis of small-cell lung cancer (5), and can also be used as a biological indicator to evaluate the efficacy and prognosis in the treatment (6). Interleukin-18 (IL-18) is an immunoregulatory factor with possible antitumor effect (7). Due to the limitations associated with single factor detection methods, combined methods are considered to be more valuable tools in early detection of lung cancer.

B-cell lymphoma-2 (Bcl-2) is an anti-apoptosis oncogene, and studies have shown that its overexpression can trigger malignant transformation of the cells and the occurrence of various types of cancers (8). This study aimed to investigate the value of the combined detection of carcinoembryonic antigen (CEA), Neuron-specific enolase (NSE) and the level of Interleukin-18 (IL-18) in the serum. We also studied the correlation between these parameters and the expression levels of Bcl-2 protein.

MATERIALS AND METHODS

Study population

Eighty patients with lung cancer were enrolled in the lung cancer group who underwent surgery in the Oncology Department of Huai'an Second People's Hospital from February 2016 to February 2018. This group included 51 males and 29 females aged 35-78 years, with a mean age of 56.46 ± 7.84 years. There were 21 patients with squamous carcinoma, 37 with adenocarcinoma, 22 with small-cell lung cancer in the lung cancer group. There were 19 patients in TNM stage I, 16 in stage II, 21 in stage III, and 24 in stage IV. During the same period, eighty patients with benign lung lesions were also selected, including 50 males and 30 females aged 32-83 years, with a mean age of 54.46 ± 8.35 years.

Eighty healthy subjects who underwent physical examination in the same period were enrolled as the control group. There were 47 males and 33 females aged 27-84 years, mean 55.54 ± 9.43 years.

The inclusion criteria were: i) patients in the lung cancer group met the pathology diagnostic criteria of lung cancer (9); ii) changes could be seen in the pulmonary veins in the chest radiograph of patients in the benign lesion group.

The possibility of lung cancer was excluded by Computed Tomography (CT), bronchoscope and other imaging data; iii) the chest radiograph of the patients in the control group showed normal cardiopulmonary function, and there were no abnormal changes in the pulmonary veins.

The exclusion criteria were: i) patients with abnormal cardiac cerebral function or hepatorenal function; ii) patients who had cancer in other parts of the body or had serious immune diseases; iii) patients who had received related treatments.

This study was approved by the medical ethics committee of The Second People's Hospital. The subjects enrolled in this study were informed of the specific contents of the experiment in detail, and they all agreed and signed the informed consent.

Collection and processing of blood samples

Ten ml of fasting venous blood was collected in the early morning using the vacuum blood collection needles. Samples were placed in the disposable vacutainer blood collection tubes for 1-2 h at room temperature. Then the samples were centrifuged for 10 min at 4°C at a speed of 1000 g. The supernatant was carefully collected, and the serum was obtained, subpackaged, and stored at -80°C .

Detection of the concentration of CEA, NSE and IL-18 in serum using Enzyme-linked immunosorbent assay (ELISA)

The serum levels of CEA, NSE and IL-18 of all experimental subjects were evaluated using double antibody sandwich ELISA. Human CEA ELISA kit (EK0904) was purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd., Shanghai, China. NSE ELISA kit (JK-(a)-1746) was purchased from Shanghai Jingkang Bioengineering Co., Ltd., Shanghai, China. IL-18 ELISA kit (BRK-0139H1) was purchased from Changsha Da Erfeng Biotechnology Co., Ltd., Hunan, China.

Samples were diluted and then the standard solutions with a concentration of 1000 ng/ml were prepared. Standard wells, sample wells, and blank control wells were set up. 100 μl of standard solution or sample were added into each reaction well, and maintained at 37°C for 2 h. The coating liquid in the well was discarded; the reaction wells were washed 4-6 times with PBST for 3 min each time; 100 μl of the primary antibody solution was added into each well followed by incubation at 37°C for 1 h. Subsequently, the coating liquid in the well was discarded and the reaction

wells were washed 4-6 times with PBST [Phosphate Buffer Solution (Shanghai Lianmai Bioengineering Co., Ltd., LM0221A)+Tween-20] for 3 min each time. Afterwards, 100 μ l of enzyme labeled antibody working solution was added in the mixture which was then incubated at 37°C for 0.5 h, and then washed again (same steps as above). Then, 100 μ l of substrate working solution was added into each reaction well, and the reaction was carried out at 37°C for 15 min in the dark. Finally, 100 μ l of stop solution was added, and the absorbance at 450 nm was read using an automatic enzyme-labeled instrument plate reader.

WB was used to detect the protein expression level of Bcl-2 in lung cancer tissue

The tumor tissue and adjacent tissues in 80 patients in the lung cancer group were collected. A small amount of the tissue was cut into small pieces with clean scissors. Protein lysate was added to the pieces. The mixture was placed on ice for 1 h, and then centrifuged for 20 min at 4°C with a centrifugal force of 12000 $\times$ g. The protein concentration was evaluated using bicinchoninic acid (BCA, Chongqing Pu Like Biotechnology Co., Ltd., 632588), and the concentration was adjusted to 6 g/L; 0.01 ml of the mixture was respectively added into each tube. Subsequently, 0.02 ml of the loading buffer was added to each tube and the mixture was heated in boiling water for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) precast gel (Shanghai Yaji Biotechnology Co., Ltd., YS-10347RJ) was then added and gel electrophoresis was conducted at 50 V, and transfer was performed at a voltage of 100 V. Solution pH was adjusted to 7.6 using Tris Buffered Saline Tween buffer solution with 0.05% Tween20. Then, 5% of skim milk was added, and then the membrane was blocked at room temperature for 1 h. Primary antibody [Bcl-2 antibody (Shanghai Qiming Biotechnology Co., Ltd., QM-10846M), β -Actin antibody (Shanghai Jingke Chemical Technology Co., Ltd., R1207-1)] was then added after incubation at 4°C overnight. Then the membrane was rinsed for 3 times with TBST buffer solution. Horseradish Peroxidase (HRP) enzyme labeled Rabbit anti-human secondary antibody (BSZH Co., Ltd., EH48690) was added. After incubation at 37°C for 1 h, it was rinsed 3 times with TBST buffer solution. The film was developed using Enhanced chemiluminescent (ECL) (Shanghai Kanglang Biotechnology Co., Ltd., KL-D2173) in a dark environment, and the gradation analysis was

performed using the Bio-Rad gel image processing system. Bcl-2/ actin ratio was used as the relative protein expression level of Bcl-2. Electrophoresis apparatus (1645050) was purchased from Shanghai Rong Weida Industrial Co., Ltd. 0.2 μ l of RIPA (Shanghai Yanjin Biotechnology Co., Ltd., RIPA20110527)

mRNA expression level of Bcl-2 in the lung cancer group was detected by real-time fluorescent quantification (qRT-PCR)

Total RNA was extracted from the cancerous tissues and normal adjacent tissue of the 80 patients in the lung cancer group using Trizol reagent (Shanghai Zeye Biological Technology Co., Ltd., ZY-1102c). The absorbance values of total RNA at 260 nm and 280 nm were detected by UV spectrophotometer (Tomorgen Biotechnology Co., Ltd., MD2000). Total RNA was reversely transcribed into cDNA using 4 μ l 5 $\times$ TransScript® All-in-One SuperMix and 1 μ l gDNA Remover. The cDNA and 10 μ l 2 $\times$ TransScript® Tip Green qPCR SuperMix (Transgen Biotech Co., Ltd., AQ201-01) were mixed, 0.4 μ l upstream and 0.4 μ l downstream primers were added, then Nuclease-Free Water (Xiamen Keyan Biotechnology Co. Ltd, P1193) was added to 20 μ l. The reaction system was 94°C for 30 s, 94°C for 5 s, 60°C for 30 s, 40-45 cycles. GAPDH was the internal reference, and the experiment was repeated 3 times. Ct values were recorded, and $2^{-\Delta Ct}$ was used to represent the relative mRNA expression level of Bcl-2. Primer design and primer synthesis were completed by Sangon Biotech (Shanghai) Co., Ltd., and real-time fluorescent quantitative PCR instrument was purchased from Applied Biosystems (Prism® 7500). The primer sequences are shown in Table I.

Observation indicators

Expression levels of CEA, NSE and IL-18 in patients' serum in the lung cancer group and the benign lesion group were evaluated. With these data, ROC curve was respectively drawn, and the diagnostic critical values of CEA, NSE and IL-18 were obtained. The diagnostic critical values were used as positive indicator. The number of positive results in the lung cancer group and the benign lesion group was analyzed. When the results of the three factors were positive at the same time, the combined detection was considered to be positive. The diagnostic value of CEA, NSE and IL-18 single detection and the combined detection were compared.

Statistical analysis

Statistical software SPSS17.0 (SPSS China, Shanghai, China) was used for statistical analysis of the experimental data. The enumeration data (%) were analyzed using χ^2 , and the measurement data were expressed using ($\bar{x} \pm sd$). The pairwise comparison was performed by independent sample *t*-test. The comparison among more than two groups was analyzed using one-way ANOVA, and expressed in *F*. ROC curves were drawn according to the expression levels of CEA, NSE and IL-18 in the serum of patients in the lung cancer group and the benign lesion group. The diagnostic values of CEA, NSE, IL-18 and the combined detection were evaluated. The correlation between CEA, NSE, IL-18 and the mRNA expression of Bcl-2 was analyzed using Pearson test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Comparison of clinical basic data

As shown in Table II, there were no differences in the factors such as age, gender, and body mass index between the lung cancer group, the benign lesion group and the control group ($p > 0.05$).

Comparison of the expression levels of CEA, NSE and IL-18 ($\bar{x} \pm sd$)

The expression levels of CEA, NSE and IL-18 in the serum of the lung cancer group were higher than those in the serum of the benign lesion group and the control group. The expression levels of CEA, NSE and IL-18 in the serum of the benign lesion group were higher than those in the serum

Table I. Primer sequences

Gene	Primer Sequences (5'-3')		bp
Bcl-2	Upstream	GAGGATTGTGGCCTTCTTTG	118
	Downstream	GTGCCGGTTCAGGTACTCA	
GAPDH	Upstream	TGCCACCCACTCCTCTACCTTCG	163
	Downstream	CCGGTGGTTTGAGGGCTCTTACT	

Table II. The comparison of general clinical basic data ($\bar{x} \pm sd$) [$n(\%)$]

Clinical factor	The lung cancer group (n=80)	The benign lesion group (n=80)	The control group (n=80)	<i>F</i> / χ^2	<i>p</i>
Age (year old)	56.46 $\pm$ 7.84	54.46 $\pm$ 8.35	55.54 $\pm$ 9.43	1.093	0.337
Body mass index (kg/m ²)	22.34 $\pm$ 1.76	22.67 $\pm$ 1.42	22.85 $\pm$ 1.26	2.396	0.093
Gender				0.458	0.795
Male	51 (63.75)	50 (62.50)	47 (58.75)		
Female	29 (36.25)	30 (37.50)	33 (41.25)		
Leukocyte				2.192	0.334
<10 $\times$ 10 ⁹ /L	72 (90.00)	71 (88.75)	76 (95.00)		
$\geq$ 10 $\times$ 10 ⁹ /L	8 (10.00)	9 (11.25)	4 (5.00)		

The enumeration data (%) were analyzed by using χ^2 , and the measurement data were expressed by using ($\bar{x} \pm s$). Pairwise comparison was performed by independent sample *t*-test. The comparison among more than two groups was analyzed by using one-way ANOVA, and expressed in *F*.

of the control group ($p<0.05$). The detailed data are shown in Table III.

Diagnostic value of CEA, NSE, IL-18 and the combination of the three factors

The area under ROC curve of CEA, NSE and IL-18 was 0.770 (0.697-0.843), 0.829 (0.767-0.890), 0.721 (0.642-0.800), respectively. The diagnostic critical values of CEA, NSE and IL-18 for the patients with

lung cancer were 16.83 $\mu\text{g/L}$, 28.17 $\mu\text{g/L}$, 299.90 $\mu\text{g/L}$ ($p<0.001$), and the diagnostic critical value was used as positive indicator. When the three factors were positive at the same time, the combined detection was considered to be positive. The sensitivity and positive predictive value of the combined detection were both higher than those of the single detection of CEA, NSE and IL-18 ($p<0.05$), and the specificity of the combined detection was lower than that of the single detection of

Table III. Comparison of the expression levels of CEA, NSE and IL-18

Group		CEA($\mu\text{g/L}$)	NSE($\mu\text{g/L}$)	IL-18($\mu\text{g/L}$)
The lung cancer group	80	18.45 $\pm$ 2.99	34.88 $\pm$ 9.58	312.43 $\pm$ 26.57
The benign lesion group	80	14.98 $\pm$ 2.91 [#]	23.28 $\pm$ 8.47 [#]	282.64 $\pm$ 28.12 [#]
The control group	80	2.14 $\pm$ 0.87 ^{#,*}	5.23 $\pm$ 1.03 ^{#,*}	131.52 $\pm$ 14.45 ^{#,*}
F		975.300	325.800	1324.00
p		<0.001	<0.001	<0.001

Measurement data were expressed by using ($\bar{x}\pm s$). The pairwise comparison was performed by independent sample t-test. The comparison among more than two groups was analyzed by using one-way ANOVA, and expressed in F. # means compared with the lung cancer group, $p<0.05$, * means compared with the benign lesion group, $p<0.05$.

Table IV. The comparison of the diagnostic value of CEA, NSE, IL-18 and the combination of the three factors

	Sensitivity(%)	Specificity(%)	Accuracy(%)	Positive predictive value(%)
CEA	73.75(59/80) [*]	73.75(59/80) ^{&}	73.75(114/160)	73.75(59/80) [#]
NSE	80.00(64/80)	73.75(59/80) ^{&}	76.88(123/160)	75.29(64/85) [#]
IL-18	72.50(58/80) [*]	68.75(55/80) ^{&}	70.63(113/160)	69.88(58/83) [#]
The combined detection	100.00(80/80)	40.00(32/80)	73.00(120/160)	62.50(42/43)

The enumeration data [$n\%(/)$] were analyzed by using χ^2 . * means compared with the sensitivity of the combined detection, the sensitivity of CEA was lower ($\chi^2 = 24.17$, $p<0.001$); the sensitivity of NSE was lower ($\chi^2 = 17.78$, $p<0.001$); the sensitivity of IL-18 was lower ($\chi^2 = 25.51$, $p<0.001$); & means compared with the specificity of the combined detection, the specificity of CEA was higher ($\chi^2 = 18.85$, $p<0.001$); the specificity of NSE was higher ($\chi^2 = 18.85$, $p<0.001$); the specificity of IL-18 was higher ($\chi^2 = 13.33$, $p<0.001$); # means compared with the positive predictive value of the combined detection, the positive predictive value of CEA was lower ($\chi^2 = 10.90$, $p=0.001$); the positive predictive value of NSE was lower ($\chi^2 = 10.05$, $p=0.002$); the positive predictive value of IL-18 was lower ($\chi^2 = 13.36$, $p=0.001$). The measurement data were expressed as ($\bar{x}\pm sd$). Independent sample t-test was used for comparison between the two groups.

CEA, NSE and IL-18 ($p < 0.05$). However, there was no difference when comparing the accuracy of the combined detection with that of the single detection of the three factors ($p > 0.05$). (Table IV, Fig. 1).

Relative expression of Bcl-2 protein and Bcl-2 mRNA

WB and qRT-PCR results showed that the relative expression levels of Bcl-2 protein and Bcl-2 mRNA in lung cancer tissues were $[(1.22 \pm 0.14) \text{ ng/L}, 1.74 \pm 0.26]$. The levels in the adjacent normal tissues were $[(0.73 \pm 0.02) \text{ ng/L}, 1.02 \pm 0.21]$. The relative

expression levels of Bcl-2 protein and Bcl-2 mRNA in the lung cancer tissues were higher than those in the paracancerous normal tissues ($p < 0.05$), and the Bcl-2 protein level was shown in Fig. 2.

Correlation between CEA, NSE, IL-18 and the protein expression of Bcl-2

qRT-PCR results showed that the expression level of Bcl-2 mRNA in the tissue in the lung cancer group was 1.74 ± 0.26 . Pearson correlation analysis was used and the result showed that the expression

Table V. The correlation between CEA, NSE, IL-18 and the mRNA expression of Bcl-2

	CEA	NSE	IL-18
r	0.065	0.069	-0.380
p	0.565	0.545	<0.001

The correlation between CEA, NSE, IL-18 and the mRNA expression of Bcl-2 was analyzed using Pearson test.

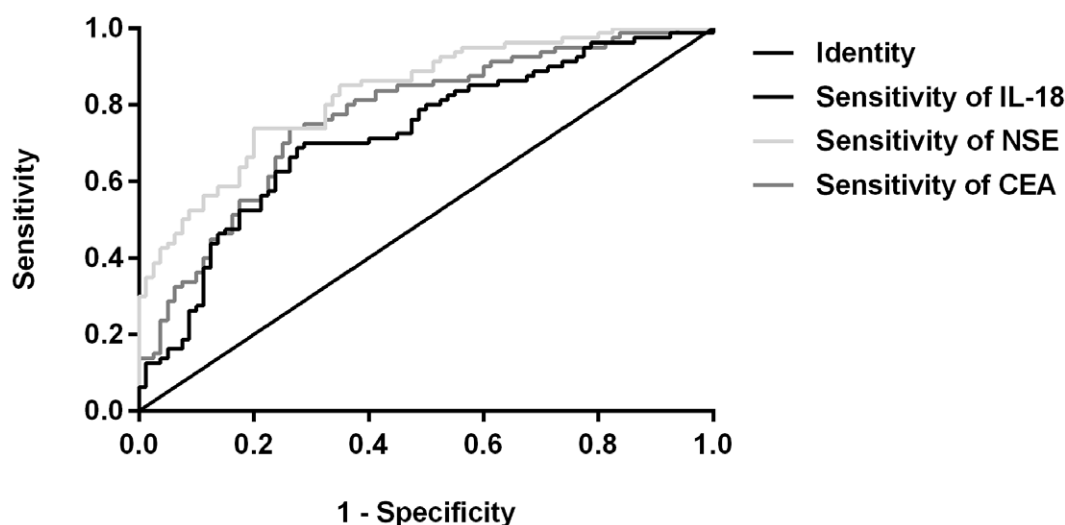


Fig. 1. ROC curve of CEA, NSE and IL-18. Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of serum CEA, NSE and IL-18, and it was repeated 3 times when reading the OD value from the microplate reader. The area under ROC curve of CEA, NSE and IL-18 respectively was 0.770 (0.697-0.843), 0.829 (0.766-0.892), 0.721 (0.642-0.800), and the diagnostic critical value of CEA, NSE and IL-18 for the patients with lung cancer was 16.83 $\mu\text{g/L}$, 28.17 $\mu\text{g/L}$, 299.90 $\mu\text{g/L}$ ($p < 0.001$).

levels of CEA and NSE in the serum were not correlated with the expression level of Bcl-2 mRNA in the tissue of the patients ($p>0.05$). IL-18 was negatively correlated with the protein expression level of Bcl-2 in the tissue of patients ($r=-0.380$, $p<0.001$). The detailed data are shown in Table V, and the correlation between IL-18 and the protein expression level of Bcl-2 is shown in Fig. 3.

DISCUSSION

Lung cancer has an extremely high degree of malignancy with poor prognosis (10). Early diagnosis can have a great impact on the prognostic survival and the patients' life quality (11). The diagnosis of lung cancer relies mainly on imagological examination, which is not very useful during the early stage (12),

and the early diagnostic rate is extremely low.

Tumor marker is a specific marker in the generation and progression of cancer cells (13), which can be used to evaluate the state of illness before surgery or treatment, as well as to observe the postoperative recurrence and evaluate the therapeutic efficacy by monitoring biological indicators in serum (14). Tumor marker can also reflect the condition of the generation and progression of cancers and the prognosis to some extent (15). CEA and NSE are two common tumor markers, and there is evidence showing that both markers are over-expressed in the serum of patients with lung cancer (16, 17). Cancer cells keep fighting with the immune system of the organism in the progression of cancer (18, 19). IL-18 is a cytokine that promotes the proliferation of immune cells and enhances their activity, therefore playing an important

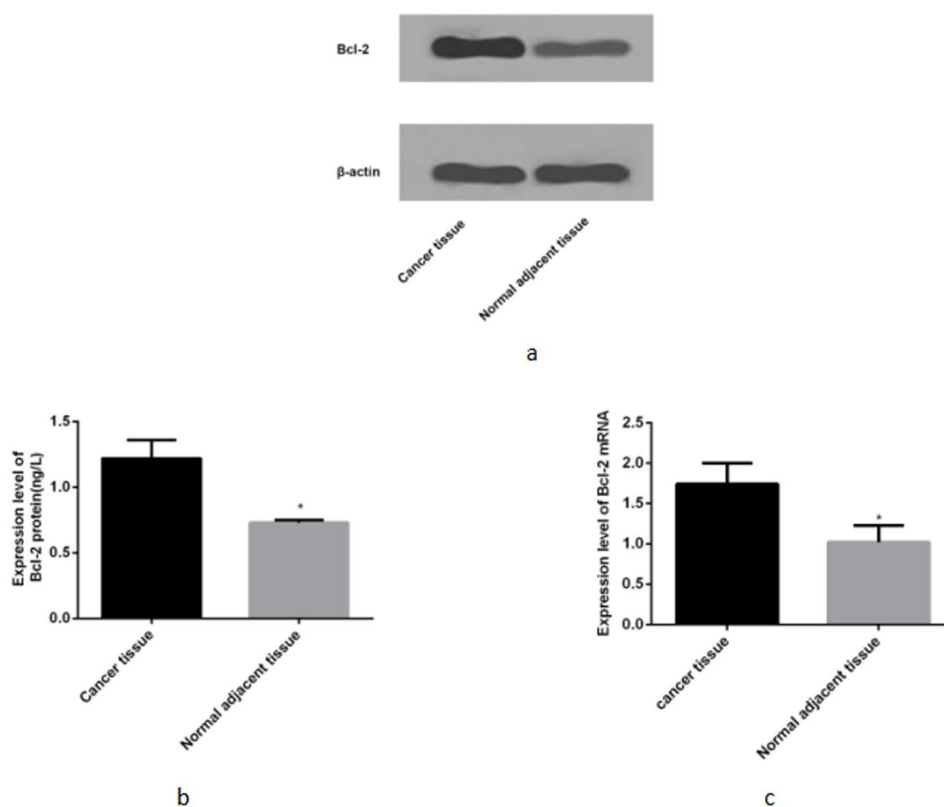


Fig. 2. *a) Relative expression of Bcl-2 protein. Western blot was used to detect the expression of Bcl-2 protein in cancerous tissues and adjacent normal tissues of 80 patients with lung cancer. The experiment was repeated 3 times and the average was taken. The results showed that there was Bcl-2 protein expression in cancerous tissues of lung cancer. b) Relative expression levels of Bcl-2 protein and Bcl-2 mRNA. The relative expression levels of Bcl-2 protein and Bcl-2 mRNA in lung cancer tissues were detected by Western blot and qRT-PCR. The relative expression levels of Bcl-2 protein and Bcl-2 mRNA in lung cancer tissues were higher than those in adjacent normal tissues ($p<0.05$).*

role in restraining the progression of tumors (20). Prior studies have revealed that Bcl-2 plays a key role in the formation of lung cancer (21). This study mainly investigated the diagnostic value of CEA, NSE, IL-18 and the combined detection in the early diagnosis of lung cancer and the relationship between the three factors and the protein expression of Bcl-2 in lung cancer tissue.

The results of this study showed that the expression levels of CEA, NSE and IL-18 in the serum of the lung cancer group were significantly higher than those in the benign lesion group and the control group. Expression levels of CEA, NSE and IL-18 in the benign lesion group were higher than those in the control group. According to ROC curve, the area under ROC curve of CEA, NSE and IL-18 in the lung cancer group were 0.770, 0.829, 0.721, respectively. The diagnostic critical value of CEA, NSE and IL-18 for patients with lung cancer was 16.83 $\mu\text{g/L}$, 28.17 $\mu\text{g/L}$, 299.90 $\mu\text{g/L}$. The sensitivity and specificity of the combined detection of the three factors were both higher than those of the single detection of CEA and IL-18, and the specificity was higher than that of the single detection of NSE. There was no difference in accuracy, which suggested that CEA, NSE and IL-18 were highly

expressed in serum of lung cancer and the application of the combined detection of CEA, NSE and IL-18 was of great significance for the early diagnosis. CEA is a common tumor marker that is over-expressed in solid tumors, such as breast cancer and lung cancer (22, 23), and can be used as an auxiliary in clinical practice. Prior study showed that the overexpression of CEA is an independent prognostic factor of non-small-cell lung cancer (NSCLC) (24), however, the sensitivity and specificity of CEA in the early diagnosis are not ideal. The expression level of CEA is also higher than the normal range in smokers without lung cancer (25). NSE has high specificity and sensitivity in small cell lung cancer, which can even be used as a therapeutic and monitoring indicator (26). However, the positive rate of NSE is not high in the diagnosis of NSCLC (27). Other studies showed that IL-18 is closely related to the generation, progression and prognosis of lung cancer (28), but the sensitivity and specificity is low. It is also over-expressed in breast cancer and systemic lupus erythematosus (29, 30). The combined detection of CEA, NSE and IL-18 can greatly improve the sensitivity and positive predictive value in the diagnosis of lung cancer, which is of great significance.

In addition, WB and qRT-PCR were used to detect the relative expression levels of Bcl-2 protein and Bcl-2 mRNA in lung cancer tissues and in the adjacent normal tissues. It was shown that the relative expression levels of Bcl-2 protein and Bcl-2 mRNA in the lung cancer tissues were higher than those in the paracancerous normal tissues ($p < 0.05$). We could not establish a meaningful relation between the expression levels of CEA and NSE and expression level of Bcl-2 in the patients' tissue using Pearson correlation analysis. IL-18 was negatively correlated with the mRNA expression level of Bcl-2 in patients' tissue ($r = -0.380$, $p < 0.001$). One study shows that Bcl-2 was highly expressed in lung cancer tissues, and its high expression is also a favorable factor for the prognosis of lung cancer patients. (31), which is consistent with our study. Studies have shown that the immune system is not always indifferent during the generation and progression of cancers. On the contrary, the immune system may make a series of immune reactions to prevent the changes caused by the invasion of tumor tissue (32). IL-18 has anti-tumor property, and its overexpression can trigger a

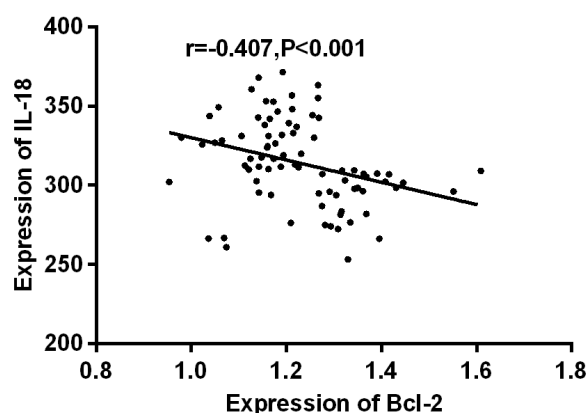


Fig. 3. The correlation between the expression of IL-18 in the serum of lung cancer and the protein expression of Bcl-2. Pearson correlation analysis was used and the result showed that the expression level of IL-18 in the serum of lung cancer was negatively correlated with the protein expression level of Bcl-2 in patients' tissue ($r = -0.407$, $p < 0.001$).

site-specific phosphorylation on Tyr 216 residue. This phosphorylation activates the expression of factors associated with cancer such as cyclinD 1, and thus promote apoptosis and inhibit the growth of cells (33). Bcl-2 is an anti-apoptotic factor through mitochondrial pathway, thereby promoting tumorigenesis (34). According to a previous study, IL-18 could act on human myocardial microvascular endothelial cells, to reduce the production of anti-apoptotic protein bcl-2 by activating NF- κ B (35). Therefore, we speculated that during the occurrence and progression of lung cancer, IL-18 may perform cellular immunity by activating some key regulatory factors such as cyclinD 1 and NF- κ B to facilitate the apoptosis of tumor cells.

However, there are still deficits to be complemented in this study. Firstly, the sample size of the study is not large enough. Secondly, the relationship between the expression levels of CEA, NSE and IL-18 and the clinical staging of patients with lung cancer as well as the classification of tumor cases should be further investigated. The relationship between the expression levels of each factor and the relevant aspects of tumors can be further investigated, and the correlation can be specifically analyzed. Finally, in terms of the relationship between the three factors and the protein expression of Bcl-2, a clearer animal model needs to be set up and the specific mechanism needs to be analyzed in detail. Due to the limited conditions, we did not have data on the interaction between CEA, NSE, IL-18. We will try to solve this problem in future studies.

In summary, the expression levels of CEA, NSE and IL-18 in the serum of patients in the lung cancer group were higher than those in the benign lesion group and the normal healthy group. IL-18, which may play a part in the pathogenesis of lung cancer, was negatively correlated with the protein expression of apoptosis gene Bcl-2. The detection of CEA, NSE and IL-18 in serum has certain diagnostic value for lung cancer, and the combined detection of the three factors can improve the sensitivity and specificity in the diagnosis of lung cancer, therefore are worthy of promotion in clinical practice.

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Remifentanyl protects neurological function of rats with cerebral ischemia-reperfusion injury via NR2B/CaMKII α signaling pathway

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The effects of remifentanyl were studied on cerebral ischemia-reperfusion injury (IRI) in rats, and its regulatory effect was explored on the N-methyl D-aspartate receptor subtype 2B (NR2B)/calcium/calmodulin-dependent protein kinase type II subunit alpha (CaMKII) signaling pathway in cerebral tissues. A total of 40 Sprague-Dawley rats were randomly assigned into sham group, model group, low-dose group (remifentanyl injected into the caudal veins at 2 μ g/kg) and high-dose group (remifentanyl injected into the caudal veins at 10 μ g/kg). Then, in the model, low-dose and high-dose groups, the rat model of cerebral IRI was established through middle cerebral artery occlusion (namely, ischemia for 1 h and reperfusion for 2 h), while no thread was inserted into the rats in the sham group. Neurological function of rats in each group was evaluated, and the cerebral infarct size was measured using 2,3,5-triphenyltetrazolium chloride (TTC) staining. Moreover, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to determine the neuronal apoptosis level, and mitochondrial membrane potential was measured via JC-1 assay. Moreover, the reactive oxygen species (ROS) level in neurons and the malondialdehyde (MDA) content in cerebral tissues were determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay and enzyme-linked immunosorbent assay (ELISA), respectively. Finally, the expression levels of apoptosis-associated proteins and the NR2B/CaMKII α signaling pathway-associated proteins in cerebral tissues were measured by Western blotting. Remifentanyl preconditioning substantially decreased the neurological score of rats ($p < 0.01$), cerebral infarct size ($p < 0.01$), neuronal apoptosis level ($p < 0.01$), ROS level in neurons ($p < 0.01$), MDA content ($p < 0.01$) and expression levels of cysteinyl aspartate specific proteinase-3 (Caspase-3), NR2B, phosphorylated CaMKII α (p-CaMKII α) and p-cAMP responsive element binding protein (p-CREB) ($p < 0.01$), but it increased the mitochondrial membrane potential ($p < 0.01$) and B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) ratio ($p < 0.01$). Remifentanyl can repress the NR2B/CaMKII α signaling pathway in the neurons of rats with cerebral IRI to decrease the p-CREB expression, ROS level and MDA content in neurons, neuronal apoptosis level and cerebral infarct size, and increase the mitochondrial membrane potential, thereby protecting the neurological function.

Key words: remifentanyl; cerebral ischemia-reperfusion injury; apoptosis; rats; NR2B/CaMKII α signaling pathway

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Stroke is a disease where cerebrovascular rupture or blockage induces loss of cerebral blood flow to damage cerebral tissues, and it falls into the categories of hemorrhagic stroke, ischemic stroke and subarachnoid hemorrhage, becoming the 2nd leading cause of death worldwide (1, 2). With the highest morbidity rate, ischemic stroke occurs in nearly 90% of stroke patients, and the blood flow in the ischemic zone can be restored through thrombolysis or thrombectomy, thereby alleviating cerebral tissue injury caused by ischemia and hypoxia (3). Ischemia-reperfusion injury (IRI) refers to the cerebral tissue injury aggravated when the blood-brain barrier is destroyed due to reperfusion in the ischemic zone, resulting in a poor prognosis of stroke patients (4). According to the study of Chen et al. (5), oxygen free radicals play vital roles in cerebral IRI, and their aggregation can contribute to mitochondrial apoptosis and neuroinflammation, thus inducing neuronal apoptosis. N-methyl D-aspartate receptor subtype 2B (NR2B), a participant in neuronal transmembrane signaling, can mediate extracellular Ca^{2+} entry into cells, thereby playing an important role in the regulation of neuronal apoptosis (6). In the case of an increase in intracellular Ca^{2+} concentration or aggravation of peripheral stimulation, the phosphorylation level of calcium/calmodulin-dependent protein kinase type II subunit alpha (CaMKII α), a multi-functional calmodulin-dependent protein kinase, will be prominently raised, thereby elevating the phosphorylation level of the downstream cAMP responsive element-binding proteins (CREBs) (7). Remifentanyl is a novel opioid receptor agonist characterized by quick onset of action and potent pharmacological action, which is often used as an anesthetic in clinics (8). Li et al. (9) researched and found that remifentanyl significantly prevents myocardial IRI by the mechanism of action that may be closely associated with the activation of the PI3K/Akt signaling pathway. There have been few studies on the influence of remifentanyl on the NR2B/CaMKII α signaling pathway and cerebral IRI, therefore the rat model of cerebral IRI was established in this study to explore the protective effect of remifentanyl against cerebral IRI and its regulatory effect on the NR2B/CaMKII α signaling pathway.

MATERIALS AND METHODS

Animals and grouping

A total of 40 male 3-4-month-old Sprague-Dawley rats weighing 280-320 g were purchased from the Animal Core Facility of Nanjing Medical University and then bred in a specific pathogen-free room with the humidity of $(45\pm 2)\%$ at $(22\pm 3)^{\circ}\text{C}$ under a circadian rhythm. They had free access to food and water and adapted to the environment for 7 d. At 12 h prior to modeling, the rats were deprived of food, then they were randomly divided into sham group (n=10), model group (n=10), low-dose group (n=10) and high-dose group (n=10).

The cerebral IRI model was constructed in the model, low-dose and high-dose groups. At 30 min preoperatively, remifentanyl (Sigma, St. Louis, MO, USA) was injected into the caudal veins at $2\text{ }\mu\text{g/kg}$ in the low-dose group and at $10\text{ }\mu\text{g/kg}$ in the high-dose group (10), while an equal volume of normal saline was injected in both the sham group and model group.

This study was approved by the Animal Ethics Committee of Xingtai People's Hospital and the experiments were undertaken as specified in the NIH Guide for the Care and Use of Laboratory Animals.

Establishment of rat model of cerebral IRI

The rats were first anesthetized by intraperitoneally injecting 2% pentobarbital sodium (Sigma, St. Louis, MO, USA) at a dose of 45 mg/kg . Then they were fixed on an operating table in a supine position and depilated at the neck, and the skin was cut open along the posterior midline of the neck to separate the cervical muscles. Subsequently, the cervical glands were pushed aside using ophthalmic forceps, and the common carotid artery, external carotid artery and internal carotid artery were bluntly separated, during which the vagus nerve was protected from being damaged. Afterwards, the common carotid artery was ligated by size 5-0 surgical sutures, the internal carotid artery was clamped by an arterial clamp, and a small incision was made at the distal end of the external carotid artery, from which a 6-0 nylon thread (Beijing Cinontech Co., Ltd., Beijing, China) was inserted to block the external carotid artery. With the arterial clamp released, a nylon thread was inserted from the common carotid artery into the internal carotid artery, and fixed using surgical sutures. After that, the skin was sutured,

and 1 h later, the thread was pulled out, and the wound was disinfected. After 2 h, the rats were sacrificed.

Evaluation of neurological function

After ischemia-reperfusion for 2 h, the neurological function in each group of rats was evaluated based on the Zea-Longa scoring system. Specifically, 4 points indicate that rats die or cannot walk with disturbances of consciousness, and 3 points represent that rats tumble towards the contralateral side of ischemia while walking. Rats that walk in circles towards the contralateral side of ischemia are scored 2 points, those able to walk but are unable to freely extend the forepaw on the contralateral side of ischemia are rated 1 point, and those able to move normally are scored 0 points.

Measurement of cerebral infarct size in rats

Upon 2 h of ischemia-reperfusion, the rats in each group were immediately sacrificed, and their cerebral tissues were isolated and rinsed by pre-cooled phosphate buffered saline (PBS) to remove blood. The cerebral tissues were then cryopreserved at -20°C for 20 min and sliced into 2 mm-thick sections in coronal plane using scalpels. Next, the cerebral tissue sections were incubated with freshly prepared 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Shanghai Sangon Biotech Co., Ltd., Shanghai, China) at 37°C in the dark for 20 min, during which they were constantly turned over for homogeneous staining. After that, the sections were taken out, and the liquid was blotted up. The resulting sections were fixed in 4% paraformaldehyde at 4°C overnight. Finally, the cerebral tissue sections were photographed, and the cerebral infarct size was calculated based on the formula: (normal cerebral tissues are stained red, while the cerebral infarct zone is white).

$$\text{Infarct size (\%)} = \frac{\text{Contralateral cerebral area} - \text{ischemic non-infarct area}}{\text{Contralateral cerebral area}} \times 100\%$$

Determination of neuronal apoptosis level in rats

After ischemia-reperfusion for 2 h, each group of rats was sacrificed immediately, and the cerebral tissues were isolated to prepare paraffin-embedded sections. Then, the neuronal apoptosis level was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining strictly according to the

instructions of the TUNEL kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). To be specific, the cerebral tissue sections were first added dropwise with proteinase K solution, digested at 37°C for 10 min and washed with PBS for 3 times. Then, the mixture containing TdT and DIG-d-UTP was added dropwise into the sections, and they were incubated at 4°C for 2 h and washed again using PBS for 3 times. The resulting sections were sealed with blocking buffer added in drops at room temperature for 30 min and cleaned using PBS for 3 times. Subsequently, biotinylated anti-digoxigenin antibody (1:100) was added dropwise to incubate the sections at 37°C for 40 min. After rinsing by PBS for 3 times, the sections were added with SABC-FITC-labeled secondary antibody (1:100) in droplets, incubated at 37°C for 40 min and washed using PBS for 3 times. Finally, the sections were sealed with anti-fade fluorescence mounting medium in drops and photographed under a confocal laser scanning microscope, and the neuronal apoptosis level therein was calculated. Apoptotic cells are positive for TUNEL staining, with yellowish green fluorescence, while normal cells are negative for TUNEL staining, with no fluorescence.

Determination of mitochondrial membrane potential in the neurons of rats

The cerebral tissues first isolated from the rats sacrificed immediately after 2 h of ischemia-reperfusion were made into paraffin-embedded sections. Then, the mitochondria were extracted from the cerebral neurons of rats in each group using the mitochondrion extraction kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) in strict accordance with the instructions. The neuronal mitochondrial membrane potential was measured using the mitochondrial membrane potential assay kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) as follows: First, 900 µL of JC-1 working solution diluted 5-fold was added into 100 µL of purified mitochondria, and the mixture was then added dropwise with anti-fade fluorescence mounting medium and observed under the confocal laser scanning microscope, with the excitation wavelength and emission wavelength adjusted to 485 nm and 500 nm, respectively. Red fluorescence represents normal mitochondrial membrane potential, while green fluorescence indicates collapse of mitochondrial membrane potential.

Measurement of reactive oxygen species (ROS) level and malondialdehyde (MDA) content in cerebral tissues of rats

In the measurement of ROS level, each group of rats was sacrificed immediately after ischemia-reperfusion for 2 h, and the cerebral tissues were isolated. Subsequently, the level of ROS in the cerebral tissues was determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA) strictly according to the instructions of the ROS assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Finally, the cerebral tissues were photographed using the confocal laser scanning microscope at the excitation wavelength of 488 nm and the emission wavelength of 525 nm.

For the measurement of MDA content, the cerebral tissues isolated from the rats were prepared into 1 mm³ blocks. Then, they were added with a moderate volume of normal saline and homogenized using an ultrasonic homogenizer until there was no macroscopic tissue debris. Subsequently, the content of MDA in the cerebral tissues was measured strictly as specified in the MDA assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Specifically, the absorbance at 532 nm in each group of samples was determined using a microplate reader and then substituted into the standard curve. Finally, the content of MDA in each group of sample solution was obtained.

Detection of relevant protein expressions via Western blotting

Following 2 h of ischemia-reperfusion, the rats were immediately sacrificed, and their cerebral tissues were extracted, cut into 1 mm³ blocks and added with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China), 1% phosphatase inhibitor and 1% protease inhibitor, followed by ultrasonic homogenization. The resulting cerebral tissues were centrifuged at 1,200 rpm and 4°C for 10 min, and the supernatant was harvested to determine protein concentration in each group using the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). To be specific, the loading buffer system with an equal concentration was first prepared through adding an appropriate volume of loading buffer into protein samples in each group, heated at 95°C for 10 min, and subjected to electrophoresis at the constant voltage of 80 V. Then, the proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at the constant voltage of 90 V for 100 min, and the membranes were blocked using

the freshly prepared 5% skim milk powder at room temperature for 1 h. Subsequently, the target bands were exercised based on their molecular weight and incubated with antibodies against cysteinyl aspartate specific proteinase-3 (Caspase-3), B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), NR2B, CaMKII α , phosphorylated CaMKII α (p-CaMKII), p-CREB, CREB and GAPDH (Cell Signaling Technology, USA, 1:1000) at 4°C overnight. Later, the bands were washed by tris-buffered saline with Tween-20 (TBST) for 3 times and incubated with the horseradish peroxidase-conjugated secondary antibodies (Beyotime Biotechnology Co., Ltd., Shanghai, China, 1:5000) at room temperature for 2 h. After rinsing with TBST for 3 times, the bands were added with freshly prepared developer for image development and exposure in a darkroom, followed by scanning. Finally, the expression levels of the corresponding proteins in the cerebral tissues were calculated.

Statistical analysis

The data in this study were presented as mean $\pm$ standard deviation, and processed using Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance was adopted for comparisons among groups, the data conforming to heterogeneity of variance were subjected to Welch's *t*-test, and the Bonferroni method was utilized for pairwise comparisons of the data meeting homogeneity of variance. $p < 0.05$ denoted that the differences were statistically significant.

RESULTS

Neurological score of rats

Following ischemia-reperfusion, the neurological function of rats was scored in each group. According to the results (Fig. 1), the neurological score of rats in the model group was prominently higher than that in the sham group ($p < 0.01$), while the rats in the low-dose and high-dose groups had a remarkably lower neurological score than those in the model group ($p < 0.05$, $p < 0.01$).

Measured cerebral infarct size

After ischemia-reperfusion, the cerebral infarct size in rats was measured *via* TTC staining, and

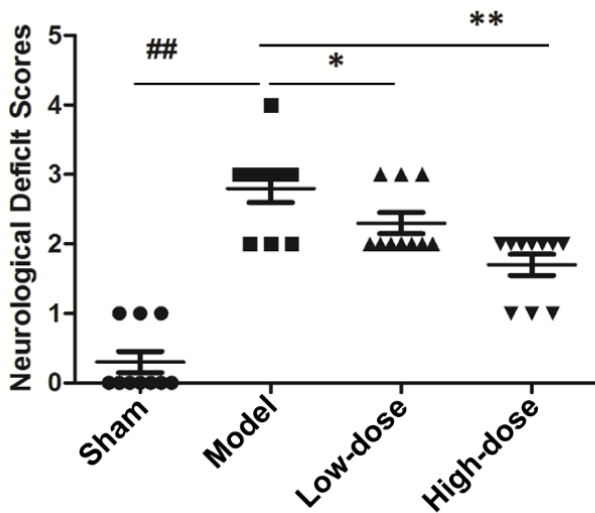


Fig. 1. Neurological score of rats in each group. The neurological score of rats in model group is dramatically higher than that in sham group, while it is considerably lower in low-dose and high-dose groups than that in model group ($^{##}p<0.01$ vs sham group, $^{*}p<0.05$ and $^{**}p<0.01$ vs model group).

it was found that in rats in the model group it was markedly larger than that in the sham group ($p<0.01$), and the low-dose and high-dose groups exhibited a prominently smaller cerebral infarct size than the model group ($p<0.05$, $p<0.01$) (Fig. 2).

Neuronal apoptosis level

The neuronal apoptosis level was determined in rats undergoing ischemia-reperfusion, and the results revealed that the neuronal apoptosis level in the model group was prominently elevated compared with that in sham group ($p<0.01$), whereas it was notably lowered in the low-dose group and high-dose group in comparison with that in the model group ($p<0.01$) (Fig. 3).

Detected neuronal mitochondrial membrane potential

After ischemia-reperfusion, the neuronal mitochondrial membrane potential was detected in rats. Based on the results (Fig. 4), the neuronal mitochondrial membrane potential in the model group was prominently decreased in contrast with that in the sham group ($p<0.01$), while the low-dose and high-dose groups displayed evidently higher neuronal mitochondrial membrane potential than the model group ($p<0.01$).

Determined ROS level and MDA content in the cerebral tissues

The ROS level and MDA content in the cerebral tissues were measured following ischemia-reperfusion, and it was discovered that the model group had a notably higher ROS level and MDA

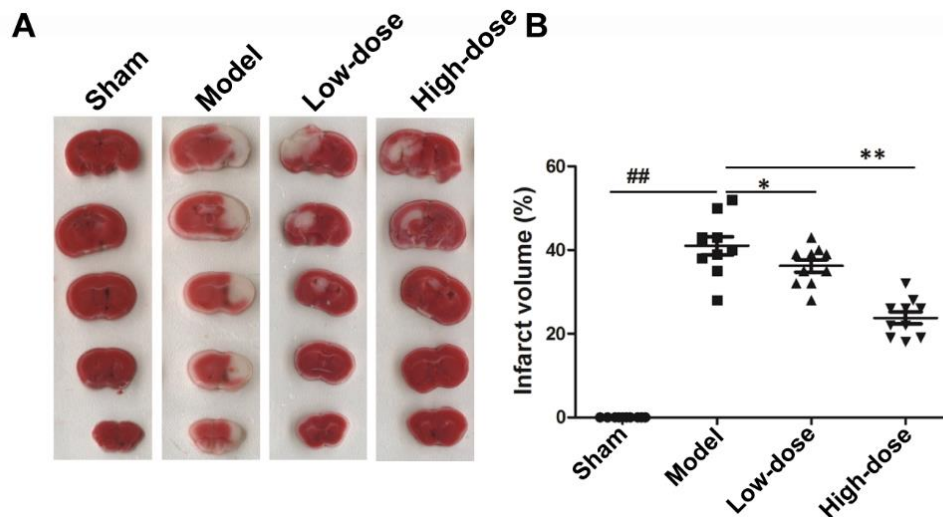


Fig. 2. Cerebral infarct size of rats. **A)** Stained cerebral tissue sections; **B)** Statistical graph of cerebral infarct size. Model group shows a considerably larger cerebral infarct size than sham group, whereas low-dose group and high-dose group exhibit a notably smaller cerebral infarct size than model group ($^{##}p<0.01$ vs sham group, $^{*}p<0.05$ and $^{**}p<0.01$ vs model group).

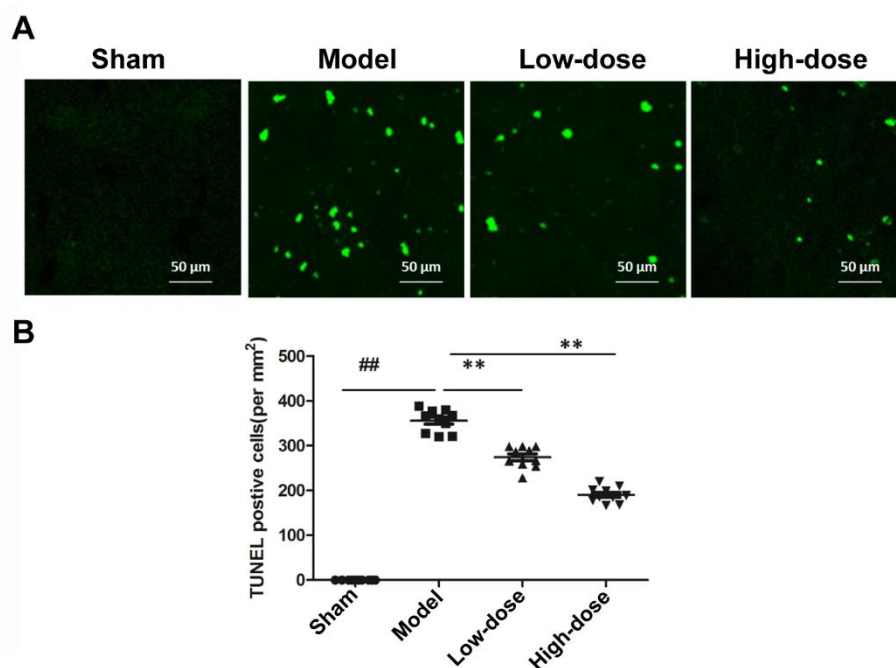


Fig. 3. Neuronal apoptosis level determined via TUNEL staining. **A)** Micrograph of TUNEL staining, (magnification: 400 $\times$); **B)** Statistical graph of apoptotic cell count (Scale bar = 50 μ m). The number of apoptotic neurons is greatly raised in model group compared with that sham group, in low-dose group and high-dose group (^{##} $p < 0.01$ vs sham group, ^{**} $p < 0.01$ vs model group).

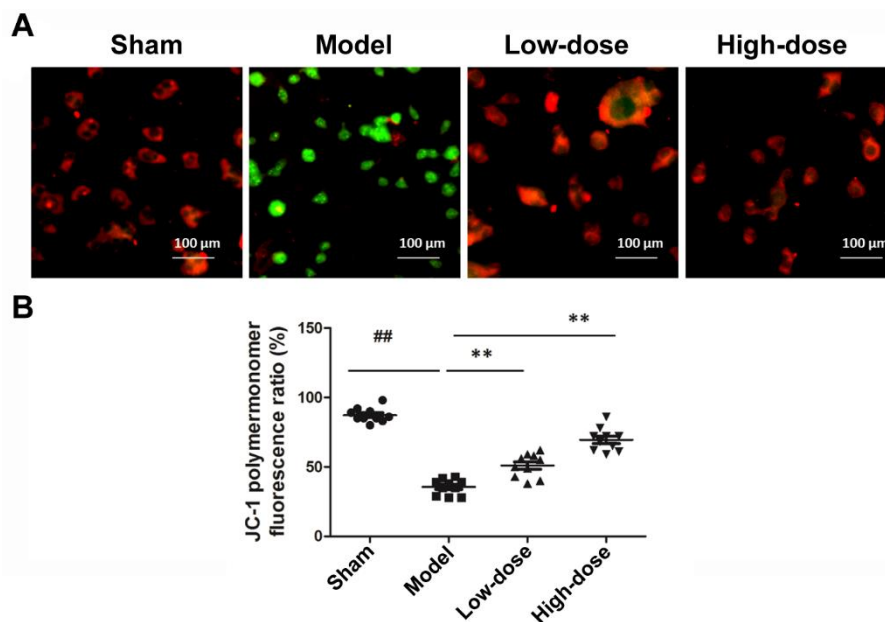


Fig. 4. Neuronal mitochondrial membrane potential in rats determined. **A)** Micrograph, (magnification: 400 $\times$). **B)** Statistical graph of mitochondrial membrane potential (Scale bar = 100 μ m). The neuronal mitochondrial membrane potential in model group is considerably lower than that in sham, low-dose and high-dose groups (^{##} $p < 0.01$ vs sham group, ^{**} $p < 0.01$ vs model group).

content in the cerebral tissues than sham group ($p < 0.01$), whereas the level of ROS and the content of MDA in the cerebral tissues in low-dose and high-dose groups were remarkably lower than those in the model group ($p < 0.01$) (Fig. 5).

Examined apoptosis-associated proteins in the cerebral tissues

The expression levels of apoptosis-associated proteins in the cerebral tissue were determined in rats after ischemia-reperfusion. It was found that the

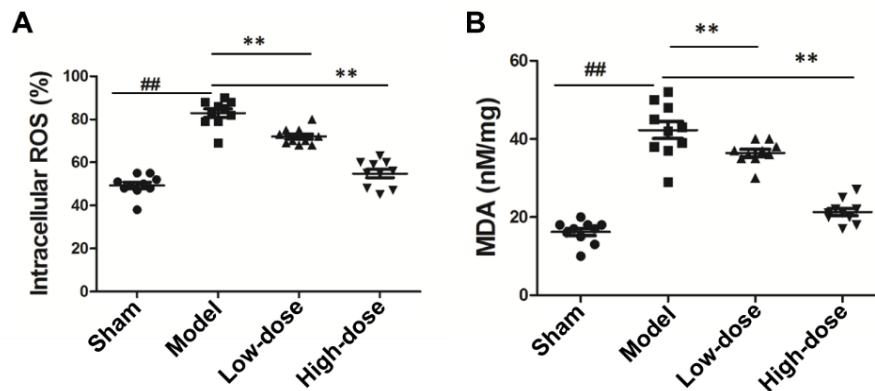


Fig. 5. Determined ROS and MDA content in the cerebral tissues of rats. **A)** ROS level; **B)** MDA content. The ROS level and MDA content in the cerebral tissues of rats in model group are distinctly elevated in contrast with those in sham, low-dose and high-dose groups ($^{##}p < 0.01$ vs sham group, $^{**}p < 0.01$ vs model group).

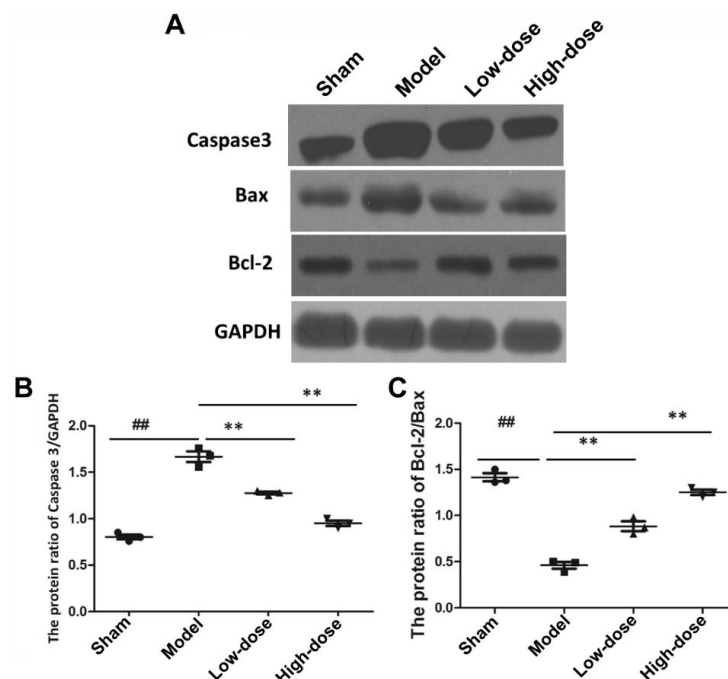


Fig. 6. Expression levels of apoptosis-associated proteins in the cerebral tissues of rats detected by Western blotting. **A)** Protein bands; **B)** Caspase-3 expression level; **C)** Bcl-2/Bax ratio. Model group exhibits a considerably higher expression level of Caspase-3 and a markedly lower Bcl-2/Bax ratio in the cerebral tissues of rats than sham group, low-dose group and high-dose group ($^{##}p < 0.01$ vs sham group, and $^{**}p < 0.01$ vs model group).

expression level of Caspase-3 in the model group was substantially higher ($p<0.01$), but the Bcl-2/Bax ratio was prominently lower ($p<0.01$) than those in the sham group, while the low-dose and high-dose groups exhibited a remarkably lower expression level of Caspase-3 ($p<0.01$) and a markedly higher Bcl-2/Bax ratio ($p<0.01$) than the model group (Fig. 6).

Expression levels of the NR2B/CaMKII α signaling pathway-associated proteins in cerebral tissues

After ischemia-reperfusion, the expression levels of the NR2B/CaMKII α signaling pathway-associated proteins in the cerebral tissues of rats were measured, and the results manifested that

the expression levels of NR2B, p-CaMKII α and p-CREB in the model group were considerably higher than those in sham group ($p<0.01$), whereas such expression levels declined remarkably in the low-dose and high-dose groups compared with those in the model group ($p<0.01$) (Fig. 7).

DISCUSSION

Cerebral IRI refers to the further damage to cerebral tissues after the blood supply to the cerebral tissues is restored following cerebral ischemia by means of surgery or medication, which seriously affects the neurological function (11). In this

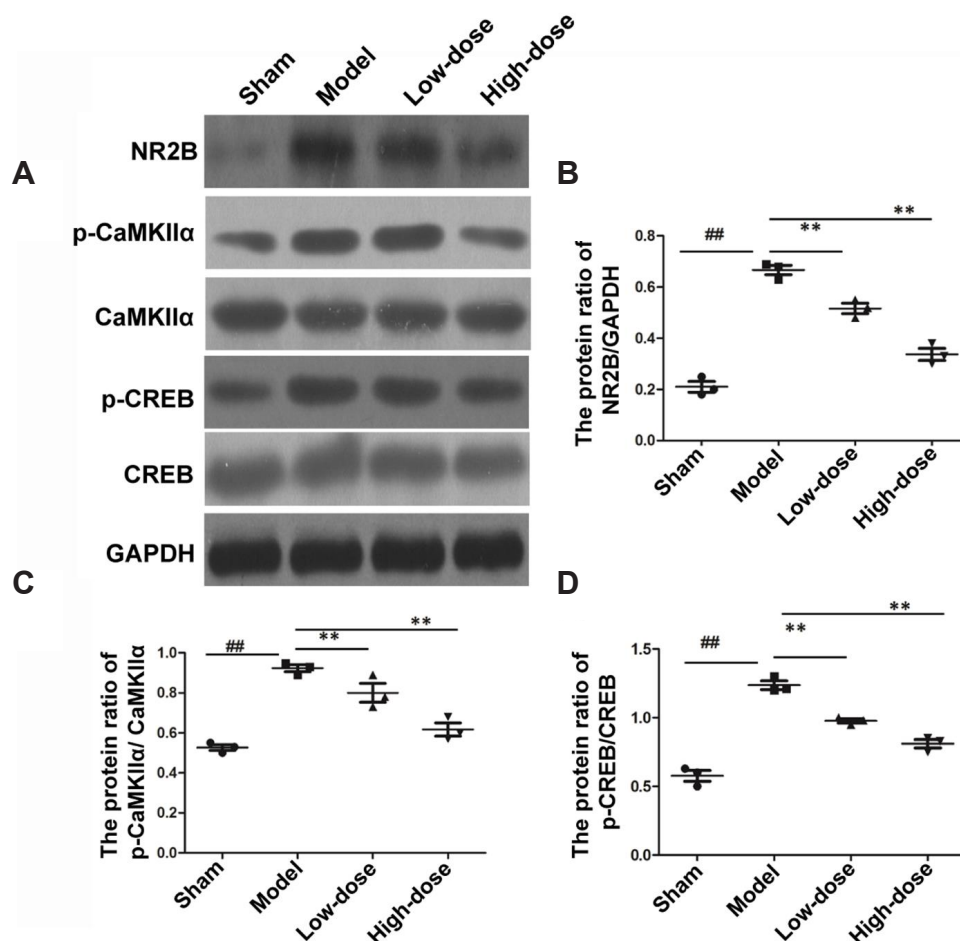


Fig. 7. Expression levels of the NR2B/CaMKII α signaling pathway-associated proteins in the cerebral tissues of rats measured via Western blotting. **A)** Protein bands; **B)** Expression level of NR2B; **C)** Expression level of p-CaMKII α ; **D)** Expression level of p-CREB. Model group has dramatically increased expression levels of NR2B, p-CaMKII α and p-CREB in the cerebral tissues of rats in comparison with sham group, low-dose group and high-dose group ($^{\#}p<0.01$ vs sham group, $^{**}p<0.01$ vs model group).

study, it was discovered that cerebral IRI gave rise to severe neurological impairment, substantially enlarged the cerebral infarct size, induced massive neuronal apoptosis, notably increased the ROS level in neurons and prominently decreased the mitochondrial membrane potential. Large numbers of studies have proven that such short-acting opioids as remifentanyl can protect the liver and kidneys against IRI (12, 13). In the present study, the rats undergoing cerebral ischemia-reperfusion were pre-treated with remifentanyl, and it was revealed that remifentanyl had an obvious protective effect against cerebral IRI in rats. Moreover, it substantially reduced the cerebral infarct size and the neuronal apoptosis and ROS levels in neurons, increased the mitochondrial membrane potential, and improved the neurological function of rats after ischemia-reperfusion.

Oxidative phosphorylation is discontinued in hypoxic mitochondria, resulting in massive lactic acid buildup. Once the oxygen is restored, the mitochondrial respiratory chain will be rebooted to produce plenty of ROS, which gives rise to imbalance between oxidation and antioxidation in cells, causes mitochondrial swelling and reduces the membrane potential, ultimately triggering the dysfunction and cell apoptosis (14). Owing to cerebral ischemia-reperfusion, massive ROS are generated in the cerebral tissues to interact with and damage intracellular deoxyribonucleic acids (DNAs), and if the DNA damage fails to be repaired, the intracellular apoptosis-associated signaling pathways will be activated, thereby inducing central neuronal apoptosis (15). Such DNA damage, milder than DNA double-strand break, can be reversed and repaired through early intervention (16). Shao et al. (17) studied and discovered that the Bcl-2 and Caspase-3 family proteins downstream of p53 can be activated by large numbers of ROS in the myocardial cells of the rats with myocardial infarction, thus causing substantial increase in apoptotic myocardial cells, and that the early intervention for reducing intracellular ROS level can effectively protect the myocardial cells. Muñoz et al. (18) found that the knockout of NADPH oxidase 1 (NOX1) in the cerebral tissues of mice can significantly decrease the middle cerebral artery occlusion-induced production

of ROS, thus effectively alleviating the neurological impairment in mice, which is consistent with the results of this study.

One study proved that local anesthetics, such as remifentanyl and lidocaine, can not only block sodium channels, but also affect Ca^{2+} transport across synaptic gaps (19). The study of Jang et al. (20) demonstrated that the down-regulation of NR2B in cells can effectively repress glutamatergic signaling, especially the NMDA-mediated glutamatergic signaling, thereby substantially relieving neuronal injury caused by external stimuli. In the present study, it was found that cerebral IRI effectively activated the NR2B/CaMKII α signaling pathway in the neurons of rats, and increased the expression of NR2B to raise the expression level of p-CaMKII α , further facilitating the increase in the p-CREB expression level and damaging the neurons of rats. Based on the results of the study of Xie et al. (21), depression can considerably increase the level of p-CREB in neurons of mice to cause a notable decline in the expression levels of the downstream brain-derived neurotrophic factors, ultimately damaging the neurons. The results of the present study revealed that remifentanyl preconditioning substantially repressed the NR2B/CaMKII α signaling pathway in the neurons of rats with cerebral IRI, thereby attenuating IRI in neurons. In conclusion, the present study uncovered that remifentanyl preconditioning can suppress the NR2B/CaMKII α signaling pathway in the neurons of rats with cerebral IRI to reduce the p-CREB expression and ROS level in neurons, increase the mitochondrial membrane potential and decrease the cerebral infarct size, thereby improving the neurological function.

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Macrophages promote cell proliferation in colorectal cancer via IL-1 β -mediated downregulation of miR-28-3p

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Infiltration of macrophages is associated with tumor progression and poor prognosis in multiple malignancies, but the underlying mechanisms by which macrophages contribute to colorectal cancer (CRC) have not yet been elucidated. The purpose of this study was to discuss the potential mechanisms of macrophages in CRC. The MTT assay was used to assess cell viability. The expression of the proliferation-related marker PCNA was detected by Western blot analysis. The 10 most important factors (PDGF, VEGF, TNF α , bFGF, IL-8, TGF- β , IFN- γ , SPARC, IL-1 β and IL-6) secreted by macrophages were knocked down by RNA interference (RNAi), and the mRNA expression levels of these 10 factors were analyzed by qRT-PCR. The effect of these factors on cell proliferation was assessed by the MTT assay. The miRNAs regulated by IL-1 β in CRC cells were identified by miRNA microarray and qRT-PCR analyses. The proliferation ability of miR-28-3p inhibitor on CRC cells was detected by colony formation assay. The association of IL-1 β and miR-28-3p expression with the clinicopathological characteristics in patients with CRC was analyzed by TCGA RNA-seq data. As a result, macrophages promoted the proliferation of CRC cells in a time- and number-dependent manner, and these effects were associated with the upregulation of PCNA and the macrophage-secreted cytokine IL-1 β , which had the most significant effect on CRC cell proliferation. Furthermore, downregulation of miR-28-3p was induced by IL-1 β in CRC cells. The miR-28-3p inhibitor promoted the proliferation in CRC cells. Moreover, upregulation of IL-1 β expression or downregulation of miR-28-3p expression was associated with poor survival in patients with CRC. Therefore, these data demonstrated that macrophages promoted CRC cell proliferation via IL-1 β -mediated downregulation of miR-28-3p.

Colorectal cancer (CRC) is the third most common cancer worldwide with high incidence and mortality according to Global Cancer Statistics 2018 (1). The prognosis of CRC in late stage is

poor, with a 5-year survival rate less than 10% due to distant metastasis (2). Typical cancer therapies, such as surgery, chemotherapy and radiation, have made progress, however, tumor invasiveness and

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metastasis contribute to the low survival rate and increase the recurrence risk of CRC (3). Thus, the exact pathogenesis of CRC needs to be elucidated.

Cancer development is a multistep process with continuous mutations in oncogenes and tumor suppressor genes, resulting in the transformation of a normal cell into a tumor cell (4). In addition to alterations in tumor cells, the microenvironment is essential to drive the development of cancers. Cells in the immune system participate in the initiation of the malignancies (5). Immune cells that have infiltrated a tumor mass can create a microenvironment by producing cytokines, chemokines, growth factors, and angiogenic factors, thereby promoting tumor progression (6, 7).

Macrophages are innate immune cells that populate all tissues and have various roles in development, homeostasis and tissue repair (8). At the early stage of tumor onset, the immune system reacts to the presence of cancer by activating macrophages and T cells, which reduce the incidence of cancer (9). However, after the initial state, the immune microenvironment is modified to promote the tumor growth (10). Accumulating evidence indicates that macrophages are abundant in most tumor types and facilitate tumor growth, angiogenesis, metastasis and immunosuppression in multiple cancers (11-13). Moreover, macrophages produce pro-inflammatory mediators, such as IL-6, tumor necrosis factor (TNF) and interferon- γ (IFN- γ), as well as growth factors, proteases, reactive oxygen species (ROS) and nitrogen species involved in tumor progression (14, 15). Therefore, targeting tumor-associated macrophages may provide a new strategy for cancer treatment.

MicroRNAs (miRNAs) are small, single-stranded noncoding RNAs that regulate gene transcription and translation in a sequence-specific manner (16). miR-21 and miR-92a have been investigated in serum from patients with CRC and adenoma (17). miR-431, miR-15b, and miR-139-39p can be used to distinguish stage IV CRC (18). In the present study, macrophages promoted the proliferation of CRC cells in a time- and number- dependent manner, and these effects were associated with the upregulation of PCNA and the macrophage-secreted cytokine IL-1 β . Furthermore, miR-28-3p downregulation

was induced by IL-1 β in CRC cells. Upregulation of IL-1 β expression or downregulation of miR-28-3p expression was associated with poor survival in patients with CRC.

MATERIALS AND METHODS

Clinical data

The clinicopathological data of 27 paired CRC and 248 unpaired CRC tissue samples as well as the relative expression levels of IL-1 β and miR-28-3p were downloaded from The Cancer Genome Atlas (TCGA) RNA sequencing database (<https://genome-cancer.ucsc.edu>).

Cell culture

Cells were stored at the Digestive Disease Laboratory of Shanghai Sixth People's Hospital. Human CRC cell lines (HCT116, SW480) and macrophages (MD, CRL-9850) were stored at the laboratory. Cells were cultured in a humidified incubator with 5% CO₂ at 37°C in RPMI-1640 medium or Dulbecco's modified Eagle's medium (DMEM; KeyGen Biotech Co. Ltd) containing 10% fetal bovine serum (10% FBS). The protocols used in the study were approved by the Ethics Committee of Shanghai Sixth People's Hospital.

miRNA inhibitors and mimics

miR-28-3p inhibitors were synthesized by GenePharma (GenePharma, Shanghai, China). qRT-PCR analysis was used to evaluate the transfection efficiency of miR-134-5p inhibitor and mimics in CRC cells. The inhibitor and mimics of miR-28-3p were transfected with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sequence of miR-28-3p inhibitor was as follows: 5'-CUCAAUAGACUGUGAGCUCCUU-3'; microRNA NC: 5'-CAGUACUUUUG UGUAGUACAA-3'. The sequence of miR-28-3p mimics was as follows: 5'-CACUAGA UUGUGAGCUCCUGGA-3', 5'-CAGGAGCUCACAAUCUAGUGUU-3'; microRNA NC: 5'-UUCUCCGAACGUCUCACGUTT-3', 5'-ACGUGACACG UUCGGAGAATT-3'.

MTT assay

CRC cells (HCT116, 2×10^3 /100 μ l/well) were seeded into 96-well plates. Cell viability was assessed by MTT assay according to a previous report (19).

Colony formation assay

Briefly, CRC cells (HCT 116, SW480, 1×10^4) were plated into 6 cm dishes and cultured in a humidified incubator with 5% CO₂ at 37°C for 15 days. Colonies were then fixed for 20 min with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min. The number of colonies containing ≥ 50 cells was counted under a microscope. Experiments were performed three times.

Quantitative reverse transcription PCR (qRT-PCR) and miRNA microarray analysis qRT-PCR and miRNA microarray analyses were performed as previously

reported (19, 20). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal controls. All qPCR assays were performed in duplicate. The primer sequences are listed in Table 1.

Western blot analysis

Total protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a 0.22 μ m polyvinylidene difluoride membrane (Millipore, Billerica, MA). Transferred membranes were blocked with 5% nonfat milk for 1 h at room temperature followed by incubation with a PCNA specific antibody (Sigma-Aldrich, USA) according to the manufacturer's protocol. A β -actin antibody (Sigma-Aldrich, USA) was used as the control.

5-Ethynyl-20-deoxyuridine (EdU) incorporation assay

EdU assays were performed using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. A total of 5×10^3 cells (HCT116, SW480) which were incubated with macrophages or IL-1 β were seeded in each well of a 96-well plate. After incubation with 50 μ M EdU for 2 h, the cells were fixed in 4% paraformaldehyde and stained with Apollo Dye Solution. Hoechst-33,342 was used to stain the nucleic acids. Images were acquired using an Olympus IX73 microscope (Olympus, Tokyo, Japan), and the percentage of EdU-positive cells was calculated using Image J (National Institutes of Health, Bethesda, MD, USA).

RNAi

Antisense oligonucleotides for miRNAs and control RNAs were synthesized by Ribobio as previously reported (21, 22).

Statistical analysis

All quantitative data were presented as the mean $\pm$ SD. Statistical analysis and graphs were achieved using SPSS v.18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). Student's *t*-test was used to compare quantitative variables. The *Chi*-squared test and Fisher's exact test were used to compare categorical variables. Pearson's correlation coefficient analysis was used to analyze the correlations between IL-1 β and miR-28-3p expression in CRC tissues. Overall survival and recurrence curves

Table I. Primers used in qRT-PCR

RNA	Sequences
Hsa-miR-28-3p	5' -CACUAGAUUGUGAGCUCCUGGA- 3'
PDGF F:	GCAGTCAGATCCACAGCATC
R:	AAATGACCGTCCTGGCTTG
VEGF F:	GGGCAGAATCATCAVGAAGT
R:	ATCTGCATGGTGATGTTGGA
TNF α F:	CCTCTCTCTAATCATACGAAGT
R:	GAGGACCTGGGAGTAGATGAG
bFGF F:	AGAGCGACCCTCACATCAAG
R:	TCGTTTCAGTGCCAVATACC
IL-1 β F:	AGCTACGAATCTCCGACCAC
R:	CGTTATCCCATGTGTGCAAGAA
IL-8 F:	TTTTCGGAAGGAGTGCTAAAGA
R:	AACCCTCTGCACCCAGTTTTC
TGF- β F:	CTAATGGTGGAAACCCACAACG
R:	TATCGCCAGGAATTGTTGCTG
IFN γ F:	TTTGGGTTCTCTGGCTGTT
R:	TCTTTTGATGCTCTGGTCA
SPARC F:	GTGCAGAGGAAACCGAAGAG
R:	AAGTGGCAGGAAGAGTCGAA
IL-6 F:	AAAGAGGCACTGGCAGAAAA
R:	AGCTCTGGCTTGTTCTCAC

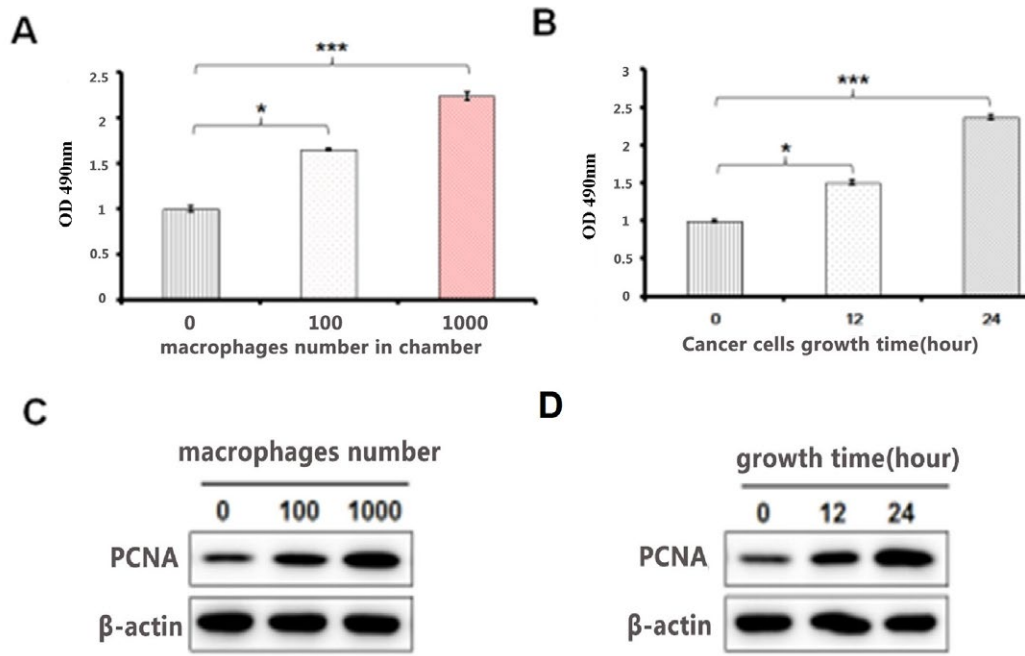


Fig. 1. Macrophages promote CRC cell proliferation. **A)** After 0, 100 and 1000 macrophages were co-cultured with tumor cells in the chamber, the cell vitality of CRC cells was examined by MTT assay. **B)** After 100 macrophages were co-cultured with tumor cells for 0, 12 and 24 h, the cell vitality of CRC cells was examined by MTT assay. **C)** PCNA expression changes were detected by Western blot analysis after treatment with different macrophage numbers. **D)** PCNA expression changes were detected by Western blot analysis after treatment with 100 macrophages at different time points. * $P < 0.05$, *** $P < 0.001$.

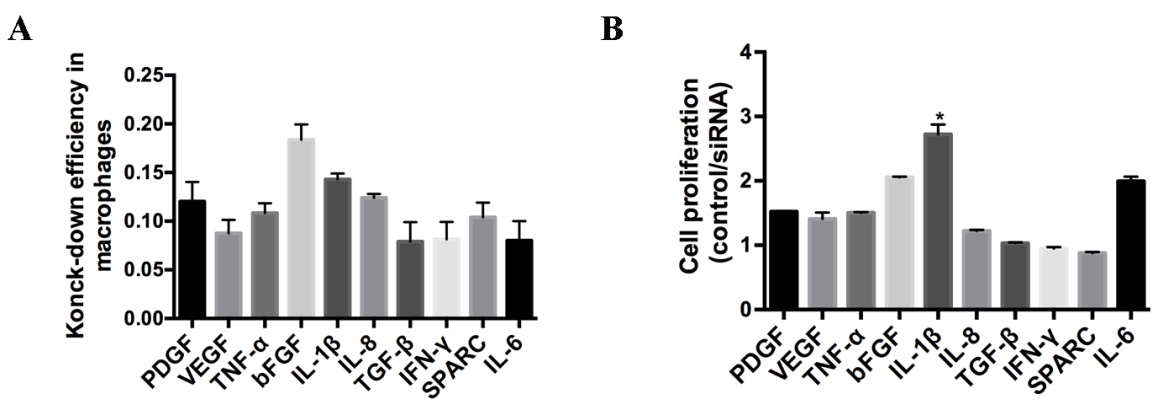


Fig. 2. **A)** The knockdown efficiency of the 10 most important factors (PDGF, VEGF, TNFα, bFGF, IL-1β, IL-8, TGF-β, IFNγ, SPARC and IL-6) in macrophages was detected by qRT-PCR. **B)** Cell proliferation (control/siRNA) in 10 factors knockdown. * $P < 0.05$.

were analyzed with the Kaplan-Meier method and log-rank test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Macrophages promote CRC cell proliferation

Tumor cells were inoculated at the bottom of the culture dish, and macrophages were then inoculated into the upper micropore chamber. Culture solution was then added for the internal fluid. When 100 macrophages were added, and the cell vitality of CRC cells after 12 h of culture was enhanced by macrophages in a number- and time-dependent manner as indicated by MTT assay (Fig. 1 A, B). The expression level of the cell proliferation marker, PCNA, as detected by Western blot analysis, was also significantly increased by macrophages in CRC

cells in a number- and time-dependent manner (Fig. 1 C, D). These results suggested that macrophages might promote the proliferation of CRC cells.

IL-1 β mediates macrophages to promote CRC cell proliferation

To identify the mechanisms by which macrophages regulate CRC cell proliferation, the 10 most important factors (PDGF, VEGF, TNF α , bFGF, IL-1 β , IL-8, TGF- β , IFN γ , SPARC and IL-6) were knocked down in macrophages by RNAi. Some studies have shown that these 10 factors are common factors secreted by macrophages and may be involved in the occurrence and development of tumors (23-25). The knockdown efficiency was confirmed by qPCR (Fig. 2A). After RNAi, the relative expression for the mRNA (PDGF, VEGF, TNF α , bFGF, IL-1 β , IL-8, TGF- β , IFN γ , SPARC and

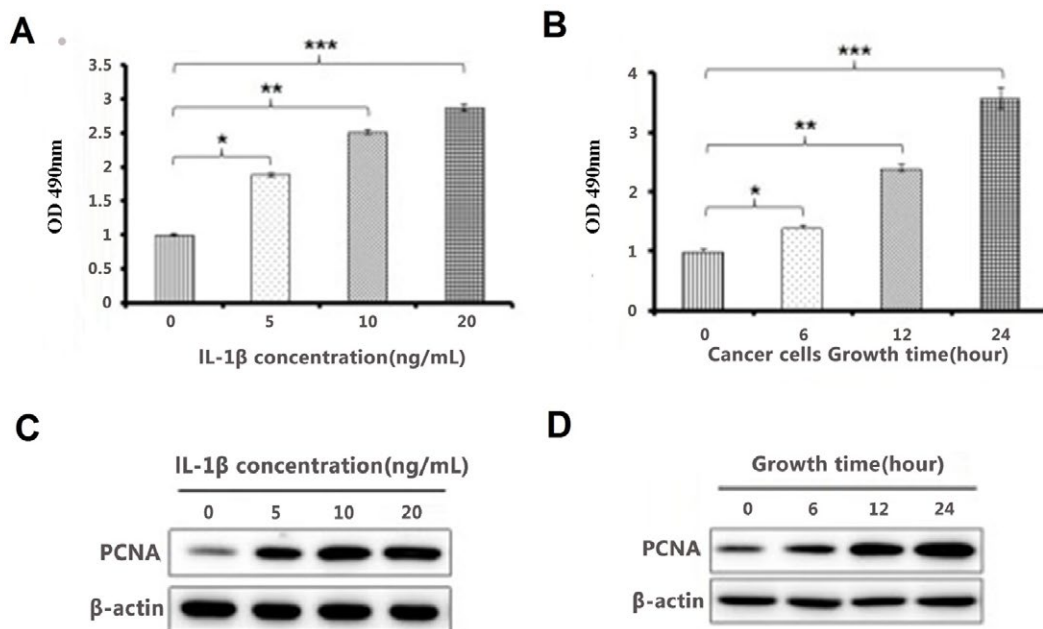


Fig. 3. *IL-1 β mediates macrophages to promote CRC cell proliferation. A)* After HCT116 cells were treated with different concentrations of IL-1 β , cell vitality was detected by MTT assay. *B)* After HCT116 cells were treated with IL-1 β at different time points, cell vitality was detected by MTT assay. *C)* PCNA expression was detected by Western blot analysis after treatment with different concentrations of IL-1 β . *D)* PCNA expression in tumor cells was detected by Western blot analysis after treatment with IL-1 β at different time points. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

IL-6) was detected (the default value for the control group is 1). IL-1 β knockdown resulted in an obvious inhibition of cell proliferation ($P < 0.05$), suggesting

that IL-1 β promoted macrophage cell proliferation (Fig. 2B). Different concentrations of IL-1 β were added to CRC cells, which were subjected to the

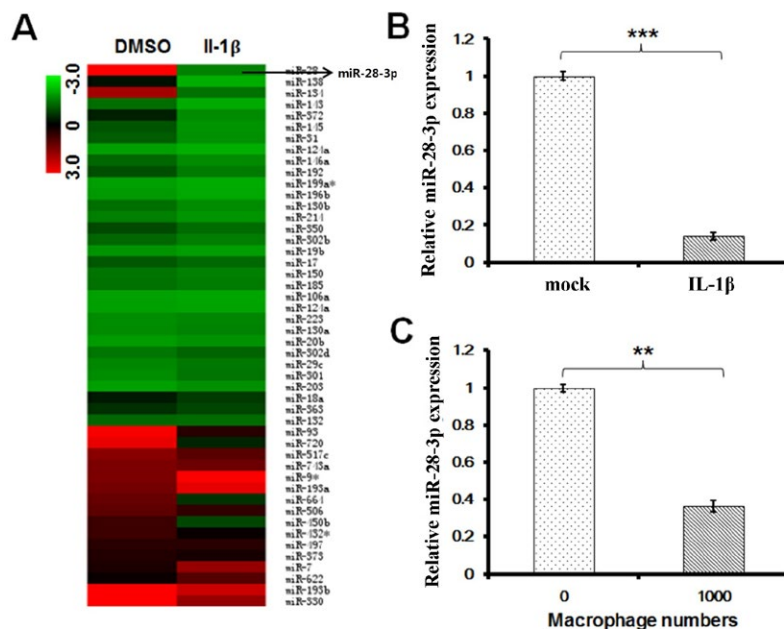


Fig. 4. IL-1 β induces the downregulation of miR-28-3p expression in CRC cells. **A)** After tumor cells were treated with IL-1 β , differentially expressed miRNAs were identified by an miRNA microarray. **B)** After HCT116 cells were treated with IL-1 β , the expression of miR-28-3p was detected by qRT-PCR. **C)** HCT116 cells were co-cultured with 1000 macrophages in a chamber for 12 h, and the expression of miR-28-3p was detected by qRT-PCR. ** $P < 0.01$, *** $P < 0.001$.

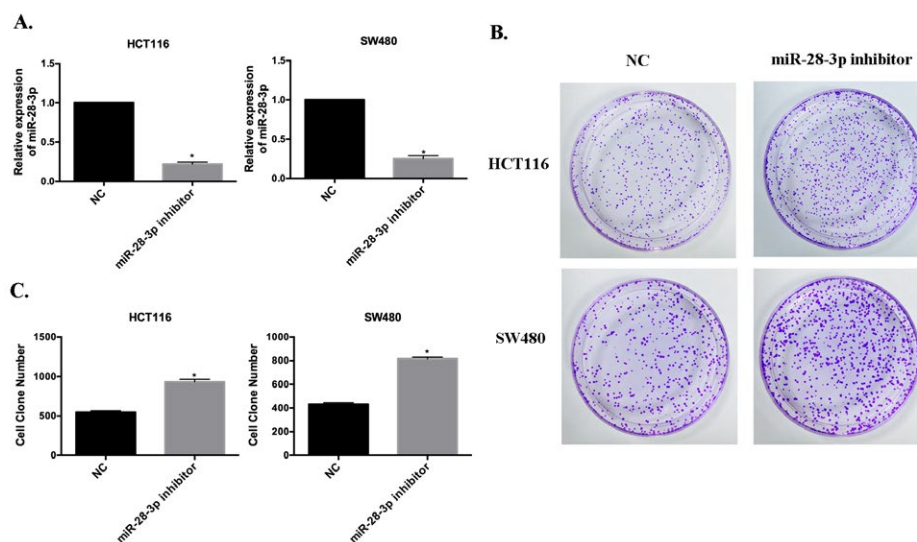


Fig. 5. miR-28-3p inhibitor promotes CRC cell proliferation. **A)** The knockdown efficiency of miR-28-3p in HCT116 and SW480 cells was confirmed by qPCR. **B, C)** Clone formation assays were conducted in HCT116 and SW480 cells transfected with miR-28-3p inhibitor. * $P < 0.05$.

MTT assay at 24h. The cell vitality of CRC cells was detected at different time points (0h, 6h, 12h, 24h) after CRC cells being treated with IL-1 β (10ng/ml). The cell vitality of CRC cells was increased in a dose- and time-dependent manner (Fig. 3 A, B). The expression level of PCNA, detected by Western blot analysis, was also significantly increased by IL-1 β in CRC cells in a dose- and time-dependent manner (Fig. 3 C, D).

IL-1 β decreases miR-28-3p expression in CRC cells

Previous studies showed that miRNAs play critical roles in the tumorigenesis of CRC (26). To confirm whether IL-1 β mediates miRNAs to regulate CRC cells, CRC cells were treated with IL-1 β (10 ng/mL)

for 12 h, and an miRNA microarray analysis was then performed. The microarray identified 37 upregulated miRNAs and 11 downregulated miRNAs, and miR-28-3p expression was substantially decreased in CRC cells compared to control cells (Fig. 4A). The expression level of miR-28-3p was further validated in HCT16 cells by qRT-PCR analysis, which indicated that the expression of miR-28-3p was decreased by IL-1 β (Fig. 4B) in CRC cells, and a similar result was confirmed in co-cultured macrophages and CRC cells (Fig. 4C).

miR-28-3p inhibitor promotes CRC cell proliferation

To evaluate the negative biological functions of miR-28-3p, miR-28-3p inhibitor was used to

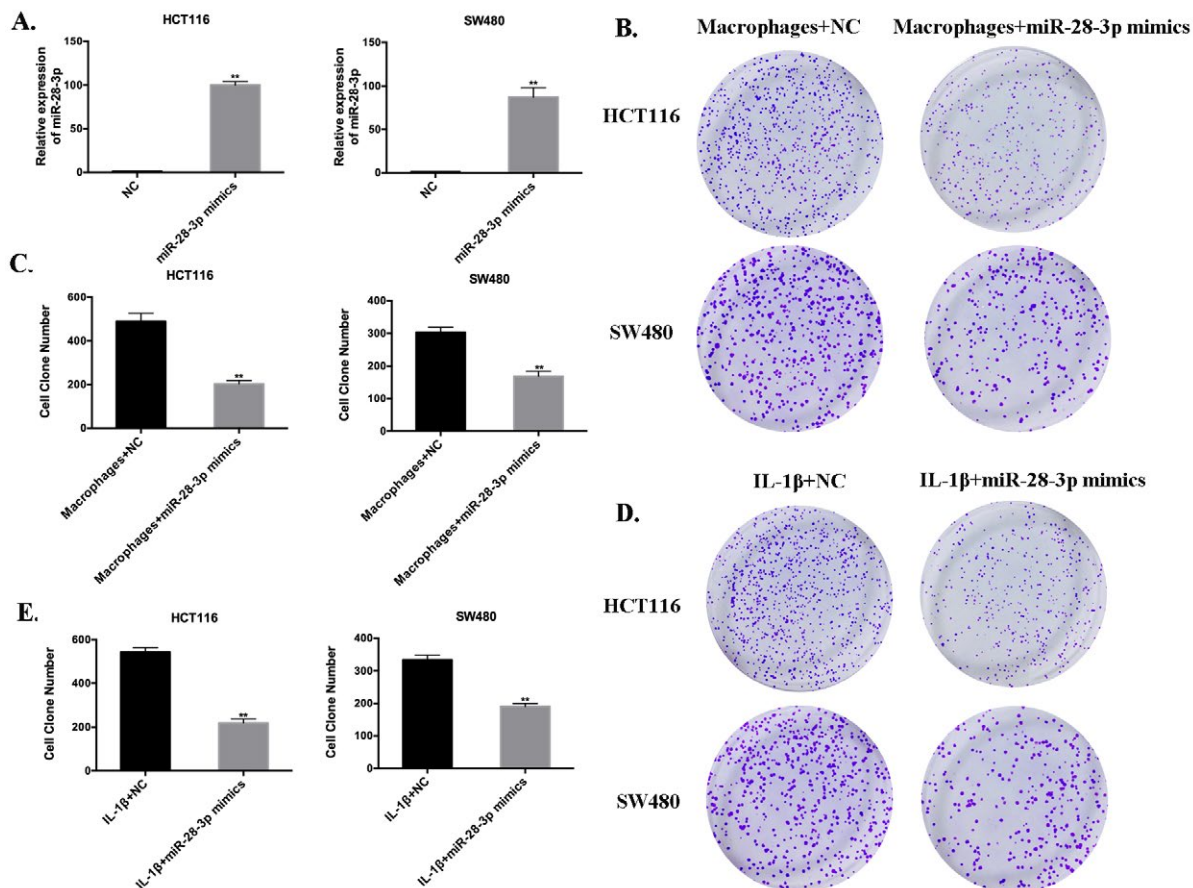


Fig. 6. miR-28-3p mimics inhibits CRC cell proliferation. **A)** The miR-28-3p expression level transfected with miR-28-3p mimics was confirmed by qPCR in HCT116 and SW480 cells. **B, C)** Clone formation assays were conducted in HCT116 and SW480 cells transfected with miR-28-3p mimics and co-incubated with macrophages (1000) for 24h. **D, E)** Clone formation assays were conducted in HCT116 and SW480 cells transfected with miR-28-3p mimics and co-incubated with IL-1 β (20ng/ml) for 24h. ** $P < 0.01$.

treat HCT116 and SW480 cells. Its knockdown efficiency was confirmed by qPCR (Fig. 5A). Clone formation assay revealed that miR-28-3p inhibitor promoted the colony-forming ability of HCT116 and SW480 cells (Fig. 5 B, C), which demonstrated that the downregulation of miR-28-3p promoted the proliferation of CRC cells.

miRNA-28-3p mimics inhibits CRC cell proliferation

Next, the biological functions of miR-28-3p were evaluated. The miR-28-3p expression level in HCT116 and SW480 cells transfected with miR-28-3p mimics was confirmed by qPCR (Fig. 6A). Clone formation assays were conducted after miR-28-3p mimics transfected HCT116 and SW480 cells were co-incubated with macrophages (1000) and IL-1 β (20ng/ml) for 24h. The results revealed that miRNA-28-3p mimics inhibited CRC cell proliferation (Fig. 6

B, C) and attenuated IL-1 β -induced cell proliferation (Fig. 6 D, E). Furthermore, the effects of macrophages or IL-1 β on DNA synthesis were determined by 5-ethynyl-2'-deoxyuridine (EdU) which showed that the activation of macrophages by IL-1 β favored CRC cell proliferation but miR-28-3p mimics reversed these effects in CRC cells (Fig. 7 A, B).

Expression of IL-1 β and miR-28-3p is associated with poor survival in patients with CRC

IL-1 β and miR-28-3p expression levels were detected in CRC tissues using TCGA data. Compared to adjacent normal tissues, IL-1 β expression was markedly increased in paired and unpaired CRC tissues (Fig. 8A), but miR-28-3p expression was dramatically decreased in paired and unpaired CRC tissues (Fig. 8B). Pearson correlation analysis showed that IL-1 β had a negative correlation with miR-28-

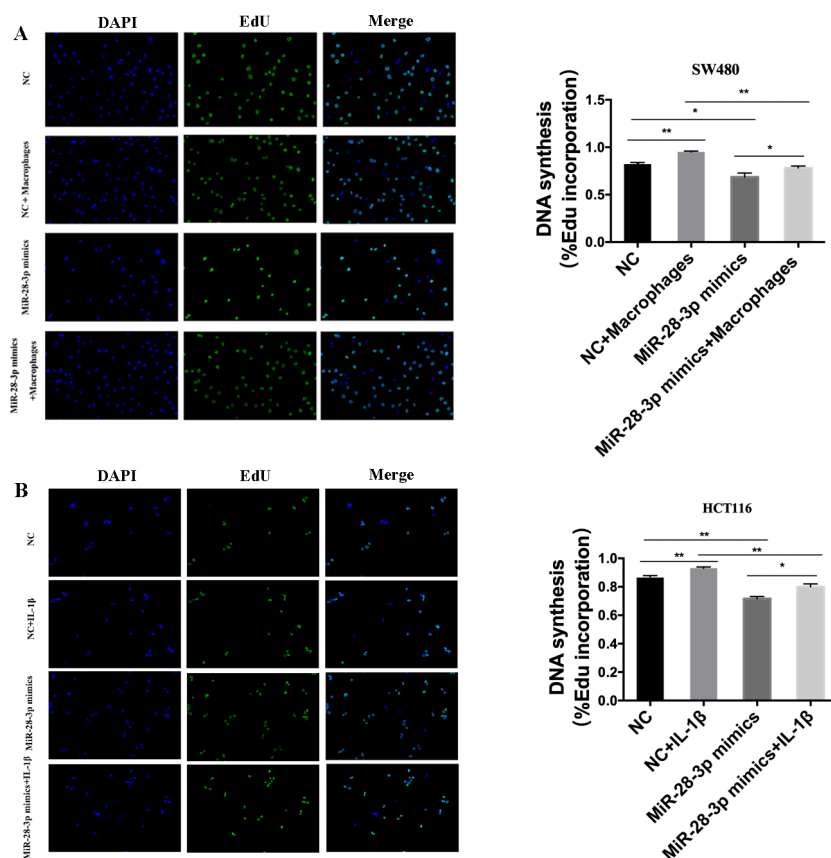


Fig. 7. miR-28-3p mimics inhibits CRC cell proliferation. **A)** EdU assays were conducted in SW480 cells transfected with miR-28-3p mimics and co-incubated with macrophages (1000) for 24 h. **B)** EdU assays were conducted in HCT116 cells transfected with miR-28-3p mimics and co-incubated with IL-1 β (20ng/ml) for 24 h. * P <0.05, ** P <0.01.

3p expression in CRC tissues (Fig. 8C). To estimate the prognostic value of IL-1 β and miR-28-3p in CRC patients, we analyzed the association of IL-1 β and miR-28-3p expression with poor survival and tumor recurrence in patients with CRC. As shown in Fig. 8 D and F, the cutoff values of IL-1 β and miR-28-3p were obtained according to their expression levels, survival time and survival status in patients with CRC, and the patients were divided into high expression and low expression groups. Kaplan-Meier analysis showed that CRC patients with high IL-1 β expression or low miR-28-3p expression had a poorer survival but showed no difference in tumor recurrence

as compared to the patients with low IL-1 β expression or high miR-28-3p expression (Fig. 8 E, G).

DISCUSSION

Macrophages are critical players in the crosstalk between cancer cells and their microenvironment (27). Several studies have indicated that high infiltration of macrophages is associated with poor prognosis in various cancers (28-30). Macrophages are implicated in inflammation-associated CRC (31) and indicate an unfavorable prognosis in CRC patients (13). However, the functional role of

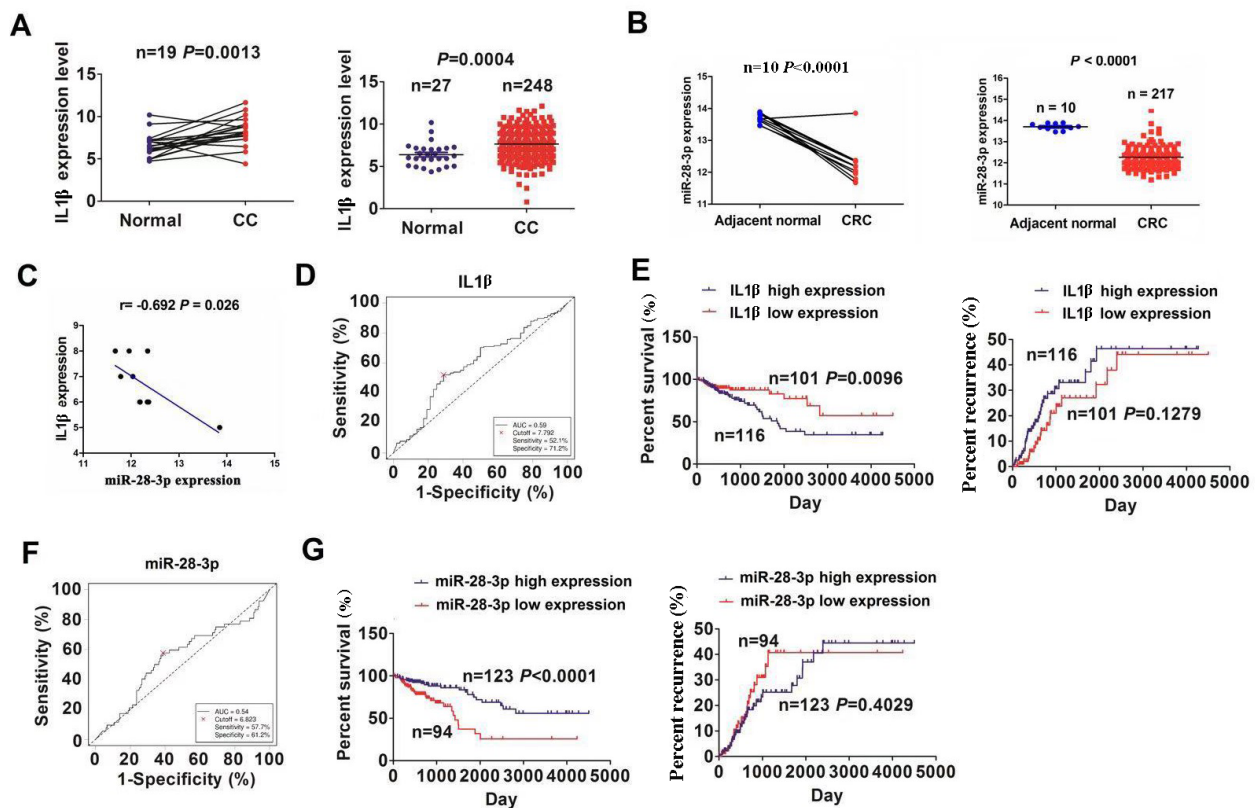


Fig. 8. The expression of IL-1 β and miR-28-3p is associated with poor survival in patients with CRC. **A)** Compared to adjacent normal tissues, IL-1 β expression was markedly increased in paired and unpaired CRC tissues according to TCGA data. **B)** Compared to adjacent normal tissues, miR-28-3p expression was decreased in paired and unpaired CRC tissues according to TCGA data. **C)** Pearson correlation analysis of IL-1 β with miR-28-3p expression in CRC tissues. **D)** ROC curve analysis of the cutoff value, sensitivity, specificity and AUC of IL-1 β in CRC patients. **E)** Kaplan-Meier analysis of the association of high or low IL-1 β expression with the poor survival and tumor recurrence in CRC patients. **F)** ROC curve analysis of the cutoff value, sensitivity, specificity and AUC of miR-28-3p in CRC patients. **G)** Kaplan-Meier analysis of the association of high or low miR-28-3p expression with the poor survival and tumor recurrence in CRC patients.

macrophages in CRC cells remains unclear. In the present study, we found that macrophages promoted the proliferation of CRC cells by upregulating PCNA expression in a number- and time-dependent manner.

Furthermore, the transition to malignancy is exacerbated by macrophages through their production of angiogenic factors, secretion of growth factors and production of proteases (16). We herein identified the expression levels of the 10 most important factors secreted by macrophages and found that IL-1 β induced an obvious macrophage cell proliferation. IL-1 β , as an inflammatory cytokine, acts in carcinogenesis and tumor progression via promoting invasiveness, tumor angiogenesis, and immune suppression (32). These biological effects induced by IL-1 β depend on the tumor types and the dose of IL-1 β administration (33). In the present study, IL-1 β induced the proliferation of CRC cells by upregulating PCNA expression in a number- and time-dependent manner. These data suggested that IL-1 β mediated macrophages to promote the proliferation of CRC cells.

Dysregulation of miRNAs is associated with tumorigenesis, angiogenesis, and metastasis (34). miR-21 is present in breast, lung, hepatocellular, ovarian and prostate cancers (35). We identified the differentially expressed miRNAs regulated by IL-1 β in CRC cells and found that IL-1 β induced a significant downregulation of miR-28-3p in CRC cells or those with co-cultured macrophages. Several studies have shown that miR-28-3p is downregulated in CRC and acts as a potential plasma marker in diagnosis of pulmonary embolism (36,37). In the present study, we found that miR-28-3p inhibitor promoted CRC cell proliferation. When co-transfected with miR-28-3p mimics with macrophages (1000) or IL-1 β (20ng/ml), respectively, miR-28-3p mimics counteracted macrophages or IL-1 β -induced CRC cell proliferation. In addition, miR-28-3p expression was decreased but IL-1 β expression was increased in CRC tissues. Low miR-28-3p expression or high IL-1 β expression was associated with poor survival in CRC patients. These findings indicated that the IL-1 β /miR-28-3p axis might mediate macrophages to regulate the proliferation of CRC cells.

However, there are several limitations to the

present study. The results were obtained entirely from *in vitro* cell culture experiments using only one CRC cell line and one macrophage cell line, which is an immortalized cell line that resembles immature monocytes/macrophages. Whether the macrophages arise from M1 or M2 polarized cell lines are unknown. Moreover, the function of the IL-1 β /miR-28-3p axis needs be validated in *in vivo* animal models.

In conclusion, macrophages promote the proliferation of CRC cells via IL-1 β -mediated downregulation of miR-28-3p, and the IL-1 β /miR-28-3p axis may provide potential markers for the poor survival of CRC patients.

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A study on protective effect of morphine against myocardial ischemia-reperfusion injury in rats via CAMP/PKA signaling pathway

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The purpose of this study was to explore the mitigating effect of morphine on the myocardial ischemia-reperfusion injury (MIRI) in rats through the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway. A total of 30 male Wistar rats were assigned into sham group, MIRI group and morphine group using a random number table. The model of MIRI was routinely established. Then, the pathological changes in the morphology of myocardial tissues were observed *via* hematoxylin-eosin (HE) staining. The levels of the oxidative stress indicators superoxide dismutase (SOD) and malondialdehyde (MDA), the content of the inflammatory cytokine tumor necrosis factor alpha (TNF- α), interleukin-1 beta (IL-1 β) and IL-6 and the quantity of glutathione peroxidase (GSH-Px), lactate dehydrogenase (LDH), creatine kinase (CK), CK-MB and cardiac troponin I (cTnI) in the myocardial enzyme spectrum were determined and analyzed through enzyme-linked immunosorbent assay (ELISA). Moreover, the messenger ribonucleic acid (mRNA) and protein expressions of cAMP, PKA, cAMP-response element binding protein (CREB) and phosphorylated CREB (p-CREB) in the cAMP/PKA signaling pathway in the myocardial tissues were measured using real-time polymerase chain reaction (PCR) and Western blotting, respectively. The results manifested that compared with those in MIRI group, the levels of myocardial infarct size, LDH, CK, CK-MB, cTnI, MDA, TNF- α , IL-1 β , IL-6 and p-CREB were decreased, while the levels of GSH-Px, SOD, PKA and CREB were increased in the morphine group. In conclusion, morphine may mitigate MIRI in rats through the cAMP/PKA signaling pathway.

The mortality rates of myocardial ischemia have been increasing yearly in middle-aged and elderly people worldwide, as the living standard has gradually improved in recent decades (1). After myocardial ischemia, effective reperfusion is an efficacious treatment for myocardial ischemia, but it increases myocardial cell death and infarct size, leading to myocardial injury. Moreover, the damaged myocardial cells and vascular endothelial cells release

a series of cytokines to induce local inflammatory responses, rupture of myocardial myofibril and sarcolemma, and mitochondrial swelling, rupture, and even disappearance in the myocardial tissues, which result in irreversible myocardial cell injury, worse cardiac hemodynamics and an increased myocardial infarct size, thus aggravating ischemic heart disease (2). Studies have revealed that biochemical indicators, such as lactate dehydrogenase (LDH), creatine

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kinase (CK), CK-MB and cardiac troponin I (cTnI), in the myocardium are released into the blood after myocardial ischemia. As a result, ischemic myocardial tissues have higher content of myocardial enzymes than normal myocardial tissues. Moreover, the detection of physiological and biochemical indicators such as LDH, CK, CK-MB and cTnI provides important clinical guidance for assessing the development and prognosis of cardiovascular disease (3). Therefore, screening efficacious drugs is a top priority for the studies on the prevention and alleviation of MIRI.

Morphine, an opioid medicine, is one of the common clinical anesthetics. A study by Kerstern et al. (4) revealed that inhalational anesthetics prevent MIRI, promoting the use of anesthetics in mitigating MIRI and broadening the application field of anesthetics. With in-depth studies, numerous scholars have proved that anesthetic drugs can alleviate MIRI (5-7). Massive opioid receptors are present in the heart. According to one study, morphine has a protective effect against MIRI (8). The cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling pathway is mainly involved in cellular material and energy metabolisms (9-11). As the second messenger, cAMP plays a pivotal role in regulating material metabolism and biochemical functions to modulate intracellular material metabolism, alter cell membrane function, inhibit production of oxygen free radicals (OFRs) and relieve inflammatory responses (12-14). The present study continued to explore the protective effect of morphine against MIRI via the cAMP/PKA signaling pathway and the mechanism therein, to provide theoretical bases for the prevention and treatment of ischemic myocardial disease.

MATERIALS AND METHODS

Laboratory animals

A total of 30 SPF-grade male Wistar rats aged 8-10 weeks and weighing 200-250 g were purchased from Liaoning Changsheng Biotechnology Co., Ltd. [license No. SCXK (Liaoning, China) 2015-0001], and they were fed in an environment with the humidity of 40-50% at 23-25°C. This study was approved by the Animal Ethics Committee of Ningbo Yinzhou Second Hospital Animal Center.

Experimental design

Thirty Wistar rats were adaptively raised for 1 week, and then divided into sham group (n=10): after the chest was opened, the left anterior descending artery (LAD) was threaded but not ligated, MIRI group (n=10): After the chest was opened, the LAD was ligated for 30 min, followed by 120 min of reperfusion to prepare an animal model, and morphine group (n=10): After the chest was opened, morphine was intravenously injected for 5 min prior to ischemia and suspended for 5 min, which was repeated for 3 times for morphine preconditioning. Finally, the MIRI model was established.

Analysis of pathological changes in the myocardium

The cardiac tissues were taken from each group of rats, fixed in 4% paraformaldehyde for 24 h and prepared into hematoxylin-eosin (HE, Boster, Wuhan, China)-stained sections through dehydration, embedding, deparaffinization, staining, transparentizing and sealing. Subsequently, the pathological changes in the myocardial tissues were observed under an upright biological microscope, and the results were recorded.

Measurement of myocardial enzyme content

Blood was drawn from the abdominal artery of rats and centrifuged at 3000 rpm for 10 min to isolate the serum. Then, the content of myocardial enzymes in the serum was determined according to the instructions of the kits. Briefly, the sample and standard zones were first marked in the enzyme-linked immunosorbent assay (ELISA) plate. Then, 50 µL of standard and 50 µL of sample were added into the corresponding wells, which were added with enzyme conjugates at 100 µL/well and incubated in a wet box at 37°C in the dark for 1 h. After the plate was washed for 3 min × 5 times, 100 µL of substrate solution was added into each well for incubation at 37°C in the dark for 15 min. Each well was then added with 50 µL of stop buffer to terminate the reaction of the enzyme conjugates and substrates. Finally, the absorbance at 450 nm of each well was measured using a multimode microplate reader, and the content of glutathione peroxidase (GSH-Px), LDH, CK, CK-MB and cTnI (Shanghai Tongwei) was calculated.

Detection of inflammatory cytokines in the myocardium

Myocardial ischemia-induced inflammatory response

impairs cardiac remodeling and accelerates the progression of heart failure. The levels of inflammatory cytokines in the myocardium were measured *via* ELISA in each group. First, the blank, control, and sample wells were set in the ELISA plate and added with diluent, standard and samples to be detected at 100 μ L/well, respectively, followed by incubation in the wet box at 37°C for 2 h. Then, the fluid in the wells was discarded, and each well was added with 100 μ L of biotin-labeled antibody working solution. The mixture was incubated in the wet box at 37°C for 1 h. With the fluid in the wells removed, the plate was washed for 3 min $\times$ 5 times, and each well was added with 90 μ L of substrate solution. After color development at room temperature for 10-15 min, the reaction was terminated by adding 50 μ L of stop buffer into each well. Finally, the absorbance at a wavelength of 450 nm of each well was read using the multifunctional microplate reader, based on which the levels of the inflammatory cytokines tumor necrosis factor alpha (TNF- α), interleukin (IL)-6 and IL -1 β (Nanjing Jiancheng) in the myocardial tissues were analyzed.

Detection of myocardial oxidative stress indicators

According to the instructions, the serum of rats was first added with xanthine oxidase or thiobarbituric acid reagents, and blank group and control group were set up. Next, the absorbance was determined by an ultraviolet spectrophotometer in each group. Finally, the activity of serum superoxide dismutase (SOD) and malondialdehyde (MDA) in each group of rats was calculated based on the formula to evaluate the oxidative stress response in the myocardium of rats in each group.

Determination of messenger RNA (mRNA) expression levels of cAMP, PKA and CREB in the myocardial tissues

After the myocardial tissues of rats in each group were ground in liquid nitrogen, total RNAs were extracted from the myocardial tissues by the column method and reversely transcribed into complementary deoxyribonucleic acids (cDNAs) using 2 μ L of 5 $\times$ PrimeScript RT Master Mix (Perfect Real Time), 5 μ L of total RNAs, 3 μ L of RNase-free dH₂O under the following conditions: reaction at 37°C for 15 min and 85°C for 5 s and preservation at 4°C. Afterwards, with the cDNAs as the template, real-time polymerase chain reaction (PCR) was performed using the primers synthesized by Sangon Biotech (Shanghai, China) Co., Ltd., and the internal

reference GAPDH (Table I) in a system composed of 12.5 μ L of 2 $\times$ TB Green Premix Ex Taq II, 1 μ L each of forward and reverse primers (10 nM), 2 μ L of cDNAs and 8.5 μ L of ddH₂O. The conditions are as follows: pre-denaturation at 95°C for 30 s, and 40 cycles of reaction at 95°C for 5 s and 60°C for 30 s. Finally, the relative mRNA expression levels of cAMP, PKA and CREB in the myocardial tissues were analyzed based on their cycle threshold (Ct) values using $2^{-\Delta\Delta C_t}$.

Measurement of expressions of the cAMP/PKA signaling pathway-associated proteins in the myocardial cells

The myocardial tissues of rats were fully ground in liquid nitrogen and added with radioimmunoprecipitation assay (RIPA) lysis buffer (Shanghai Beyotime Biotechnology) to extract proteins. The content of the total proteins was then determined by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). A total of 30 μ g of total proteins were taken from each group for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), after which the proteins were transblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) *via* wet transfer. Then, the PVDF membrane was sealed in the blocking buffer [5% skim powder diluted in tris-buffered saline with Tween-20 (TBST)] at room temperature (18-22°C) for 1-2 h, rinsed by TBST for 5 min $\times$ 3 times and incubated with the diluted primary antibody solution (PKA: 1:3000, CREB: 1:800, p-CREB: 1:500) at 4°C overnight. After washing with TBST for 5 min $\times$ 3 times, the PVDF membrane was incubated with the secondary antibody diluent (1:5000) at room temperature for 1 h, rinsed again with TBST for 5 min $\times$ 3 times and incubated with electrochemiluminescence (ECL) color development solution for 1 min. Finally, the experimental results were recorded using the multifunctional imager, and the ratios of gray values of PKA, CREB and pCREB protein bands to the gray value of GAPDH protein band were calculated using ImageJ software to analyze the changes in the expressions of the cAMP/PKA signaling pathway-associated proteins in each group of rats.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis of the obtained experimental data, and the

analysis results were presented as mean $\pm$ standard error. Differences between two groups were assessed using the Student's *t*-test. Comparison among multiple groups was made using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $P < 0.05$ represented statistically significant differences.

RESULTS

Pathological examination results of rat myocardium

The myocardial tissues of rats in each group were fixed, sectioned and subjected to HE staining. Through the comparisons of the pathological changes in the myocardial tissues of rats among the 3 groups, it was found that in the sham group, the myocardial cells were long-rod-shaped and regularly arranged, with uniform cell space, and the nuclei were central and round. Moreover, under the microscope, the myocardial tissue sections in the MIRI group exhibited swollen and necrotic myocardial cells and disordered myocardial fibers, with a higher level of inflammatory cell infiltration and karyopyknosis,

while the myocardial cells were orderly arranged, with a lower level of inflammatory cell infiltration in morphine group (Fig. 1).

Influence of morphine on the content of serum LDH, GSH-Px, CK, CK-MB and cTnI

The myocardial enzymes LDH, GSH-Px, CK, CK-MB and cTnI are the markers for myocardial injury, so the myocardial injury and the prognosis were assessed through their content changes. The content of serum myocardial enzymes in rats was determined according to the instructions of the ELISA kit. Based on the results (Table II), the content of serum LDH, CK, CK-MB and cTnI in the myocardium of rats in the MIRI group was higher than that in the sham group, while the content of serum GSH-Px was lower, showing a statistically significant difference ($P < 0.05$). The results suggested that ischemia-reperfusion causes myocardial injury in rats, the changes in the permeability of myocardial cell membrane and the release of myocardial enzymes from the tissues to the blood, further indicating the

Table I. *Primer sequences for real-time PCR*

Gene	Primer sequence	Genbank accession No.
GAPDH	F: 5' AAG GTC GGT GTG AAC GGA TTT 3'	NM_017008.4
	R: 5' AGA TGA TGA CCC TTT TGG CTA 3'	
CAMP	F: 5' TCT TTC CTA CAA CAG CCT CCG 3'	NM_001100724.1
	R: 5' TAA ATG CTC GCT TCA AAC TCA G 3'	
PKA	F: 5' TCC CTT CTC ACT TCA GTT CA 3'	NM_001100922.1
	R: 5' TAA ATA GCA ATC CAG TCT GTT GT 3'	
CREB1	F: 5' GAA GCA GCA CGA GAG TGT C 3'	NM_134443.1
	R: 5' GTC CTT TTC CCA CCA TCA TA 3'	

successful establishment of the rat MIRI model. Moreover, in comparison with the treatment in the MIRI group, morphine preconditioning protected the myocardial tissues and substantially reduced the content of serum CK, CK-MB and cTnI in the rats ($P<0.05$).

Effects of morphine on the activity of serum oxidative stress indicators

The activity of SOD and the content of MDA in the serum of rats were determined using xanthine oxidase method and thiobarbituric acid method, respectively. According to the results (Table III), compared with the sham group, the activity of SOD declined, while the content of MDA was raised in the MIRI group, showing statistically significant differences ($P<0.05$). In comparison with the MIRI group, the morphine group exhibited raised SOD activity and decreased MDA content, with statistically significant differences ($P<0.05$). It can be inferred that morphine preconditioning reduces the

myocardial ischemia-reperfusion-induced oxidative stress responses in rats.

Impact of morphine on the levels of serum inflammatory cytokines

After myocardial injury, large numbers of inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, were released by vascular endothelial cells. It was found through the ELISA that the content of the inflammatory cytokines TNF- α , IL-1 β and IL-6 in the serum of rats in the MIRI group was substantially higher than that in the sham group ($P<0.05$), and their content declined obviously in the serum of rats in the morphine group compared with that in the MIRI group ($P<0.05$) (Table IV), implying that morphine preconditioning reduces the content of serum inflammatory cytokines in the rats with MIRI.

Influence of morphine on the mRNA expression levels of cAMP, PKA and CREB

The total RNAs of the myocardial tissues were

Table II. Comparison of serum myocardial enzyme content in rats ($\bar{x}\pm s$, $n=10$)

Group	LDH (IU/mL)	GSH-Px (U/mL)	CK (U/L)	CK-MB (U/mL)	cTnI (pg/mL)
Sham group	5.197 $\pm$ 0.151	49.774 $\pm$ 1.924	69.677 $\pm$ 1.667	5.481 $\pm$ 0.128	176.295 $\pm$ 3.184
MIRI group	8.393 $\pm$ 0.153*	21.484 $\pm$ 1.407*	207.505 $\pm$ 9.037*	9.467 $\pm$ 0.231*	538.412 $\pm$ 11.118*
Morphine group	6.024 $\pm$ 0.063*#	33.002 $\pm$ 1.837*#	129.077 $\pm$ 4.099*#	7.491 $\pm$ 0.158*#	205.552 $\pm$ 6.146*#

*Statistically significant difference, vs sham group; #statistically significant difference, vs MIRI group

Table III. Content of serum SOD and MDA in rats ($\bar{x}\pm s$, $n=10$)

Group	SOD (U/mL)	MDA (U/mL)
Sham group	310.430 $\pm$ 11.960	59.678 $\pm$ 2.437
MIRI group	186.997 $\pm$ 9.227*	78.687 $\pm$ 3.397*
Morphine group	248.981 $\pm$ 6.621*#	64.444 $\pm$ 1.818*#

*Statistically significant difference, vs sham group; #statistically significant difference, vs MIRI group

extracted and reversely transcribed into cDNAs. Then, with the cDNAs as the template, real-time PCR amplification was performed to detect the changes in the relative mRNA expression levels of cAMP, PKA and CREB in the myocardial tissues of rats in each group. Based on the results (Fig. 2), the mRNA expression levels of cAMP, PKA and CREB in the myocardial tissues of rats in the MIRI group were distinctly lower than those in the sham group ($P<0.05$), while their mRNA expression levels rose evidently in the morphine group compared with those in the MIRI group ($P<0.05$).

Influence of morphine on the expression levels of the cAMP/PKA signaling pathway-associated proteins

The expression levels of the cAMP/PKA signaling pathway-associated proteins in the myocardial tissues were measured *via* Western blotting. Compared with those in the sham group, the protein expression levels of PKA and CREB were notably

decreased ($P<0.05$), and the protein expression level of p-CREB was considerably raised ($P<0.05$) in the MIRI group. Moreover, in comparison with the MIRI group, the morphine group exhibited raised protein expression levels of PKA and CREB ($P<0.05$) and an obviously lowered expression level of p-CREB ($P<0.05$) (Fig. 3). The above results suggest that morphine may protect the ischemic myocardial cells against reperfusion injury through the cAMP/PKA signaling pathway.

DISCUSSION

Cardiovascular disease gravely endangers human health, with the deaths accounting for as high as 40% of the total in urban and rural residents in China. Ischemic heart disease, the leading cause of cardiovascular disease, causes the decline in the blood supply to the myocardium and insufficiency of cardiac oxygen supply in patients, further

Table IV. Content of serum inflammatory cytokines in rats ($\bar{x}\pm s$, $n=10$)

Group	TNF- α (pg/mL)	IL-1 β (pg/L)	IL-6 (pg/mL)
Sham group	56.635 $\pm$ 1.265	48.395 $\pm$ 1.316	108.57 $\pm$ 5.451
MIRI group	86.213 $\pm$ 2.628*	136.340 $\pm$ 2.972*	195.005 $\pm$ 7.356*
Morphine group	64.055 $\pm$ 1.580 [#]	87.842 $\pm$ 6.983 [#]	131.996 $\pm$ 3.893 ^{*#}

*Statistically significant difference, vs sham group; [#]statistically significant difference, vs MIRI group

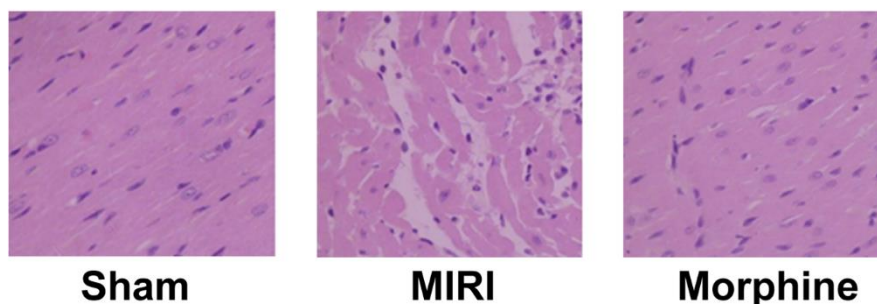


Fig. 1. Pathological sections of myocardial tissues in rats observed via HE staining (magnification: 400 $\times$).

leading to a train of severe complications such as a large myocardial infarct size, arrhythmia and the weakening of ventricular systolic function. Moreover, the development of ischemic heart disease and its complications are associated with such factors as myocardial calcium overload, oxidative stress injury, mitochondrial injury, release of inflammatory cytokines and myocardial energy metabolism disorder. In the early stage of myocardial ischemia, the treatment measures including restoration of coronary blood flow and relief of hypoxia in myocardial tissues lower the incidence rate of myocardial infarction in the patients. However, after restoration of blood supply to the heart, the myocardium further suffers from injuries, such as destruction of myocardial cell structure, impairment of cardiac function and myocardial metabolic disorders. Therefore, the studies on the strategies to protect the myocardium have laid stress on the screening of effective drugs in recent years. Medication protects and maintains cardiac function, lowers the incidence rate of ischemic heart disease, prevents various complications and improves the prognostic quality of life of patients. According to the results of this study, the rats in the MIRI group had swollen and necrotic myocardial cells, disorderly myocardial fibers and a higher level of inflammatory cell infiltration, while morphine preconditioning alleviated the pathological injuries in the myocardial cells, with more regularly arranged myocardial cells

and a lower level of inflammatory cell infiltration. It could be inferred from these results that morphine can obviously attenuate myocardial tissue injury in rats.

Opioids, widely used analgesics in cardiac anesthesia, are able to reduce myocardial stunning and lower the incidence rate of heart failure after coronary artery bypass graft (15). One study demonstrated that morphine can be used to treat heart failure by lowering peripheral vascular resistance, blood pressure and heart rate (16). Opioid preconditioning can inhibit neutrophil infiltration, reduce lipid peroxidation, activate the signaling pathways, like the mitogen-activated protein kinase pathway, and decrease intracellular calcium overload, thus protecting the myocardium (17-19). Oxidative stress is a pathogenesis of myocardial ischemia. As the intermediates of aerobic metabolisms in organisms, OFRs can be removed by SOD, GSH-Px and glutathione at the physiological state, thereby maintaining the balance between their generation and elimination in organisms. In MIRI, OFRs impair the integrity and mobility of myocardial cell membranes, thus causing strengthened permeability of cell membranes and cell edema and further leading to the loss of cytoplasm. Since the permeability of cell membranes are strengthened after myocardial cell injury, LDH, with a high content in myocardial tissues, passes through the cell membrane and enters the blood, and its content is substantially elevated in the blood. Moreover, LDH is present in the blood for a longer time than other myocardial enzymes and expands the time window for the diagnosis, so it is commonly used as a marker for the detection of myocardial injury. Massive reactive oxygen species (ROS) are produced in myocardial cells during ischemia-reperfusion. The increase in ROS content promotes the activation of neutrophils that further facilitates the release of large numbers of inflammatory cytokines $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 by myocardial vascular endothelial cells, thereby aggravating myocardial vascular endothelial injury. Additionally, the increase in inflammatory factors is a marker for myocardial cell injury (20). In the present study, the levels of LDH and MDA declined in the serum of rats after morphine pre-conditioning, suggesting that morphine has a certain protective

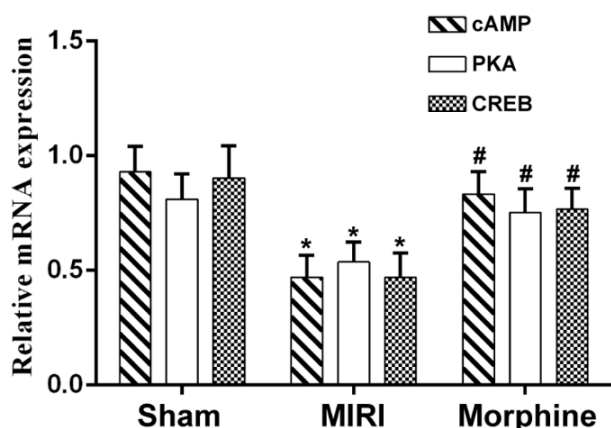


Fig. 2. *MRNA expression levels of cAMP, PKA and CREB in the myocardial tissues in each group of rats (* $P < 0.05$ vs sham group, # $P < 0.05$ vs MIRI group)*

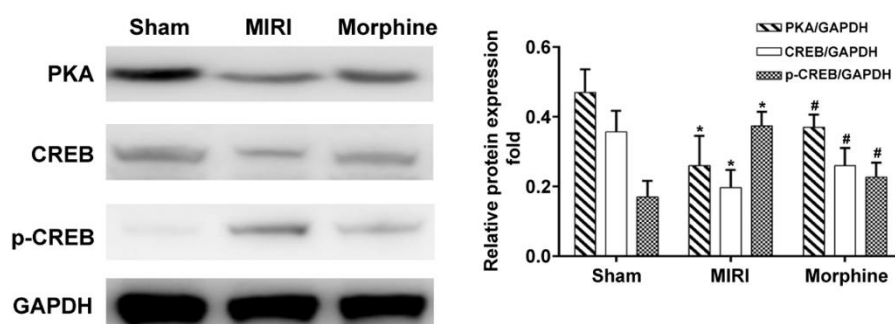


Fig. 3. Expression levels of PKA, CREB and p-CREB in the myocardial tissues of rats (* $P < 0.05$ vs sham group, # $P < 0.05$ vs MIRI group).

effect against MIRI. When the myocardial cell injury was relieved, the activity of SOD became stronger, and massive superoxide anions were removed from the injured myocardial cells, thereby effectively protecting the myocardial tissues from being attacked by OFRs. Morphine inhibited the peroxidation in myocardial tissues to reduce the content of inflammatory cytokines TNF- α , IL-1 β and IL-6 in the serum. Moreover, the maintained myocardial cell membrane integrity hindered the release of inflammatory factors into the blood and suppressed the production of myocardial enzymes. The above results implied that morphine enhances the anti-oxidative stress ability of myocardial tissues to prevent peroxidative damage to the myocardium and lower the content of serum inflammatory factors and myocardial enzymes, thereby effectively protecting against MIRI.

CAMP, the second messenger in myocardial cells, activates PKA to induce the phosphorylation of L-Ca²⁺ on the cell membranes and accelerate Ca²⁺ inflow, thereby exerting positive inotropic action (21). Intracellular cAMP stimulates the release of lysosomal enzymes to increase the permeability of cell membranes (22). PKA, a main effector of cAMP in cells, can be activated by cAMP at a high concentration in the cells. Furthermore, PKA can phosphorylate CREB at serine 133, and p-CREB is involved in regulating the expression of genes related to cellular metabolism and proliferation, further playing an important role in gene modulation, cell apoptosis and cell proliferation. Therefore,

the changes in intracellular cAMP level affect the expressions of the related genes in the cells, thereby regulating cellular functions. It was discovered through the real-time PCR and Western blotting that morphine raised the expression levels of PKA and CREB proteins in the myocardial cells and reduced the level of p-CREB in cells compared with the treatment in MIRI group. Hence, morphine suppresses the release of inflammatory factors and mitigates oxidative stress damage through regulating the expression of cAMP/PKA signaling pathway-related proteins, thereby protecting myocardial cells.

In summary, morphine probably stunts oxidative stress injury and represses the release of inflammatory factors and myocardial enzymes through the cAMP/PKA signaling pathway in myocardial cells, thereby exerting a protective effect against MIRI in rats.

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Fibrogrowth factor-2 protects against acute lung injury by activating the PI3K/Akt signaling pathway

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Acute lung injury (ALI)/Acute respiratory distress syndrome (ARDS) is a very dangerous disease. The purpose of this study was to investigate the effects of fibrogrowth factor-2 (FGF-2) on lipopolysaccharide (LPS)-induced lung injury and its mechanisms. C57/BL6 mice were used in the study and LPS was used to construct the ALI/ARDS model. In addition, human normal lung epithelial cell line BEAS-2B was cultured to investigate the effect of FGF-2 on the lung and its mechanism of action *in vitro*. FGF-2 significantly reduced wet/dry weight ratio of mice, the number of cells and inflammatory factors in BALF, and MPO activity in lung tissue. In addition, FGF-2 also reduced the level of oxidative stress in mouse lung tissue. *In vitro*, FGF-2 effectively reduced LPS-induced inflammatory and apoptotic levels of BEAS-2B cells and increased the activity of the PI3K/Akt signaling pathway. However, LY294002, an inhibitor of the PI3K/Akt signaling pathway, alleviated the protective effect of FGF-2 on lung tissue. Therefore, FGF-2 attenuated inflammation, oxidative stress and apoptosis in alveolar epithelial cells by activating the PI3K/Akt signaling pathway.

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a type of respiratory disease characterized by respiratory distress, hypoxemia, chronic persistent inflammatory reaction and increased vascular permeability (1). In recent years, due to changes in air pollution and living habits, the incidence of ALI/ARDS has increased. The main causes of ALI/ARDS include pneumonia, shock, severe infection, and pulmonary embolism (2). At present, the main clinical treatment strategies include treatment of primary disease, respiratory support, sedation treatment, and circulatory function support, but there is no specific drug for treating ALI/ARDS (3). Recent statistics showed that the mortality rate of ALI/ARDS is approximately 30~40%, and the mortality rate of elderly patients over 85 years of

age can be as high as 60%. ALI/ARDS has a severe impact on the respiratory system, especially lung tissue, and greatly reduces the patient's quality of life (4). Therefore, the development of anti-ALI/ARDS drugs to alleviate pain and improve the long-term prognosis has become a problem to be solved.

The pathogenesis of ALI/ARDS is still unclear, but there is increasing evidence that multiple signaling pathways and related proteins can modulate the inflammatory response of ALI/ARDS. Among them, PI3K/Akt signaling pathway plays an important role in the pathogenesis (5). Studies have confirmed that LPS can induce pulmonary microvascular endothelial injury in LPS-induced ALI/ARDS model, but activation of PI3K/Akt can increase the content of cyclic adenosine monophosphate in

Key words: fibrogrowth factor-2; acute lung injury; acute respiratory distress syndrome; lipopolysaccharide; PI3K/Akt signaling pathway

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alveolar cells, protect lung vascular endothelial cells and maintain the stability of the environment inside the lung and the dynamic balance inside and outside the blood vessels (6).

Fibroblast growth factor-2 (FGF-2), one of the members of the fibroblast growth factor family, is widely involved in various physiological and pathological processes, such as neurodevelopment, wound healing, cell differentiation, tissue carcinogenesis, etc., and is widely distributed in various organs (7). FGF-2 was first discovered to stimulate the proliferation of murine fibroblast NIH3T3 cells. In subsequent studies, it was found that FGF-2 not only promotes fibroblasts, but also promotes the mitosis of various cells, and stimulates the proliferation and differentiation of these cells (8). With the continuing research, an increasing number of physiological effects of FGF-2 have been discovered. Now FGF-2 is considered to be a multi-effect factor with extremely broad biological effects.

Previous studies have found that FGF-2 has a significant activation effect on the PI3K/Akt signaling pathway (9). Therefore, we suspected that FGF-2 may have a therapeutic effect on ALI/ARDS by activating PI3K/Akt signaling pathway. To verify our hypothesis, we used mice to make an ALI/ARDS model and treated them with FGF-2, and cultured the human normal lung epithelial cell line BEAS-2B to investigate the effect of FGF-2 on ALI/ARDS.

MATERIALS AND METHODS

Animals and grouping

A total of 60 C57/BL6 male mice were used for this study. The mice were housed in barrier facilities. The mice were divided into 3 groups: normal control group, lipopolysaccharide (LPS) group and LPS+FGF-2 group. LPS (5 mg/kg, Sigma, St. Louis, MO, USA) was used to induce ALI/ARDS in mice. Recombinant mouse FGF-2 (25 µg/kg, Promocell, Heidelberg, German) was used to treat the mice (10). This study was approved by the Animal Ethics Committee of Fujian Medical University Animal Center.

Operative procedure of mice ALI/ARDS

After anesthetizing with 4% chloral hydrate (10 µL/g), the mice were fixed in the supine position and the fur

of the neck was carefully removed using scissors. Then the skin of the neck was cut and the lung was gradually exposed. LPS or a mixture of LPS and FGF-2 was then injected into the trachea using a microsyringe. After 24 h, the mice were sacrificed by cervical dislocation and the serum or lung tissue was collected.

Wet/dry weight ratio (W/D) of lung tissue

After the mice were sacrificed, the left lung lobes were collected, and then the surface of the lung tissue was blotted gently with filter paper. The lung was weighed and marked as wet weight. The lung tissue was then placed in an oven at a temperature of 60°C. After 48 h of baking, the lung tissue was again weighed and recorded as dry weight.

Myeloperoxidase (MPO), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity assay

The lung tissue was pulverized into powder and dissolved in PBS. Then MPO kit (Lianke, Hangzhou, China), MDA kit (Lianke, Hangzhou, China), SOD kit (Lianke, Hangzhou, China) and GSH-Px kit (Lianke, Hangzhou, China) were used to detect the activity of MPO, MDA, SOD and GSH-Px, respectively, in lung tissue according to the manufacturer's instructions.

Preparation of bronchoalveolar lavage fluid (BALF)

After the mice were sacrificed, the lung tissue was lavaged with PBS. The BALF was then collected and the pellet was removed by centrifugation. We observed and counted the total number of cells and the number of neutrophil granulocytes in BALF by microscopy.

Enzyme linked immunosorbent assay (ELISA)

After collecting the BALF from the mice, we used the ELISA kits (Lianke, Hangzhou, China) to detect the IL-1β, IL-6 and TNF-α levels in the BALF according to the manufacturer's instructions. Standard substance with known concentrations in the ELISA kit was used to make a standard curve, and then we calculated the concentration of the sample based on the absorbance of the sample and the standard curve.

Hematoxylin-eosin (HE) staining

After the mice were sacrificed, the lung tissue was fixed in 4% paraformaldehyde for 24 h. Then the lung

tissue was embedded in paraffin and made into paraffin sections. After paraffin sections were dewaxed and hydrated, they were soaked in hematoxylin for 5 min. After washing for 3 min with water, the sections were soaked in 1% hydrochloric acid alcohol for 30 s and then rinsed with water for 3 min, after which they were placed in 0.5% Eosin solution for 3 min. After washing for 3 min with water, the sections were dehydrated and sealed.

Cell culture and treatment

The human normal lung epithelial cell line BEAS-2B [American Type Culture Collection (ATCC) (Manassas, VA, USA)] was used in this study. BEAS-2B cells were cultured using Dulbecco's modified eagle medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin plus streptomycin (Gibco, Rockville, MD, USA). The cells were placed in an incubator at 37°C and 5% CO₂. LY294002 (Selleck, Houston, TX, USA) was used to inhibit the PI3K/Akt signaling pathway in BEAS-2B cells. Recombinant human FGF-2 (Promocell, Heidelberg, German) was used to treat BEAS-2B cells.

Western blot

RIPA lysates (NCM Biotech, Newport, RI, USA) were used to lyse mouse lung tissue or BEAS-2B cells. Bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China) was used to detect protein concentration. Thirty µg of total protein was added to each well of the electrophoresis gel. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred to the polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Non-specific antigens were blocked with 5% skim milk. Then the PVDF membrane was incubated at 4°C overnight with primary antibody dilution (IL-1β 1:3000, Rabbit, Abcam, Cambridge, MA, USA; IL-6, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; TNF-α, 1:2000, Rabbit, Abcam, Cambridge, MA, USA; Bax, 1:1000, Rabbit, Abcam, Cambridge, MA, USA; Bcl-2, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; PI3K, 1:2000, Rabbit, Abcam, Cambridge, MA, USA; p-PI3K, 1:1000, Rabbit, Abcam, Cambridge, MA, USA; Akt, 1:5000, Rabbit, Abcam, Cambridge, MA, USA; p-Akt, 1:5000, Rabbit, Abcam, Cambridge, MA, USA; β-actin, 1:3000,

Rabbit, Abcam, Cambridge, MA, USA). After washing the PVDF membrane the next day, secondary antibody dilution (Goat anti-rabbit, 1:3000, Abcam, Cambridge, MA, USA) was used to incubate the PVDF membrane for 2 h at room temperature. Finally, chemiluminescence was used to detect protein expression.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract RNA from mouse lung tissue and BEAS-2B cells. The mRNA was reversed to complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then cDNA was amplified using the SYBR Green kit (Invitrogen, Carlsbad, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. 2^{-ΔΔCt} method was used to calculate relative expression level. The primer sequences of mRNA are shown in Table I.

Cell counting kit-8 (CCK8) assay

The CCK8 kit (Yifeixue, Shanghai, China) was used to detect the viability of BEAS-2B cells. Cells were cultured in 96-well plates and were treated with different concentrations of FGF-2. After 24 h, 10 µL of CCK8 reagent was added into each well and the cells were re-incubated in the incubator for 2 h. Then a microplate reader was used to measure the absorbance of each well.

Immunocytofluorescence (IF) staining

Twelve-well plates were used to culture BEAS-2B cells. After the cell growth density reached 60%, the cells were treated with LPS (10 µM) and FGF-2 (100 ng/mL). After washing the cells with PBS, they were treated with 4% paraformaldehyde and 0.5% Triton-PBS. Then the non-specific antigen in the cells were blocked with 10% goat serum. The cells were incubated with primary antibody dilution (caspase3, 1:500, rabbit, Abcam, Cambridge, MA, USA) at 4°C overnight. Fluorescent secondary antibody (Goat anti-rabbit-FITC, 1:500, Abcam, Cambridge, MA, USA) was used to incubate the cells for 1 h in the dark. Then the cells were incubated for 10 min using 4',6-diamidino-2-phenylindole (DAPI). Finally, we observed and recorded the staining results using a fluorescence microscope.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) were used to analyze the data of this study. Measurement data were represented as mean $\pm$ standard deviation. Comparison between multiple groups was made using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p < 0.05$ was considered to be statistically significant. All experiments were repeated more than 3 times.

RESULTS

Exogenous FGF-2 attenuates LPS-induced mouse ALI/ARDS

To determine effect of FGF-2 on ALI/ARDS, we used LPS to induce ALI/ARDS in mice and intraperitoneally injected FGF-2. The results showed that the lung tissue W/D of the LPS group was significantly higher than that of the control group, while the LPS+FGF-2 group had lower lung tissue W/D than the LPS group (Fig. 1A). This suggested

that FGF-2 can attenuate LPS-induced lung tissue edema. In addition, the number of cells in BALF was examined. The results showed that FGF-2 can significantly reduce the total number of cells (Fig. 1B) and the number of neutrophils (Fig. 1C) in BALF. In addition, the activities of MPO (Fig. 1D), MDA (Fig. 1E), SOD (Fig. 1F) and GSH-Px (Fig. 1G) in mouse lung tissues were examined, and it was found that FGF-2 can significantly reduce the level of oxidative stress in lung tissue. The results of HE staining (Fig. 1H) also showed that the lung interstitial of the LPS group showed obvious edema and alveolar epithelial tissue discontinuity, while the LPS+FGF-2 group had better lung tissue morphology than the LPS group.

Exogenous FGF-2 reduces LPS-induced inflammation in mouse lung tissue

The expression of IL-1 β , IL-6 and TNF- α in mouse BALF were examined. The results indicated that FGF-2 can significantly reduce LPS-induced inflammation and reduce the expression of IL-1 β (Fig. 2A), IL-6 (Fig. 2B) and TNF- α (Fig. 2C). The results of Western blot (Fig. 2D) and RT-PCR (Fig. 2 E, F) showed that the expression of IL-1 β and TNF- α in lung tissue of LPS group was significantly increased and FGF-2 could decrease their expression. The results of IF staining also indicated that FGF-2 reduces IL-1 β expression.

FGF-2 reduces LPS-induced inflammation and apoptosis in BEAS-2B cells

BEAS-2B cells were cultured to examine the effects of FGF-2 on alveolar epithelial cells at the cellular level. The optimal concentration of FGF-2 for BEAS-2B cells was determined by CCK8 assay. The results of CCK8 assay indicated that 100 ng/mL was the optimal concentration of FGF-2 to stimulate BEAS-2B cells (Fig. 3A). The results of Western blot (Fig. 3B) and RT-PCR (Fig. 3 C-G) showed that FGF-2 significantly decreased the expression of IL-1 β , IL-6, TNF- α and Bax and increased the expression of Bcl-2. The results of IF staining (Fig. 3H) also indicate that FGF-2 can reduce the expression of caspase3. These results indicated that FGF-2 can reduce inflammation and apoptosis of BEAS-2B cells *in vitro*.

Table 1. RT-PCR primer sequences

Name	sense/anti-sense	Sequence(5'-3')
Human		
IL-1 β	sense	AGCTATCGATCATGCTACGACG
	anti-sense	ACGATCGACGTACGTACGTATCC
IL-6	sense	ACTCACCTCTTCAGAACGAATTG
	anti-sense	CCATCTTTGGAAGGTTTCAGGTTG
TNF- α	sense	CTACCATCACCCTGACTGAGAT
	anti-sense	GGTCACTTCACCATAGTGGACA
Bax	sense	CAGTTGAAGTTGCCATCAGC
	anti-sense	CAGTTGAAGTTACCATCAGC
Bcl-2	sense	GACTGAGTACCTGAACCGGCATC
	anti-sense	CTGAGCAGCGTCTTCAGAGACA
PI3K	sense	GGTGACTGTGTGGGACTTATTGA
	anti-sense	CTGATGTAGTGTGTGGCTGTTGA
Akt	sense	CAGTTTACCCAGTGACAATCA
	anti-sense	CACGAGACAGGTGGAAGAAGAGC
GAPDH	sense	ACAACCTTGGTATCGTGAAGG
	anti-sense	GCCATCACGCCACAGTTTC
Mouse		
IL-1 β	sense	CAGAATCATAAGCCCCTGGA
	anti-sense	TCTGCGAGTCAGGCATTG
TNF- α	sense	TTCTTGAGCAACACCCTC
	anti-sense	CGCATACACTGTCTACCT
PI3K	sense	AGTCTAGCACCTTACTATCGA
	anti-sense	CTAGCGATCGAGCTGACG
Akt	sense	CTAGCTAGCATCTATAAAGCGT
	anti-sense	GTAGATTAGCGATTACGTAGCAC
GAPDH	sense	AGCGACATCCTAGTACGGACAT
	anti-sense	CGATCTAGCTGACAGCGATA

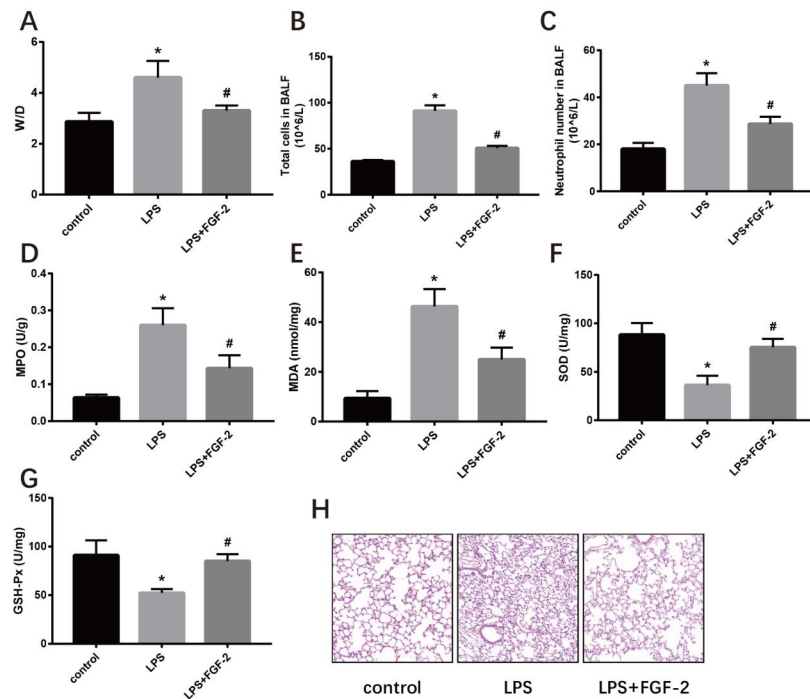


Fig. 1. Exogenous FGF-2 attenuates LPS-induced mouse ALI/ARDS. **A**, W/D in three groups was detected. Total cells number (**B**) and neutrophil number (**C**) in BALF were detected. MPO (**D**), MDA (**E**), SOD (**F**) and GSH-Px (**G**) activity in three groups. **H**) HE staining of mice lung tissue (magnification: 400 $\times$). *statistical difference with the control group; #statistical difference with the LPS group

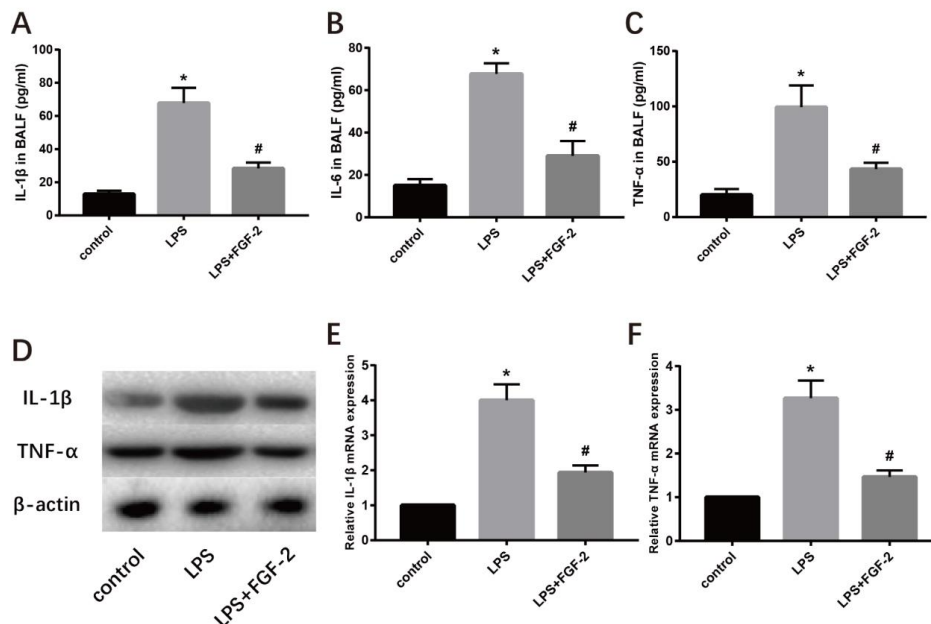


Fig. 2. Exogenous FGF-2 reduces LPS-induced inflammation in mouse lung tissue. Expression of IL-1 β (**A**), IL-6 (**B**), and TNF- α (**C**) in BALF was determined by ELISA. Expression of IL-1 β and TNF- α in three groups was determined by Western blot (**D**) and RT-PCR (**E**, **F**). *statistical difference with the control group; #statistical difference with the LPS group

FGF-2 increases the activity of PI3K/Akt signaling pathway in BEAS-2B cells

To verify the mechanism of action of FGF-2, changes were examined in the expression of the PI3K/Akt signaling pathway. The results of Western blot (Fig. 4A) and RT-PCR (Fig. 4 B, C) showed that the expression levels of PI3K and Akt and phosphorylated in the lung tissues of FGF-2 treated mice were increased. Similar results also occurred in cell experiments (Fig. 4 D-F). These results indicated that FGF-2 increased the activity of the PI3K/Akt signaling pathway in lung tissue.

Inhibition of PI3K/Akt signaling pathway attenuates the protective effect of FGF-2 on BEAS-2B cells

To verify that FGF-2 protects lung tissue by activating the PI3K/Akt signaling pathway, the inhibitor of PI3K/Akt signaling pathway, LY294002, were used to inhibit the activity of the PI3K/Akt signaling pathway in BEAS-2B cells. The results of Western blot (Fig. 5A) and RT-PCR (Fig. 5 B-F) showed that LY294002 increased the expression of IL-1 β , IL-6, TNF- α and Bax in BEAS-2B cells and decreased the expression of Bcl-2. The results of IF staining (Fig. 5G) also indicated that LY294002

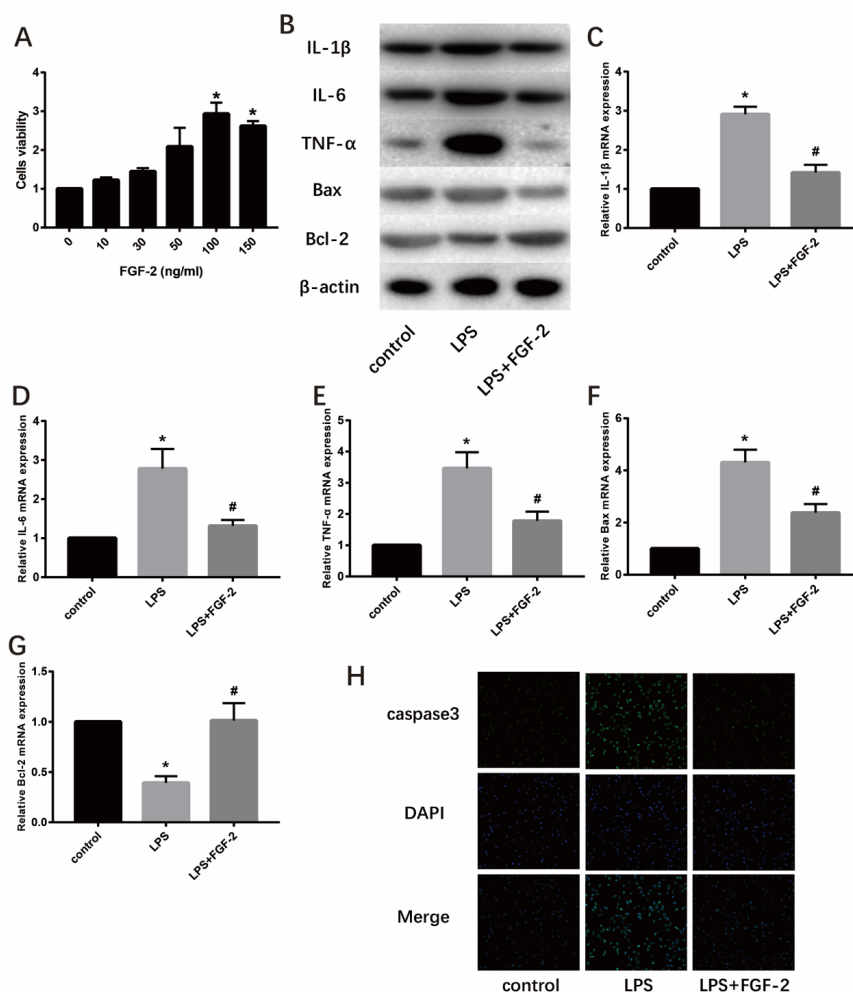


Fig. 3. FGF-2 reduces LPS-induced inflammation and apoptosis in BEAS-2B cells. **A)** CCK8 found that 100 ng/ml was optimum concentration. Expression of IL-1 β , IL-6, TNF- α , Bax and Bcl-2 in three groups was determined by Western blot (**B**) and RT-PCR (**C-G**). **H)** IF staining detected the expression of caspase3 (magnification: 400 $\times$). *statistical difference with the control group; #statistical difference with the LPS group

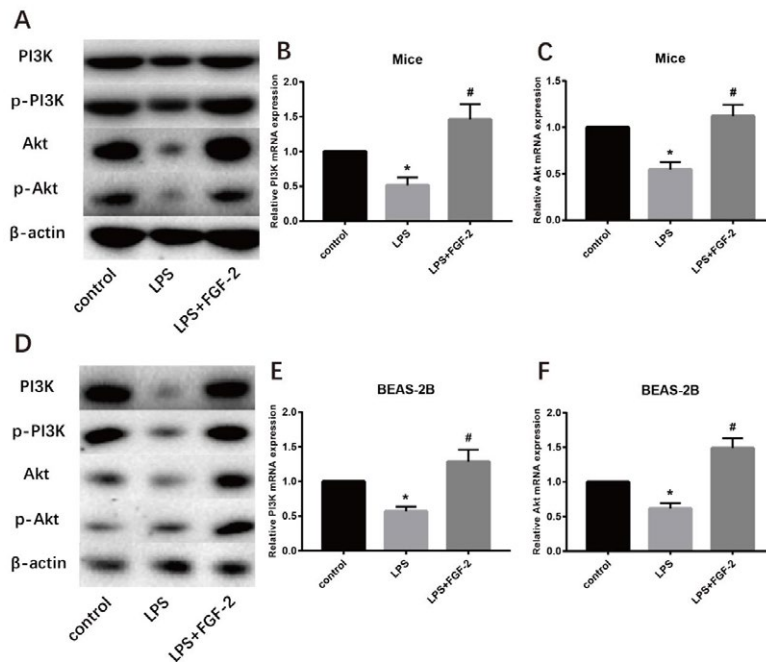


Fig. 4. FGF-2 increases the activity of PI3K/Akt signaling pathway in BEAS-2B cells. **A-C**, Expression of PI3K, p-PI3K, Akt and p-Akt in mice was determined by Western blot (**A**) and RT-PCR (**B**, **C**). In addition, expression of PI3K, p-PI3K, Akt and p-Akt in BEAS-2B cells was determined by Western blot (**D**) and RT-PCR (**E**, **F**). *statistical difference with the control group; #statistical difference with the LPS group

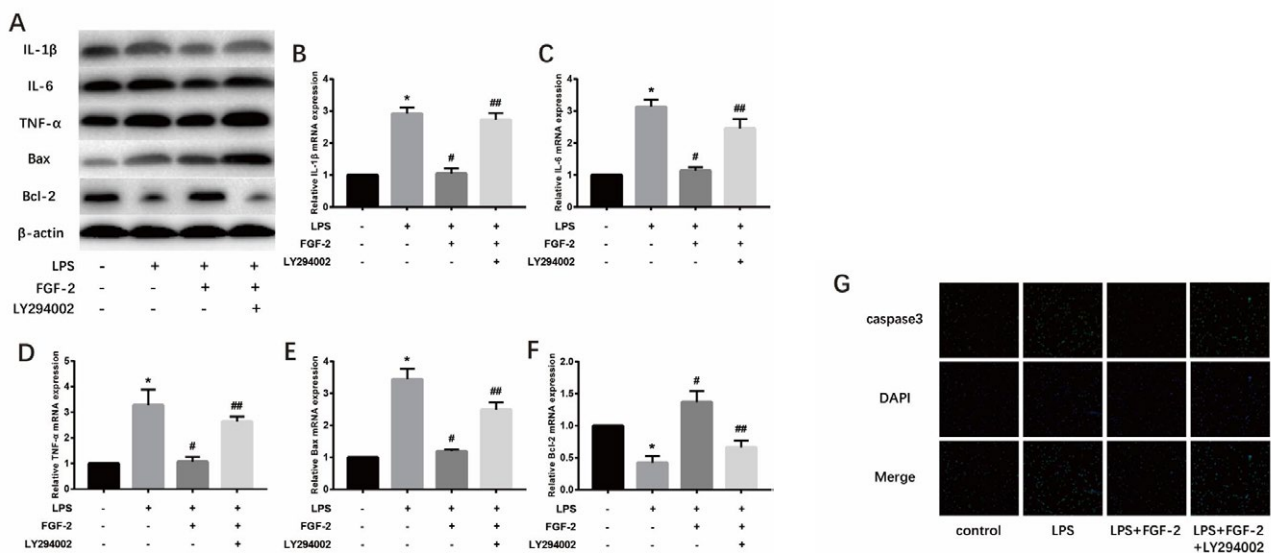


Fig. 5. Inhibition of PI3K/Akt signaling pathway attenuates the protective effect of FGF-2 on BEAS-2B cells. Expression of IL-1β, IL-6, TNF-α, Bax and Bcl-2 in four groups was determined by Western blot (**A**) and RT-PCR (**B-F**). **G**) IF staining detected the expression of caspase3 (magnification:400×). *statistical difference with the control group; #statistical difference with the LPS group; ##statistical difference with the LPS+FGF-2 group).

increased the expression of caspase3. These results indicated that the protective effect of FGF-2 on BEAS-2B cells was reduced after inhibition of the PI3K/Akt signaling pathway.

DISCUSSION

Studies on the pathogenesis of ALI/ARDS have shown that ALI/ARDS was essentially an uncontrolled inflammatory response that occurs in lung tissues. The pathological level of ALI/ARDS is characterized by impaired alveolar epithelial barrier, increased permeability of basement membrane, pulmonary interstitial edema, pulmonary hypoxia, macrophages, neutrophils and other inflammatory properties (11). The inflammatory cells accumulate in the lungs and trigger the inflammatory factor-like release effect. When the initial inflammatory response to ALI occurs, the etiology is often infection with gram-negative bacteria. LPS is a major component of the cell wall of gram-negative bacteria widely reported in the literature (12). The intranasal induction of LPS can effectively simulate the pathogenesis of ALI/ARDS and is a widely-used animal model. LPS can activate a variety of inflammatory cells and release a large number of inflammatory mediators, thereby promoting the development and progression of ALI/ARDS (13). Inflammation refers to a series of defensive pathophysiological reactions that occur when the body is stimulated by internal and external sources. Under the stimulation of adverse stimuli, mononuclear macrophages will produce and release a large number of pro-inflammatory factors that can cause further tissue damage. One study showed that the number of neutrophils increased in BALF and the concentration of neutrophil protease increased in the early stage of ALI/ARDS. The number of neutrophils and protease are directly related to the degree of ALI/ARDS and the prognosis of patients (14). In addition, when stimulated by LPS, alveolar macrophages release inflammatory mediators such as leukotriene, IL-1 β , and TNF- α , triggering a strong inflammatory response (15). Therefore, by inhibiting the excessive activation of inflammatory cells and controlling the release of inflammatory factors, the deterioration of ALI/ARDS conditions can be

alleviated to some extent (16). Our study found that in LPS-induced mice, there was significant edema in the lungs and a marked increase in lung inflammation and oxidative stress levels. In mice given FGF-2 pretreatment, W/D and the number of cells in BALF was significantly reduced. FGF-2 also reduced MPO activity and MDA content. HE staining also found that FGF-2 significantly improved alveolar morphology and pulmonary interstitial. These results indicated that FGF-2 effectively ameliorated LPS-induced lung injury in mice.

One study indicated that activation of the α_2 -adrenergic receptor can trigger the transduction of the downstream PI3K/Akt signaling pathway, resulting in the opening of the sodium channel, which can clear the edema fluid and protect the lung tissue (17). Anjum et al. (18) pointed out that after the lung microvascular endothelium was damaged by external factors, the alveolar capillary barrier was also damaged, which induced alveolar collapse, altered ventilation/blood flow ratio, and even led to respiratory failure. In the experiment of inhaling anesthetic isoflurane to prevent lung injury in mice, some scholars have pointed out that isoflurane has a good protective effect on lung injury, and directly confirms that activation of PI3K/Akt signaling pathway can effectively treat lung damage. When sevoflurane and heme oxygenase-1 (HO-1) were administered in mouse with LPS induction, activation of the PI3K/Akt cascade signal can control ALI. In the mechanism experiment of oxidative damage of lung tissue caused by hyperoxia, the researcher treated the animal with edaravone, and observed that the lung injury in rats and the injury of human alveolar epithelial cells *in vitro* were all improved. Up-regulation of PI3K/Akt inhibited pathological damage in lung tissue, reduced peroxidation and DNA damage, and then protected lung tissue (19). Wang et al. (20) explored the damage of alveolar epithelial cells induced by *Staphylococcus aureus* and confirmed that PI3K/Akt cascade signal had a certain regulatory effect on cytopathic changes, while the increase of p-Akt was positively related with the degree of cell injury. Our results also found that FGF-2 can increase the expression and phosphorylation of PI3K and Akt in

lung tissue or lung epithelial cells, both in animal and in cell experiments. In addition, LY294002 can also attenuate the protective effect of FGF-2 by inhibiting the PI3K/Akt signaling pathway. These results suggest that the protective effect of FGF-2 on lung tissue may be related to the PI3K/Akt signaling pathway.

FGF-2 is a multifunctional, broad-ranging cell growth factor. It is closely related to the formation and development of many organs and diseases (21). For example, FGF-2 is closely related to the formation of teeth and is also associated with the deterioration of glioma. FGF-2 is involved in the disorder of glucose metabolism in diabetes, which affects the occurrence and development of complications in diabetic patients. In addition, FGF-2 is involved in the process of fat formation and fat metabolism (22). FGF-2 has also been found to be a target of Mir-424 to regulate apoptosis and inflammation of alveolar epithelial cells (23). Therefore, FGF-2 has a wide application prospect for apoptosis and inflammatory-related diseases. To our knowledge, this is the first study to report the effects of FGF-2 on ALI/ARDS. We believed that our results can provide a new direction and theoretical basis for clinical treatment of ALI/ARDS.

In conclusion, FGF-2 effectively attenuated LPS-induced lung injury, lung inflammatory response and oxidative stress levels, thereby reducing apoptosis of alveolar epithelial cells. In addition, FGF-2 can activate the PI3K/Akt signaling pathway, and this may be the mechanism of FGF-2 to protect lung tissue.

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β -Carotene improves intestinal barrier function by modulating proinflammatory cytokines and improving antioxidant capacity in β -lactoglobulin-sensitized mice

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Increased intestinal permeability due to barrier dysfunction is supposed to cause several gastrointestinal diseases. We have previously demonstrated that a single β -carotene (BC) dose protects against increase in anaphylactic response in β -lactoglobulin (BLG)-sensitized mice with no effect on the epithelial permeability and weak recovery of villi length. Utilizing the same murine *ex vivo* intestinal model, the aim of this study was to investigate the effect of different BC doses on BLG-mediated intestinal epithelial barrier disturbances. Jejunum was harvested from BLG-sensitized mice pretreated with either one of three different doses of BC (5, 10 and 20 mg/kg body weight) and mounted on Ussing Chambers. Transepithelial electrical resistance (TER) and short-circuit current (*I*_{sc}) were recorded as indicators of intestinal epithelial barrier function. Histopathological analysis of the intestine was carried out for the control and experimental mice. TNF- α and IL-6 levels were determined in serum using ELISA, and the analysis of antioxidant activity was performed for reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). BC was capable of enhancing the intestinal barrier function, as indicated by the increased TER and the decreased *I*_{sc}. Intestinal damage characterized by the shortening of villi and infiltration of intestinal lymphocytes was significantly reversed by BC pretreatment. Such effects of BC were accompanied by a reduction in the levels of IL-6 and TBARS and an increase of GSH. TNF- α levels were reduced only at the lowest BC dose. These findings may encourage the use of BC-based therapies for controlling the breakdown of the intestinal barrier *in vivo*.

Intestinal barrier dysfunction initiated by various etiologies is a main contributing factor in several pathological conditions involving the gastrointestinal tract (1). A breakdown or impairment of the epithelial barrier has been implicated as a critical determinant in the predisposition to intestinal inflammation and

a number of gastrointestinal diseases including inflammatory bowel disease and food allergy (2). While increased intestinal epithelial permeability can be a consequence of disease exacerbation, clinical evidence suggests that it may be a primary etiologic factor predisposing to disease development (3).

Key words: β -carotene; transepithelial resistance; TNF- α ; IL-6; oxidative stress

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Proinflammatory cytokines such as TNF- α and IL-6 play a crucial role in the modulation of the inflammatory response in the gastrointestinal tract (4). Proinflammatory cytokines can induce the endocytosis of tight junction proteins resulting in increased intestinal permeability (5).

The role of the immune system is likely to generate an aggressive physiological response to the imbalance induced by the barrier dysfunction (6). Increased immune cell activity has been reported to result in significant free radical production (7) that may negatively impact barrier function. Excessive levels of reactive oxygen species (ROS) damage cellular proteins including cytoskeletal proteins (8) and, ultimately, disrupt gastrointestinal tract barrier to increase gut permeability which contributes to inflammation in a variety of gastrointestinal diseases (9). In this respect, antioxidants may reduce increased intestinal permeability by regulating ROS signaling.

Several studies have shown that diets rich in β -carotene (BC) are beneficial to the health of humans and may prevent the development of inflammation associated diseases. Such an effect has been attributed to the various bioactivities of BC, including antioxidant activity (10). In an earlier publication from our laboratory, we showed that supplementation with a single BC dose resulted in a significant decrease in secretory response with no effect on the epithelial permeability and weak recovery of villi length in a murine model of food allergy (11). Therefore, in the current study, we questioned whether different BC doses could improve β -lactoglobulin (BLG)-mediated intestinal barrier dysfunction. Towards this goal, we used the same *in vivo* model employed above.

MATERIALS AND METHODS

Animals and housing conditions

Female Balb/c mice (16.80 ± 1.95 g, $n = 40$) were maintained at the animal facility of nutrition physiology and food safety laboratory-university Oran 1 with a 12-h light–dark cycle at $23 \pm 2^\circ\text{C}$ and free access to standard laboratory feed and water. All experimental procedures involving animals were approved by the current Algerian legislation covering the protection of animals.

Experimental protocol

At 4 weeks of age, mice were randomly divided into four groups of ten animals each: one control group (Vehicle) and three experimental groups (5BC, 10BC and 20BC, respectively). The experimental groups received daily doses of 5, 10, and 20 mg/kg of body weight/day of BC (Sigma, France), respectively, for 2 weeks through the gavage method; while the control group was given only corn oil (Sigma, France) over the same period of time. BC was prepared as suspension in 0.2 ml corn oil used as vehicle. On the 16th day of the experiment, mice in all groups were sensitized intraperitoneally with BLG, as described previously (11). During the 50-day experimental period, the mice body weights were monitored weekly, however, there were no significant differences in body weight among the groups in the study (data not shown).

It has to be noted that the applied doses exceed the levels from any natural sources of BC. Although mice readily convert BC to vitamin A, the bioavailability of the molecule is low in case of absorption through the gut, requiring administration of BC at higher doses than physiologically required (12). The experiments were made to partly offset this limitation

Sample collection and processing

On day 50, the mice were anesthetized by pentobarbital sodium (5 mg/kg) and sacrificed. The small intestine and the liver were carefully collected. After discarding the duodenum and the ileum, the intestine was rinsed with phosphate buffer saline (PBS) solution in order to remove fecal content. A 5 cm portion of the middle of the intestine was placed in cassettes and fixed in 10% buffered formalin solution pH 7.2, and the consecutive following segment was opened lengthwise along the mesenteric line and immediately mounted as sheets in Ussing chambers. Serum samples, collected after removing blood cells, and liver were stored at -80°C until analysis.

From each mouse, 4 to 8 pieces of intestine were used for *ex vivo* Ussing chamber experiments as previously described by our group (11, 13, 14). Briefly, the spontaneous potential difference (PD) was monitored via agar-salt bridges connected to calomel electrodes, and the appropriate short-circuit current (I_{sc}) was added via Ag-AgCl electrodes to maintain a zero PD through an automatic voltage clamp device (model VCC MC8; Physiologic Instruments, San Diego, CA, USA). The

transepithelial electrical resistance (TER) was calculated from the spontaneous PD and *I*_{sc} by means of Ohm's law. The transmembrane resistance is a sensitive marker of the epithelial barrier function, and a decline of the value reflects a break in the barrier (15). *I*_{sc} is considered an index of electrogenic ion movement. Changes in TER and *I*_{sc} during experimental conditions were calculated as a percentage of corresponding basal values.

Intestinal tissues were dehydrated by gradually soaking in alcohol and xylene, paraffin-embedded, and stained with hematoxylin and eosin for histological analysis. Tissues were visualized for changes in intestinal pathology. Villus length was measured using an optical microscope equipped with a micrometer (Optica Axiom 5000, Beijing, China).

Serum IL-6 (Cat. No. RAB0308) and TNF- α (Cat. No. RAB0477) were analyzed by using commercial ELISA kits according to the manufacturer's protocol and instruction. The levels of TBARS in serum and liver were determined according to previously described methods (16). The results are expressed as μ mol malondialdehyde (MDA)/mg protein. GSH content was determined by 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as an indicator (17). GSH content was expressed as μ mol/mg protein. The protein content was determined using Lowry's method measuring absorbance at 750 nm (18). Bovine serum albumin was used as a standard.

Statistical analysis

Results are given as mean $\pm$ standard error of the mean (SEM). Differences between means were determined using one-way ANOVA (analysis of variance) with Tukey's post-hoc test using GraphPad Prism 5 software. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

BC improved BLG-induced disruption of barrier function ex vivo

Three concentrations of BC were tested, and their effects on transepithelial electrical resistance and short current circuit were measured. *I*_{sc} and TER were followed over time.

Sensitization with BLG induced significant decline of transepithelial resistance (Fig. 1a, b) and increase of short current circuit (Fig. 1d, e), whereas

BC pretreatment at different doses significantly inhibited these effects. By increasing concentrations of BC, the peak amplitude of Δ TER increased by 45.27, 61.62 and 59.45%, respectively, and Δ *I*_{sc} decreased by 16.37, 29.16 and 24.92%, respectively.

The jejunal sheets isolated from vehicle-sensitized control mice had a basal TER of $45.38 \pm 9.96 \Omega \cdot \text{cm}^2$ and a basal *I*_{sc} of $75.84 \pm 11.10 \mu\text{A} \cdot \text{cm}^{-2}$. Oral administration of BC at doses of 5, 10 and 20 mg/kg induced a significant increase in the mean value of basal TER (63.82 ± 4.29 , 68.98 ± 5.40 and $68.69 \pm 3.30 \Omega \cdot \text{cm}^2$, respectively) and decrease of *I*_{sc} (66.33 ± 6.23 , 48.33 ± 3.35 and $46.22 \pm 4.28 \mu\text{A} \cdot \text{cm}^{-2}$, respectively) (Fig. 1 c, f).

BC ameliorated BLG-induced mucosal damage

Histological examination of the control group showed in most cases preservation of structure (Fig. 2 a, b). In the intestinal tissues of BLG-sensitized mice receiving or not corn oil, there was obvious intestinal inflammation characterized by an increased lymphocytes infiltration and remarkable villus atrophy (Fig. 2 c-f). After two weeks of treatment, BC improved the mucosal inflammation induced by BLG (Fig. 2 g-i). The decrease of the villus length induced by BLG was significantly inhibited by BC pretreatment with a maximal inhibition at BC 20 mg/kg (Fig. 2j). Villus height was increased by 23.54, 30.92 and 62.76% in the jejunum of mice receiving 5, 10 and 20 mg/kg of BC respectively.

As shown in Fig. 3a, the serum level of IL-6 was markedly inhibited by all the investigated doses of BC. For TNF- α , only pretreatment of BLG-sensitized mice with 5 mg/ml of BC per day significantly attenuated the level of this cytokine, whereas 20 mg/kg BC significantly increased TNF- α content in serum (Fig. 3b). BC treatment significantly decreased TBARS levels and elevated GSH content in the liver when compared with vehicle-sensitized control mice (Fig. 4a, c), whereas there was no effect on the serum TBARS level (Fig. 4b).

DISCUSSION

We have previously shown that supplementation

with a single BC dose resulted in a significant decrease in secretory response with no effect on the epithelial permeability and weak recovery of villi length (11). Therefore, in the present study, we investigated whether different BC doses could regulate intestinal barrier function in our mouse model of food allergy by examining changes in the transepithelial electrical resistance and short current

circuit. An important factor is the poor bioavailability of carotenoids, including BC, in case of the absorption from the gut requiring the administration of BC at higher doses than physiologically relevant (12). In our study, three concentrations were tested (5, 10 and 20 mg/kg) in order to achieve maximal barrier enhancement. The basal values for *I*_{sc} and TER recorded in our experiments are well within

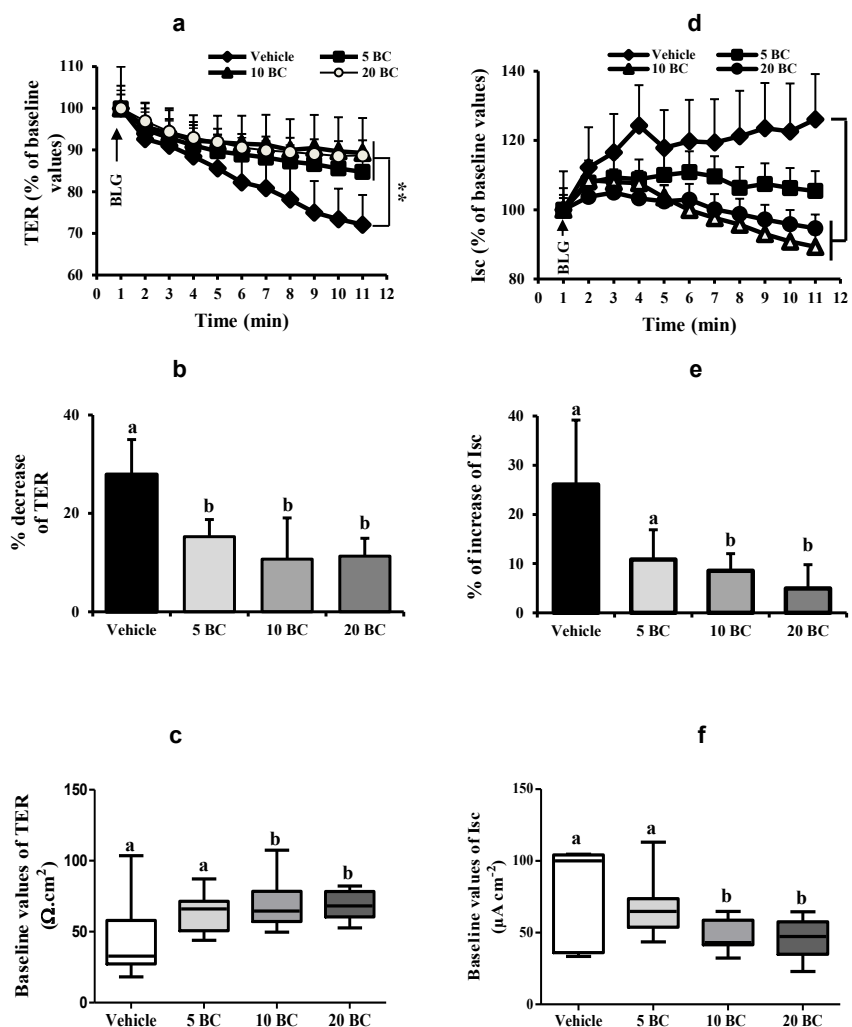


Fig. 1. Effects of BC administration on intestinal barrier function during serosal side exposure to BLG. Murine intestine pretreated with either one of three different doses of BC (5, 10 and 20 mg/kg body weight) was placed on Ussing Chambers and the TER and I_{sc} were measured over a 10-minute period after BLG (60 μg/ml) challenge. **A)** Percentage (%) of baseline TER values measured at various time points after BLG challenge (**p < 0.01). **B)** Percentage (%) TER decrease after BLG challenge; the decrease in TER is the difference between the peak value after BLG challenge and the baseline value. **C)** The mean value of basal TER of tissues from different groups. **D)** Percentage (%) of baseline I_{sc} values measured at various time points after BLG challenge (***p < 0.001). **E)** Percent (%) I_{sc} increase after BLG challenge; the increase in I_{sc} is the difference between the peak value after BLG challenge and the baseline value. **F)** The mean value of basal I_{sc} of tissues from different groups. Values (means ± SEM, n = 10) not sharing a common letter are significantly different (*p < 0.05). Comparisons were made between different doses of the same treatment and all doses vs Vehicle

the range found by our group and other groups working with mouse intestine (19, 20). Interestingly, all BC concentrations were optimal in terms of both elevating TER and decreasing *I_{sc}*. More specifically, TER significantly increased at BC 10 mg/kg. TER is one of the commonly useful indicators for permeability of intestinal epithelial cells. It could reflect the opening of the tight junctions between

epithelial cells and the paracellular permeability of the intestinal mucosa (21). The present study supports previous observations in that low serum carotenoids were suggested as good markers for the intestinal barrier function (22, 23). Vieira et al. (23) suggested that carotenoids ameliorate disrupted intestinal barrier function, via scavenging free oxygen radicals or via maintenance of tight

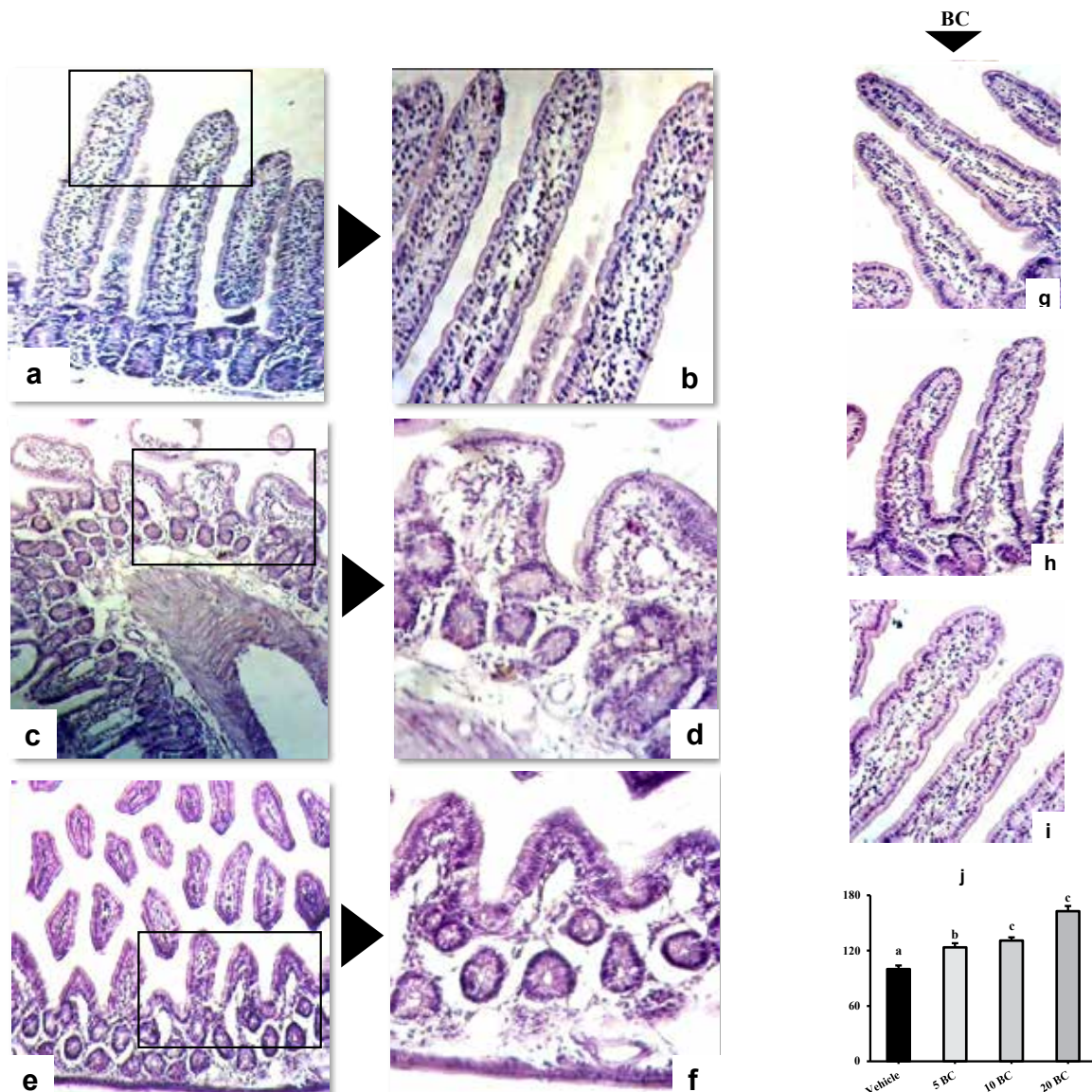


Fig. 2. H&E staining of small intestine section from a representative mouse from each group. **A, B**) Naïve mice (X 100, X 400, respectively). **C, D**) BLG-sensitized mice (X 100, X 400, respectively). **E, F**) Vehicle-sensitized control mice (X 100, X 400, respectively). **G-I**) Mice receiving 5, 10 and 20 mg/kg of BC, respectively (X 250). **J**) Effect of BC pretreatment on villus length, measured from villus-crypt junction to villus tip ($n = 21$). n is the number of tissues studied from four mice per group. Values (means ± SEM) not sharing a common letter are significantly different ($p < 0.05$). Comparisons were made between different doses of the same treatment and all doses vs Vehicle

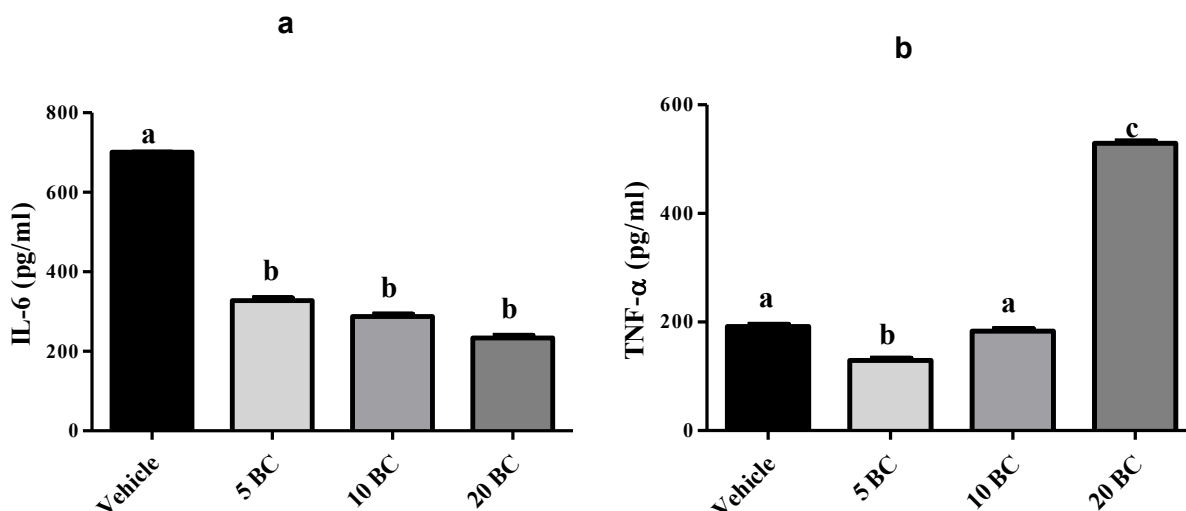


Fig. 3. Effects of BC administration on proinflammatory cytokines. **A)** IL-6 levels. **B)** TNF- α levels. Values (means $\pm$ SEM, $n = 10$) not sharing a common letter are significantly different ($*p < 0.05$). Comparisons were made between different doses of the same treatment and all doses vs Vehicle

junctions and adherence junction proteins. The underlying mechanisms for this remain unknown at present; however, it is speculated that the beneficial effects of consumed BC are thought to be due to its ability to be converted to vitamin A (24). A study by He et al. (25) showed that vitamin A improves intestinal epithelial barrier function by enhancing the expression of tight junction proteins. Vitamin A metabolites could also affect some aspects of the epithelial barrier function. Retinoic acid, the most biologically active form of vitamin A (26), was found to be partially but significantly able to attenuate the disruption of barrier properties of MDCK monolayer (1). Interestingly, Rybakovsky et al. (27) have emphasized the ability of certain micronutrients to enhance one or more aspects of tight junction barrier function in Gie-3B11 human epithelial cell culture model. At concentrations that produced optimal improvement of barrier function, retinoic acid produced significant decreases in claudins-1 and -2, and significant increases in claudins-4 and -5.

As stated above, villi length increased as treatment concentration increased. To our knowledge, our study is believed to be the first to show that BC is effective

in ameliorating epithelial damage and restoring epithelial function in *in vivo* model. Whereas, in a series of experiments aimed at examining the impact of BC on oxidative stress-induced cerebral, cardiac and hepatic damage, Esrefoglu et al. (28) demonstrated that BC could reduce stress-induced organ damage by both inhibiting lipid oxidation and supporting the cellular antioxidant defense system.

To determine whether these BC-induced improvements in barrier function were due in part to changes in proinflammatory cytokines, we examined the BC effects on TNF- α and IL-6 levels. As indicated above, BC pretreatment significantly inhibited BLG-induced IL-6 increase in a dose-dependent manner, while TNF- α levels decreased only at BC 5 mg/kg. TNF- α and IL-6 are believed to be among the most important in inducing the intestinal epithelial barrier dysfunction and leading to increased intestinal permeability (29). BC exerted an inhibitory effect on proinflammatory cytokines. It blocked nuclear translocation of the NF- κ B p65 subunit which is correlated with its inhibitory effect on phosphorylation and degradation of the NF- κ B inhibitor (30). Recently, a major advance in

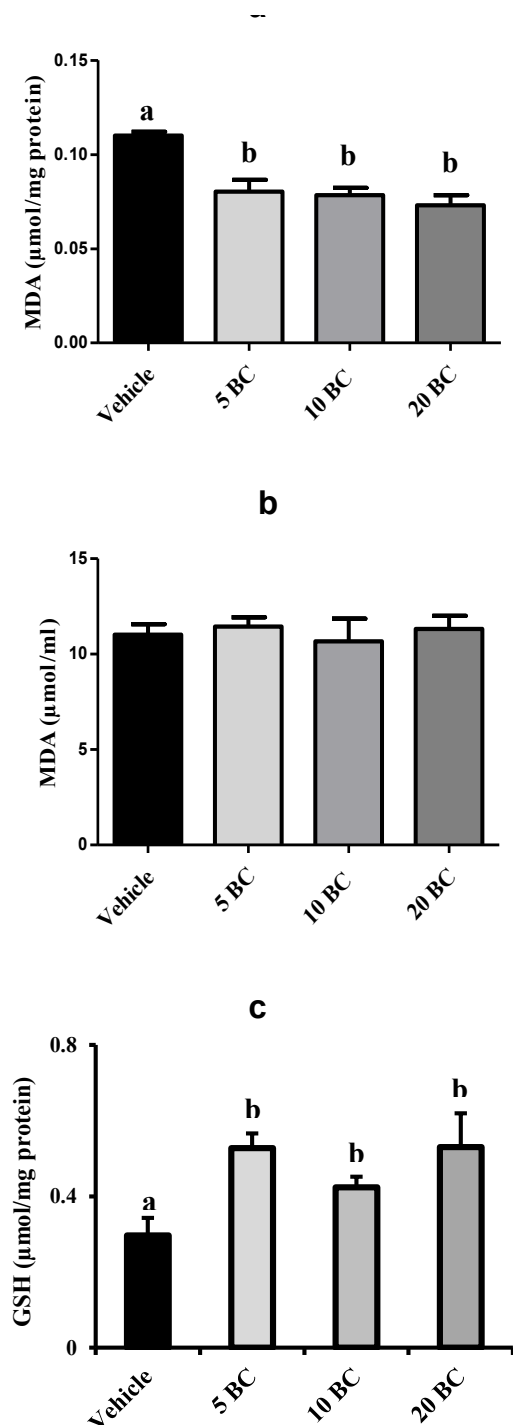


Fig. 4. Effects of BC administration on the antioxidant status of BLG-sensitized mice. **A, B)** TBARS levels in the liver and the serum, respectively. **C)** Hepatic GSH content of BLG-sensitized mice fed either one of three different doses of BC. Values (means $\pm$ SEM, $n = 10$) not sharing a common letter are significantly different (* $p < 0.05$). Comparisons were made between different doses of the same treatment and all doses vs Vehicle

understanding the anti-inflammatory activities of BC was made by the work of Li et al. (31). They reported that the anti-inflammatory effect of BC is achieved by inhibiting multiple signaling pathways, such as JAK2/STAT3, NF- κ B and JNK/p38 MAPK but not ERK1/2. Our results revealed that BC at dose of 20 mg/kg per day in the given model, and using the described protocol, increased TNF- α content in serum. Due to the complexity of the immune system and response, the anti-inflammatory effect of BC requires further investigation in our future study.

It has been postulated that the role of BC as an anti-inflammatory agent may be due to its capacity to scavenge ROS and may be attributed to the electrophilicity of ROS-induced carotenoid intermediates (32). Given this possibility, we felt it necessary to examine the antioxidant redox activity of BC. Thus, we assessed TBARS levels as a marker of oxidative stress. In our study, BC significantly decreased the TBARS content in the liver. The inhibition of oxidative stress was reportedly closely related to the improvement in the non-enzymatic and enzymatic antioxidant status. To our knowledge, GSH is one of the most important non-enzymatic antioxidants (33). As alluded to above, the GSH levels were significantly restored in BC-treated mice. The activity of GSH is quite important for immunological functions. In an *in vitro* cell culture model using the human alveolar macrophages, it was shown that the production of TNF- α , IL-6, and IL-8 was inhibited by addition of GSH to the medium (34). In addition, chronic depletion of mucosal GSH by buthionine sulfoximine, a specific inhibitor of glutamate cysteine ligase, has recently been shown to cause severe degeneration of epithelial cells from jejunum and colon, which was prevented by oral GSH or GSH monoester (35).

On the basis of the results discussed in this study, we believe that BC improved BLG-induced intestinal barrier disruption, through reducing epithelial permeability, decreasing intestinal mucosa atrophy, modulating the cytokine levels, and enhancing intracellular antioxidative protection. Further studies in this domain, examining different carotenoids, and perhaps mixtures representing more natural conditions, are warranted.

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Punicalagin suppresses osteosarcoma growth and metastasis by regulating NF- κ B signaling

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Osteosarcoma is the most prevailing malignant bone tumor among adolescents. Punicalagin, a polyphenolic compound extracted from pomegranate, possesses many functions such as anti-oxidation, anti-bacterial, anti-virus, and immunosuppression, which can counter the aggressiveness of a variety of cancers such cervical, ovarian and prostate. This study aimed to investigate the inhibitory effect of punicalagin on the proliferation and metastasis of osteosarcoma cells and its potential regulatory mechanisms. Osteosarcoma cell lines (HOS cells, U2OS cells and MG63 cells) were treated with different doses of punicalagin, and the effects on osteosarcoma cell activity were examined *in vitro* using cell counting kit-8 (CCK-8), colony formation and apoptosis assays. The mobility, migration and invasion abilities of osteosarcoma cells were detected by wound healing and Transwell assays. NF- κ B activity was explored by the NF- κ B p65 luciferase reporter assay. Western blot was used to investigate the expressions of downstream proteins. We found that punicalagin inhibited the viability of osteosarcoma cells *in vitro* in dose-dependent and time-dependent manners and promoted apoptosis. In addition, punicalagin could significantly impede the mobility, migration and invasion abilities of osteosarcoma cells. In terms of mechanism, punicalagin down-regulated the expressions of p65, survivin, XIAP, CIAP2 and other proteins, and suppressed the proliferation and metastasis of osteosarcoma cells by repressing NF- κ B signaling pathway. In conclusion, it is concluded that punicalagin restrains the growth and metastasis of osteosarcoma by obstructing the NF- κ B signal transduction pathway.

Osteosarcoma, a common primary malignant bone tumor in children and adolescents, widely exists in the distal femur, proximal tibia and proximal humerus (1). Despite the utilization of current treatment strategies, including surgery and chemotherapy, the long-term disease-free survival rate of patients is approximately 60-75% (2), with the 5-year survival rate of patients with local recurrence and distant metastasis merely accounting for 27-55% (3). To effectively improve the treatment

of osteosarcoma, it is urgent to explore new drugs.

Based on the increase in tumor research, an increasing number of active ingredients in plants have been tested for the treatment of various tumors (4). For example, astragalus plays an anti-tumor role by inhibiting the growth of osteosarcoma cells and inducing apoptosis (5). Baicalin induces apoptosis of osteosarcoma cells via the ROS-mediated mitochondrial pathway (6). The above studies suggest that active ingredients from plants may provide new

Key words: punicalagin; osteosarcoma; apoptosis; NF- κ B

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treatment options for patients with osteosarcoma. Pomegranate (*Punicagranatum*), a common fruit in Asia, has been demonstrated to exert anti-tumor effects in many studies. With cell and animal models, the anticancer effects of pomegranate are confirmed in colon cancer, lung cancer and liver cancer (7-9). Punicalagin (2,3-hexahydroxydiphenoyl-gallagyl-D-glucose, PUN) is the main bioactive component of pomegranate. Moreover, previous research confirms that it has anti-oxidant, anti-viral, anti-proliferative and anti-tumor functions (10-13). For example, PUN induces apoptosis of colon cancer and glioma cells (10, 14). However, whether PUN can inhibit the proliferation and metastasis of osteosarcoma cells remains unclear.

In recent years, some investigations suggest that NF- κ B signaling pathway plays an important role in the occurrence and development of osteosarcoma (15, 16). For example, it is indicated that Hsa-let-7g activates the NF- κ B pathway by decreasing HOXB1, and promotes the progression of osteosarcoma (17). In addition, SND1 activates NF- κ B pathway by up-regulating the expression of COX2/PGE2, and has an impact on promoting proliferation of osteosarcoma cells (18). Many drugs, such as raddeanin and xanthatin, that act on the NF- κ B pathway have been shown to impede osteosarcoma processes (19, 20). However, whether PUN can suppress the NF- κ B pathway in osteosarcoma has not yet been fully elucidated.

In the present study, we demonstrated that PUN repressed the proliferation and metastasis of osteosarcoma by suppressing the NF- κ B signaling pathway. Our research provided a potential insight into the treatment of patients with osteosarcoma and could improve their therapeutic outcomes in the future.

MATERIALS AND METHODS

Cell counting kit-8 (CCK-8) assay

Osteosarcoma cells (HOS cells, U2OS cells, and MG63 cells) were purchased from the Shanghai Cell Library of the Chinese Academy of Sciences. All cells were then cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 0.1mg/mL streptomycin and 100U/mL penicillin (Hyclone, Logan,

UT, USA) in 5% CO₂ at 37°C. Subsequently, the cells were seeded in 96-well plates at a density of 2×10^3 cells/well and incubated for 24 h. After the cells were treated with different concentrations of punicalagin (PUN, N1687, Apexbio Biotechnology Co., Ltd, China) (0, 25, 50, 100, and 200 μ M) for 24 h, 36 h, 48 h, 60 h, and 72 h, the cell viability was detected by CCK-8 assay, followed by the addition of 10 μ l of CCK-8 solution (Baiaolaibo, Beijing, China) to each well. Finally, the absorbance of cells in each well was measured using a microplate reader with a wavelength of 450 nm, and the absorbance value was used as an index for evaluating cell viability.

Colony formation assay

Osteosarcoma cells were inoculated in 6-well plates at a density of 1×10^3 /well, and then treated with 50 μ M PUN for 48 h. PUN was not added to the control group. After being cultured for 10 d at 37°C in 5% CO₂, each group of cells was rinsed with cold PBS solution, fixed with formaldehyde, and then stained with 0.1% crystal violet. The number of formed colonies was recorded.

Apoptosis analysis

Drug-induced apoptosis was detected using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA). After the treatment with PUN, osteosarcoma cells were collected and assayed according to the manufacturer's instructions with a flow cytometry (BD Biosciences, San Jose, CA, USA).

Dual-luciferase reporter gene assay

Osteosarcoma cells were transfected with NF- κ B luciferase reporter plasmids (pNF- κ B-luc, Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) employing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. After 24 h, the medium was removed. Osteosarcoma cells were then treated with 50 μ M of PUN for 12 h, and luciferase activity was measured using a luciferase activity detection kit (Beyotime Institute of Biotechnology, Nantong, China).

Western blotting

After being rinsed with PBS, the cells were lysed with RIPA lysis buffer containing protease inhibitor (Thermo Fischer Scientific, Rockford, IL, USA). The supernatant was collected after high-speed centrifugation, and heated

in water bath to denature the protein. After quantified by the BCA method, proteins were then separated by SDS-PAGE and transferred to a nitrocellulose (NC) membrane (Millipore, Bedford, MA, USA). Then, the membrane was blocked with 5% defatted milk for 30 min at 37°C. After that, the primary antibodies were added, with which the cells were incubated overnight at 4°C. The NC membrane was rinsed with the TBST solution and then incubated with the secondary antibody (Goat Anti-Rabbit IgG, 1:2000) for 1 h at room temperature. Moreover, chemiluminescence was developed using a hypersensitive ECL (Baiaosi, Wuhan, China). All antibodies (anti-Bax antibody (ab53154), anti-Bcl-2 antibody (ab196495), anti-N-cadherin antibody (ab18203), anti-E-cadherin antibody (ab15148), anti-MMP-2 antibody (ab37150), anti-MMP-9 antibody (ab73734), anti-TIMP1 antibody (ab61224), anti-TIMP2 antibody (ab53730), anti-Survivin antibody (ab469), anti-XIAP antibody (ab21278), anti-CIAP2 antibody (ab137393), anti-NF- κ B p65 antibody (ab86299), anti- β -actin (ab179467) and goat anti-rabbit IgG H&L (HRP, ab205718)) were from Abcam (Shanghai, China).

Wound healing assay

Osteosarcoma cells were seeded in 6-well culture plates at a density of 1×10^6 /ml, and cultured until the confluence reached about 90%. Then the scratches were made on the monolayer cells with a sterile pipette tip and rinsed several times with PBS to remove cell debris. After the treatment of 50 μ M PUN, the width of the scratches was recorded at different times (0 h, 24 h, 36 h, and 48 h) and the rates of scratch healing were calculated.

Transwell migration assay

Migration and invasion abilities of cells were assessed with Boyden Transwell chambers (Corning, NY, USA) with an 8.0 μ m pore size polycarbonate membrane. In migration experiments, 200 μ L osteosarcoma cell suspension (without FBS) with a density of 2×10^5 cells/ml and PUN (0 or 50 μ M) were added into the upper chamber, and a medium supplemented with 10% FBS was added to the lower chamber. After being cultured at 37°C for 24 h, the medium was removed. The filter was rinsed twice with PBS. The cells remaining on the upper surface of the membrane were removed, and migrated and invaded cells were fixed with 4% paraformaldehyde for 15 min, and the cells were then stained with Giemsa

solution. Finally, the number of cells was recorded. The Matrigel was covered on the surface of the membrane for invasion experiments, and the remaining steps were the same as for the migration experiments.

Statistical analysis

The statistical processing software used in this study was Graphpad prism 7. All data were expressed as mean $\pm$ standard deviation. Student's *t*-test was used to compare the measurement data between the two groups, and *P* < 0.05 was considered statistically significant.

RESULTS

PUN suppressed the viability of osteosarcoma cells

Previous research indicates that PUN can inhibit the growth of colon cancer, glioma, and papillary thyroid carcinoma cells (10, 11, 14). To assess the role of PUN in the proliferation of osteosarcoma cells, we treated osteosarcoma cells with different concentrations (0, 25, 50, 100, and 200 μ M) of PUN, and detected the viability of cells in each group by CCK-8 assay at different time-points (24, 36, 48, 60, and 72 h). We observed that the viability of osteosarcoma cells was significantly impeded in dose-dependent and time-dependent manners compared to that of the control group (Fig. 1A). We then performed a colony formation assay to record the number of colony formations in each group of cells treated with PUN at different concentrations, the results of which suggested that with the higher concentration of PUN, the number of formed colonies became smaller (Fig. 1B). These results demonstrated that PUN could inhibit the proliferative viability of osteosarcoma cells, indicating that PUN had the effect of suppressing the growth of osteosarcoma.

PUN induced the apoptosis of osteosarcoma cells

After the clarification that PUN inhibited the proliferation of osteosarcoma cells, we further explored whether PUN could promote the apoptosis of osteosarcoma cells. Osteosarcoma cells were treated with different concentrations of PUN, and the proportion of apoptotic cells in each group of osteosarcoma cells was determined. The results suggested that, compared with that of the control

group (0 μM), the percentages of apoptotic cells in treatment groups were markedly increased (Fig. 2A). Subsequently, Western blot was performed to

detect the expression levels of Bcl-2 and Bax in osteosarcoma cells. The results showed that Bcl-2 expression in osteosarcoma cells treated with PUN

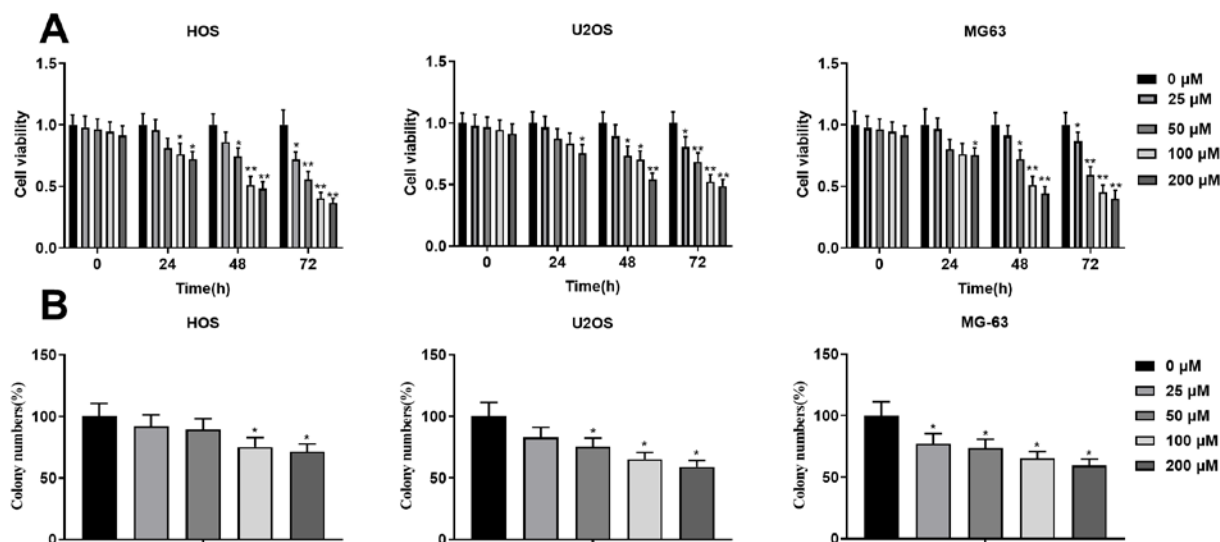


Fig. 1. Punicalagin inhibited the proliferation of osteosarcoma cells. Osteosarcoma cells were treated with different concentrations of punicalagin for the indicated times, and the viability of these cells was measured using CCK8 assay. **A)** Punicalagin inhibited osteosarcoma cell (HOS, U2OS and MG63) viability in time-dependent and dose-dependent manners. **B)** Colony formation assay was used to assess cell proliferation capacity in a dose-dependent manner. PUN, punicalagin. * $P < 0.05$; ** $P < 0.01$.

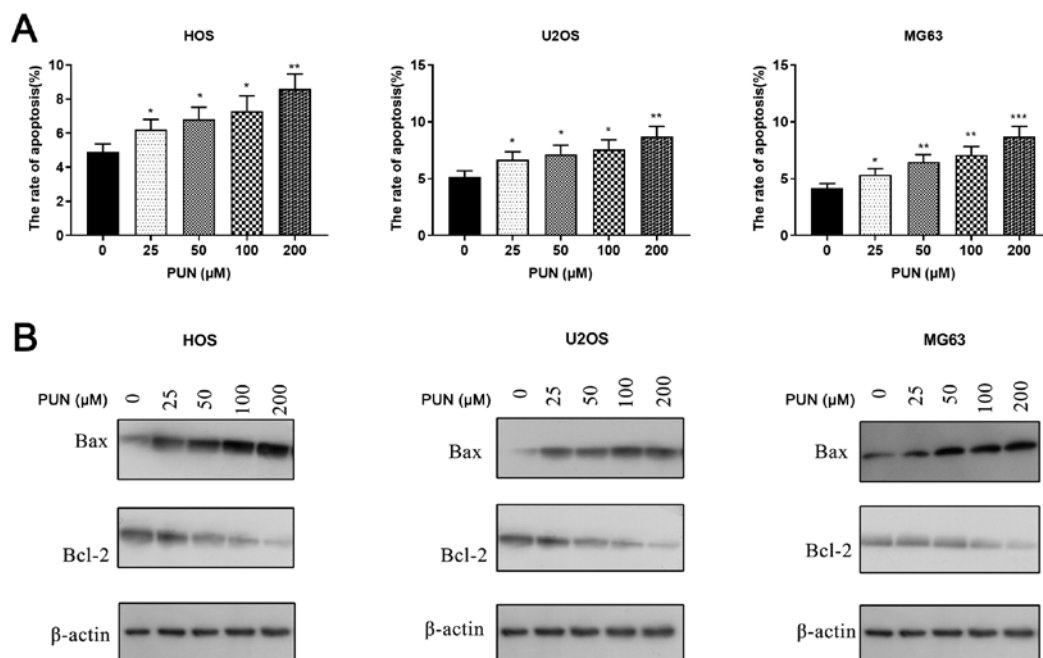


Fig. 2. Punicalagin promoted the apoptosis of osteosarcoma cells. **A)** Osteosarcoma cells were treated with different concentrations of punicalagin for 48 h. The percentages of cellular apoptosis were shown. **B)** Western blot was used to detect the changes of apoptosis-associated protein levels (bcl-2 and bax) in osteosarcoma cells treated with increasing concentrations of PUN (0, 25, 50, 100 and 200 μM) for 24 h. PUN, punicalagin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

was observably down-regulated, and Bax expression was signally up-regulated compared with that of osteosarcoma cells without the treatment of PUN (Fig. 2B). Our data suggested that PUN facilitated the apoptosis of osteosarcoma cells.

PUN inhibited the mobility, migration and invasion of osteosarcoma cells

Next, we used a wound-healing assay to examine the function of PUN in regulating the mobility of osteosarcoma cells. After the osteosarcoma cell lines were treated with 50 μ M PUN, the scratch width was recorded at 0 h, 24 h, 36 h, and 48 h, respectively. By calculating the scratch healing rate, it was demonstrated that PUN significantly repressed the motility of osteosarcoma cells (Fig. 3A). Then, we

detected the metastatic ability of osteosarcoma cells by Transwell assay, and the results demonstrated that the ability of osteosarcoma cells to migrate and invade was significantly lower than that of the control group (Fig. 3B-C). These results illustrated that PUN suppressed the mobility, migration and invasion of osteosarcoma.

PUN treatment changed the expression levels of proteins related to migration and invasion

The above experiments confirmed that PUN inhibited the metastasis of osteosarcoma cells. To pinpoint the inhibitory effect of PUN on osteosarcoma cells, the expression levels of proteins related to migration and invasion were detected by Western blot. The results verified that when osteosarcoma

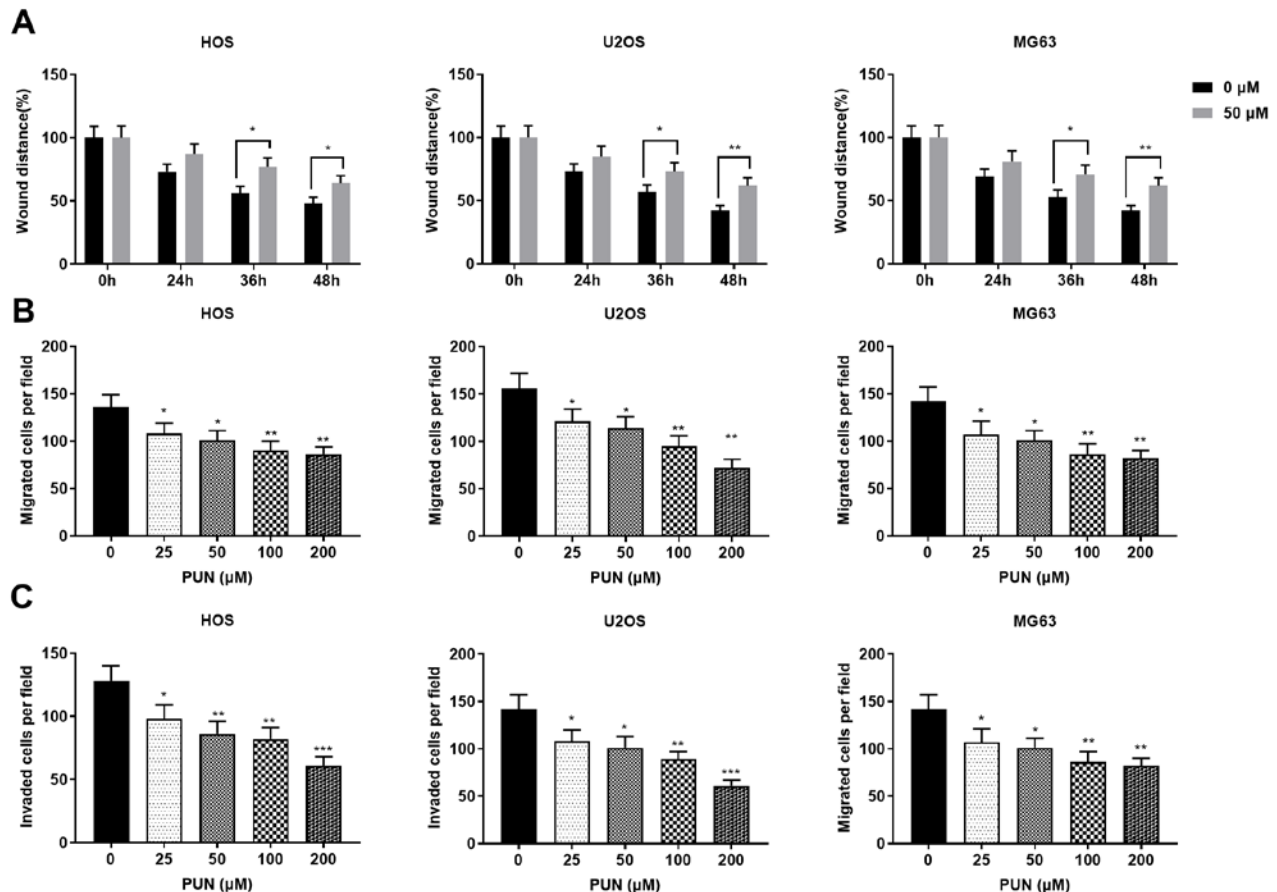


Fig. 3. The effects of punicalagin on the mobility, migration and invasion of osteosarcoma cells. **A)** Wound healing assay was performed to evaluate the mobility of osteosarcoma cells. **B)** The migration of HOS, U2OS and MG63 cells was investigated with Transwell migration assays. **C)** The effects of punicalagin treatment on the invasiveness of HOS, U2OS and MG63 cells were detected by Transwell assay with Matrigel. PUN, punicalagin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

cells were treated with 50 μ M PUN at different time-points (0, 24, 36, and 48 h), the expression level of N-cadherin was significantly decreased. In contrast, E-cadherin expression was signally upregulated (Fig. 4A). The expression levels of MMP-2 and MMP-

9 were markedly decreased in osteosarcoma cells treated with different concentrations, while those of TIMP1 and TIMP2 were observably increased (Fig. 4B). The above results further manifested that PUN inhibited the metastasis of osteosarcoma cells.

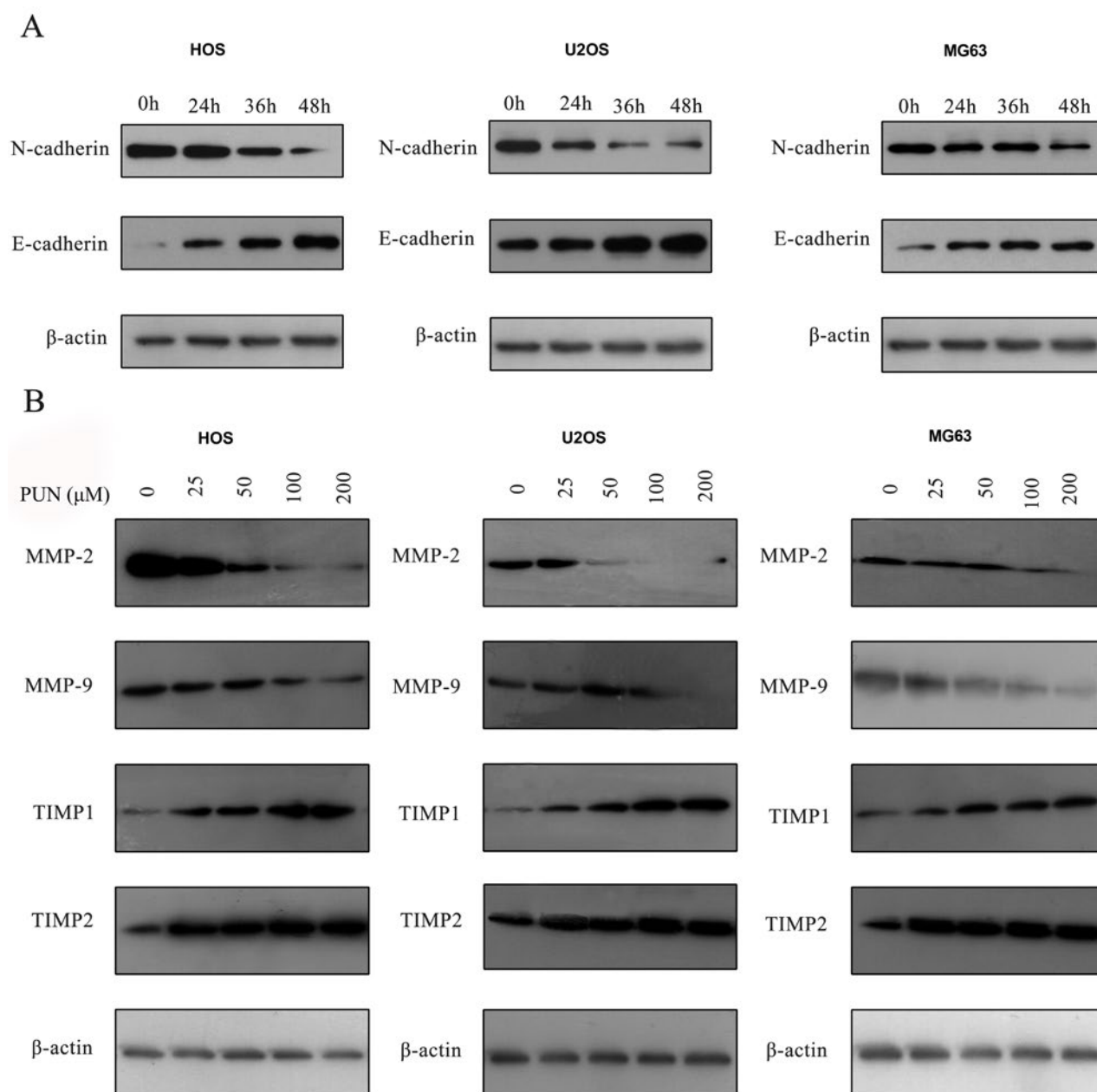


Fig. 4. The effects of punicalagin on the expression levels of proteins related to migration and invasion of cancer cells. **A)** Western blot was used to detect the expression levels of N-cadherin and E-cadherin in osteosarcoma cells treated with 50 μ M PUN for different times (0, 24, 36 and 48 h). **B)** The protein expressions of MMP-2, MMP-9, TIMP1 and TIMP2 were analyzed by Western blot. PUN, punicalagin.

PUN suppressed the proliferation and metastasis of osteosarcoma cells by inhibiting NF- κ B pathway

Previous research reports that drugs inhibiting the NF- κ B signaling pathway can be used to treat osteosarcoma (16, 19). Therefore, we wondered whether PUN suppressed the growth and metastasis of osteosarcoma through impeding NF- κ B signaling. To explore the mechanism of action of PUN in inhibiting the progression of osteosarcoma, we transfected osteosarcoma cells with NF- κ B luciferase reporter vector and then treated them

with PUN for 24 h. As shown, p65 NF- κ B luciferase reporter activity was significantly downregulated by PUN treatment (Fig. 5A). In addition, the expression of p65 in the nucleus of osteosarcoma cells was detected by Western blot, the findings of which demonstrated that it was significantly inhibited by PUN treatment (Fig. 5B). Consistently, we further detected the expression levels of NF- κ B target genes, validating that the expression levels of survivin, XIAP, and CIAP2 were also down-regulated by PUN (Fig. 5C).

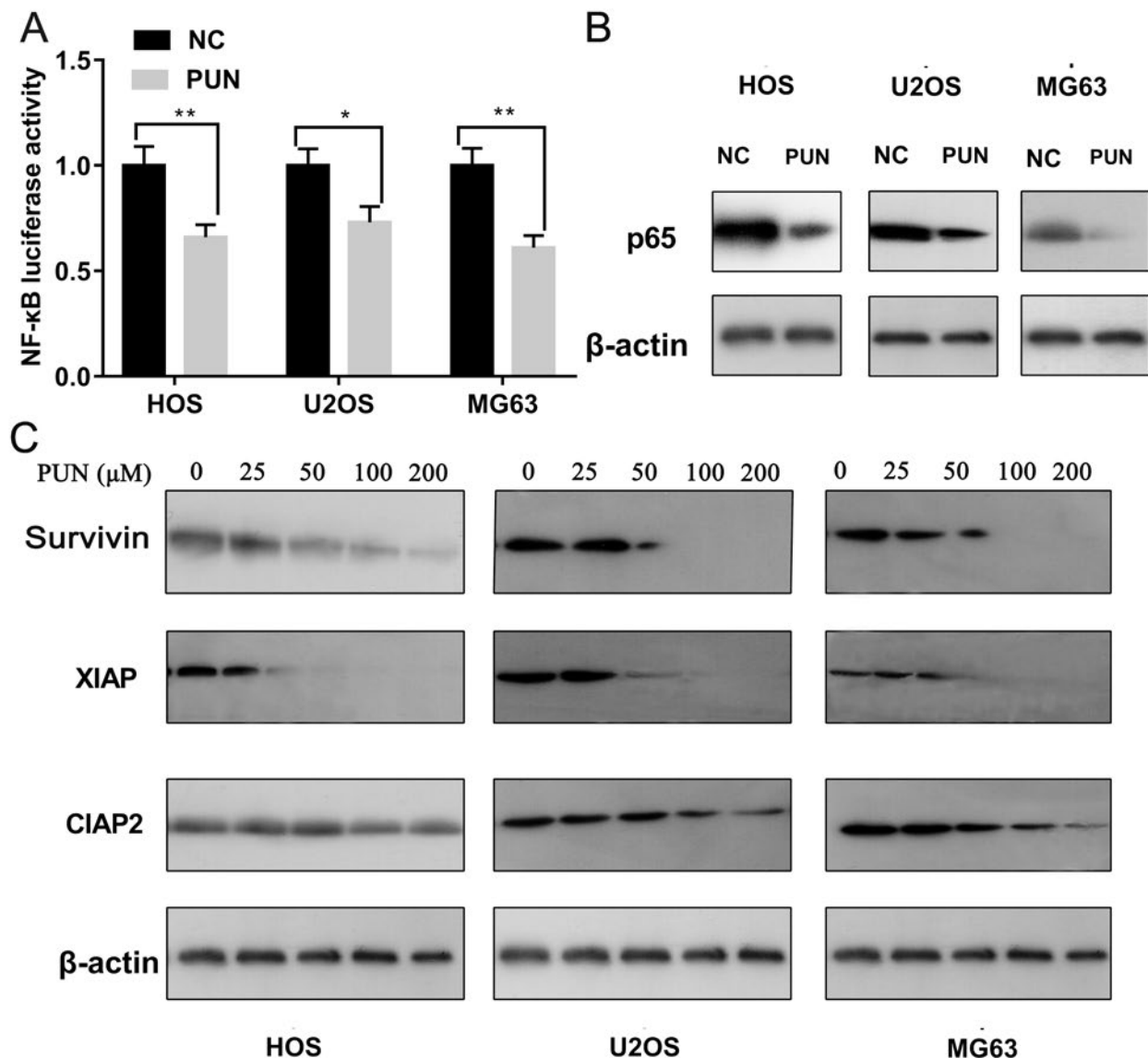


Fig. 5. Punicalagin inhibited the activity of NF- κ B signaling. **A)** The inhibitory function of punicalagin on the transcriptional activity of NF- κ B was detected by luciferase reporter assay. **B)** Western blot was used to detect the expression of p65 in osteosarcoma cells after punicalagin treatment. **C)** Western blot was used to detect the expressions of downstream genes of NF- κ B signaling, including survivin, XIAP and CIAP2 after punicalagin treatment. * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

A considerable number of studies point out that many ingredients extracted from plants may exert an anti-tumor effect, such as curcumin treatment of colon cancer, berberine treatment of esophageal cancer, and baicalein treatment of gastric cancer (21-23). It has been confirmed that these natural compounds will provide a potential strategy for the treatment of tumors. This study demonstrated that PUN suppressed proliferation and metastasis of osteosarcoma by inhibiting the NF- κ B signaling pathway.

Existing studies authenticate that PUN has an anti-tumor effect (24). For example, it promotes apoptosis of prostate cancer cells by up-regulating Bax expression and down-regulating Bcl-2 expression (25, 26). PUN reduces the expression of Bcl-2 in glioma cells and increases activated caspase-9 (10). In this study, we confirmed *in vitro* that PUN inhibited the proliferation of osteosarcoma cells and induced the apoptosis of osteosarcoma cells. Furthermore, it also repressed the mobility, migration and invasion abilities of osteosarcoma cells. In addition, we further examined the expression of related proteins by Western blot. Our data indicated that PUN significantly increased the expression of pro-apoptotic Bcl-2 in osteosarcoma cells, and reduced the expression level of anti-apoptotic Bax. In addition, PUN could also diminish the expression of N-cadherin, facilitate the expression of E-cadherin, and lessen the expressions of MMP-2 and MMP-9, while improving the expressions of TIMP1 and TIMP2. These data indicated that PUN inhibited the malignant biological behaviors of osteosarcoma cells.

In terms of mechanism, PUN can treat a variety of diseases by suppressing multiple signaling pathways. For example, PUN represses the proliferation and invasion of cervical cancer cells by suppressing the Wnt/ β -catenin pathway (26). *Punica granatum* (pomegranate) induces the apoptosis of non-small cell lung cancer cells by promoting the mitochondrial intrinsic pathway (27). In addition, PUN can inhibit the progression of some disease models by suppressing the NF- κ B pathway. For example, PUN may block the progression of Alzheimer's disease by regulating NF- κ B signaling (28). PUN

also regulates neuroinflammation by inhibiting nuclear translocation of p65 and p50 (29). It is widely accepted that NF- κ B is an intracellular signal transduction protein involved in various biological behaviors of cells (15-17, 19, 20). Therefore, it is necessary to investigate whether PUN can inhibit the progression of osteosarcoma by suppressing the NF- κ B signal pathway. In this study, it was observed that PUN inhibited the transcriptional activity of NF- κ B in osteosarcoma cells and the expression of p65. Furthermore, we detected the expression of NF- κ B downstream molecules by Western blot and found that the expression levels of survivin, XIAP and CIAP2 were down-regulated by PUN treatment. Therefore, we confirmed that PUN could reduce the malignancy of osteosarcoma cells by suppressing the NF- κ B signaling pathway.

In conclusion, this study demonstrates that PUN suppresses the growth and metastasis of osteosarcoma cells by inhibiting the NF- κ B signal pathway. In subsequent studies, animal experiments can help further confirm the role of PUN in treating osteosarcoma. As a natural component of fruit, PUN has the advantages of small toxic and side effects and low cost, showing good clinical application prospects. In the following work, it is also worth studying whether it can improve the clinical treatment of osteosarcoma when combined with radiotherapy and chemotherapy.

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MiR-20b is implicated in preeclampsia progression via the regulation of myeloid cell leukemin-1

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Preeclampsia (PE) may induce gestational failure, threatening a significant number of pregnant women. Recently, microRNAs (miRNAs) have been reported to participate in PE progression, whereas the precise functions and potential mechanisms of miR-20b in placental trophoblast cells as well as in PE progression remain poorly understood. In the present study, real-time quantitative polymerase chain reaction (RT-qPCR) analysis was used to detect expressions of miR-20b and myeloid cell leukemin-1 (MCL-1) mRNA. Cell viability was investigated by cell counting kit-8 (CCK-8) assays. Cell invasion and migration abilities were determined by Transwell assays. Western blot was performed to detect MCL-1 protein expressions. The interaction between miR-20b and MCL-1 was investigated by bioinformatics analysis and luciferase activity assay. The results of the study demonstrated that miR-20b was highly expressed in placental tissues of patients with PE. Moreover, miR-20b overexpression inhibited HTR8/SVneo cell proliferation, invasion and migration. Furthermore, MCL-1 was targeted by miR-20b, and MCL-1 restoration could partially attenuate the effect of miR-20b on HTR8/SVneo cells. In conclusion, the results indicate that miR-20b may contribute to PE through inhibiting proliferation, invasion and migration of placental trophoblast cells by targeting MCL-1. Therefore, miR-20b may be used as a notable biomarker for the diagnosis, prevention, and treatment of PE. MiR-20b targeting MCL-1 deserves further investigation in order to explore their potential role in PE.

Preeclampsia (PE) is a serious pregnancy syndrome which usually occurs after 20 weeks of gestation. PE may cause gestational failure, with an incidence of 5-8% (1). PE is characterized by hypertension and proteinuria which is still the main factor for maternal and perinatal morbidity and mortality (2, 3). Mothers and newborns with PE history are prone

to an augmented future threat with type 2 diabetes or cardiovascular disease (4). Abnormal placentation has been confirmed to be associated with PE development (5). Moreover, according to previous studies, inhibition of trophoblast cell invasion may promote PE progression via different pathways (6). PE leads to risks for fetuses and pregnant women. However, the

Key words: preeclampsia; miR-20b; MCL-1

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precise molecular pathogenesis of PE remains largely unknown. Hence, various biomarkers of pregnancy disorders have received considerable attentions.

Investigation of the epigenetic and genetic changes involved in the behavior of trophoblast cells may help to develop promising therapeutic biomarkers for PE treatment (7, 8). MiRNAs are conserved, non-coding RNAs which could regulate their target genes via inhibiting post-transcription (9). Specifically, miRNAs could base pair with the target mRNAs, resulting in translational degradation (10). Moreover, both in normal development and disease onset, miRNAs could regulate varieties of cellular processes via regulation of target genes (11). Emerging studies have shown that miRNAs participated in trophoblast cell proliferation, invasion, migration and apoptosis of human placental trophoblasts (12, 13). Therefore, clarifying the potential roles of miRNAs in regulating behaviors of trophoblasts may be conducive to explore novel biomarkers for PE diagnosis and therapy.

MiR-20b has been reported to serve as an important regulator in various diseases (14, 15). However, whether miR-20b is implicated in trophoblast behaviors and PE pathogenesis still remains unclear. Therefore, the present study focused on the potential roles and mechanisms of miR-20b in PE. In brief, significantly upregulated miR-20b was identified in placental tissues of PE patients. Additionally, results also revealed that miR-20b overexpression could inhibit trophoblast cell proliferation, invasion and migration by targeting MCL-1. In short, the study indicated that miR-20b/MCL-1 axis may serve as a therapeutic target in PE treatment by regulating trophoblast cell proliferation, invasion and migration.

MATERIALS AND METHODS

Tissue samples

A total of 30 pairs of placental tissue samples were collected from pregnant women with PE or normal full term pregnancy females who underwent cesarean section at Jining No.1 People's Hospital. 24 h urine protein ≥ 0.3 g and systolic/diastolic blood pressure ($>140/90$ mm Hg) were diagnosed as PE. All enrolled participants in our study had no history of other systemic illnesses.

The collected placental tissue samples were immediately frozen in liquid nitrogen for further assays. This study was approved by the Institutional Ethics Committee of Jining No.1 People's Hospital, and written informed consent was obtained from each participant.

Cell lines and cell culture

HTR8/SVneo cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) containing 10% FBS. The cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C.

Cell transfection

MiR-20b mimic, inhibitor and the respective negative controls (NC) were designed and synthesized by Ribobio (Guangzhou, China). MCL-1 overexpression vector (pcDNA3.1 MCL-1) was synthesized by Gene pharma (Shanghai, China). Lipofectamine 2000 (Invitrogen) was used to transfect the above vectors into HTR8/SVneo cells referring to the manufacturer's instructions.

RT-qPCR assay

TRIzol reagent (Invitrogen, Carlsbad, USA) was utilized to isolate the total RNAs from cultured HTR-8/SVneo cells and placental tissue samples following the manufacturer's protocol. Prime Script II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) or PrimeScript RT Master Mix (Takara) was used for cDNA synthesis for miR-20b or MCL-1 mRNA. Then, RT-qPCR was performed on an ABI 7900 real-time PCR system (Applied Biosystems, Foster City, USA) with SYBR Premix ExTaq RT-PCR kit (Takara). Thermal conditions were as follows: 95°C for 10 min, and then 40 cycles of 95°C for 10 s and 60°C for 35 s. U6 and GAPDH were used as internal controls for miR-20b and MCL-1, respectively. The relative expression levels were calculated by the 2^{- $\Delta\Delta C_t$} method.

CCK-8 assay

To detect cell viability, CCK-8 assay was performed. The transfected cells were placed into a 96-well plate and CCK-8 solution was added into each well at 24, 48, 72, 96 h post-transfection according to the manufacturer's instructions. Then, the cells were incubated at 37°C for another 2 h. The absorbance at 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell assay

Transwell assays were carried out to determine the influence of miR-20b on HTR8/SVneo cell invasion and migration abilities. In brief, for invasion assay, the chamber was pre-coated with Matrigel (BD, San Jose, CA, USA), and the transfected cells in serum-free medium were inserted into the top chamber. In the meantime, the bottom chamber was filled with medium containing 10% FBS. After the cells had been incubated for 48 h at 37°C, the non-invasive cells were carefully removed by cotton swabs. Afterwards, the invasive cells were fixed and stained. Then, the stained cells were photographed and quantified in 5 random fields under a light microscope (Olympus, Tokyo, Japan). For cell migration assay, the top chamber was not coated with Matrigel.

Western blot

Total proteins in transfected HTR8/SVneo cells were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Then, a BCA assay kit (Thermo Fisher) was used to quantify the protein concentration. Subsequently, 30 µg total proteins were subjected to separation by 10% SDS-PAGE, and then, the proteins were transferred onto PVDF membrane. Subsequently, the membranes were blocked with 5% non-fat milk and incubated with suitable primary antibodies: anti-MCL-1 (ab32087, 1:500; Abcam) and anti-GAPDH (ab9485, 1:2,000; Abcam) at 4°C overnight. Thereafter, membranes

were incubated with HRP-conjugated IgG secondary antibody (ab6721, 1:2,000; Abcam) at room temperature for 2 h. The Pierce ECL kit (Thermo Fisher Scientific, Inc.) was used to detect protein bands. GAPDH was used as an internal control.

Luciferase reporter assay

Wide-type or mutant (WT or MUT) MCL-1 3'-UTR was amplified and inserted into pGL3 vectors (Promega, Madison, WI, USA), respectively. HTR8/SVneo cells were co-transfected with the above luciferase reporter vectors and miR-20b mimics by Lipofectamine 2000. Finally, luciferase activity was determined using Dual-Luciferase Assay Kit (Promega).

Statistical analysis

All the above experiments were performed in triplicate. The SPSS 17.0 version (SPSS Inc. Chicago, IL, USA) was used to perform the statistical analysis. Data was tested using Student's *t*-test. $P < 0.05$ was considered as statistically significant difference.

RESULTS

Analysis of clinical characteristics

As shown in Table I, the weights of newborns in the PE group were lighter than those in the normal group (2123 ± 356.52 g vs 3230 ± 264.85 g, $p = 0.013$).

Table I. Basic information of normal and PE pregnancies

Characteristics	Normal n=30	PE n=30	p-Value
Maternal age (years)	30±1.62	29±1.53	0.512
Birth weight (g)	3230±264.85	2123±356.52	0.013
GA at delivery (weeks)	38.50±1.50	34.30±2.70	0.021
Systolic BP (mmHg)	116.60±6.35	168.70±7.85	<0.001
Diastolic BP (mmHg)	73.79±5.53	112.30±8.62	<0.001
Proteinuria (g/24 hr)	None	4.85±0.73	<0.001
Smoking (n)	0	0	NA

BP: blood pressure; GA: gestational age; NA: not analyzed; PE: preeclampsia.

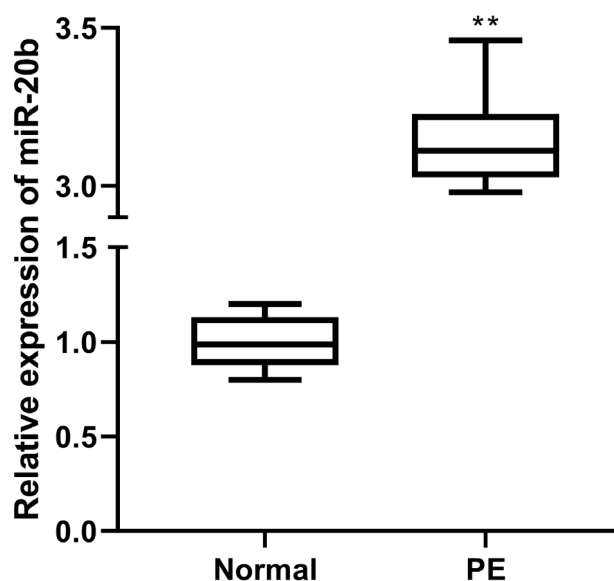


Fig. 1. MiR-20b expression levels were increased in placentas of patients with PE. ** $p < 0.01$.

Furthermore, the gestational ages of delivery in the PE group were smaller than those in the normal group (34.30 ± 2.70 weeks vs 38.50 ± 1.50 weeks, $p = 0.021$). In the PE group, the systolic blood pressure, diastolic blood pressure, and urine protein were higher than those in the control group.

MiR-20b expressions were increased in placentas of patients with PE

A total of 30 pairs of PE and normal pregnant women were enrolled in this study. In particular, patients with PE were diagnosed by systolic/diastolic blood pressure and proteinuria. Firstly, to investigate the functions of miR-20b in PE progression, the levels of miR-20b were measured in the placental tissues of PE patients and healthy subjects. Results showed that miR-20b expression was significantly increased in PE tissues compared with that in the

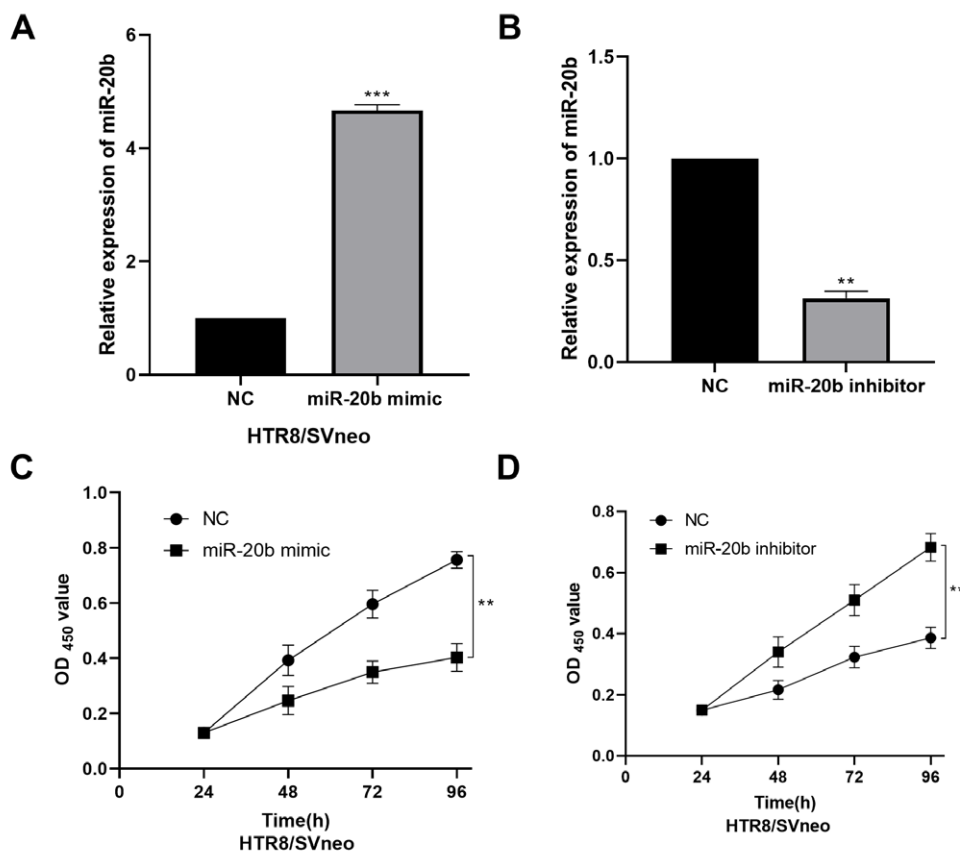


Fig. 2. MiR-20b overexpression could inhibit trophoblast cell proliferation. **A, B**) RT-qPCR was performed to determine the transfection efficiencies of miR-20b mimics or inhibitor in HTR8/SVneo cells. **C, D**) The influence of miR-20b mimics or inhibitor on HTR8/SVneo cell proliferation was detected by CCK-8 assays. ** $p < 0.01$, *** $p < 0.001$.

samples of normal subjects (Fig. 1). These data suggested that dysregulated miR-20b might be required for PE progression.

MiR-20b overexpression inhibited trophoblast cell proliferation

As ectopic miR-20b had been identified in PE, we wondered whether miR-20b could affect placental trophoblast cell proliferation, invasion and migration. Firstly, HTR8/SVneo cells were transfected with miR-20b mimic or inhibitor to overexpress or inhibit

miR-20b expressions. RT-qPCR analysis was used to confirm the transfection efficiencies. As a result, miR-20b was overexpressed by miR-20b mimic and downregulated by miR-20b inhibitor (Fig. 2A and 2B). Thereafter, we performed CCK-8 assays to determine the functions of miR-20b in HTR8/SVneo cell proliferation. Results showed that enrichment of miR-20b effectively inhibited HTR8/SVneo cell viability when compared to the control group (Fig. 2C). Moreover, silence of miR-20b significantly elevated HTR8/SVneo cell viability (Fig. 2D).

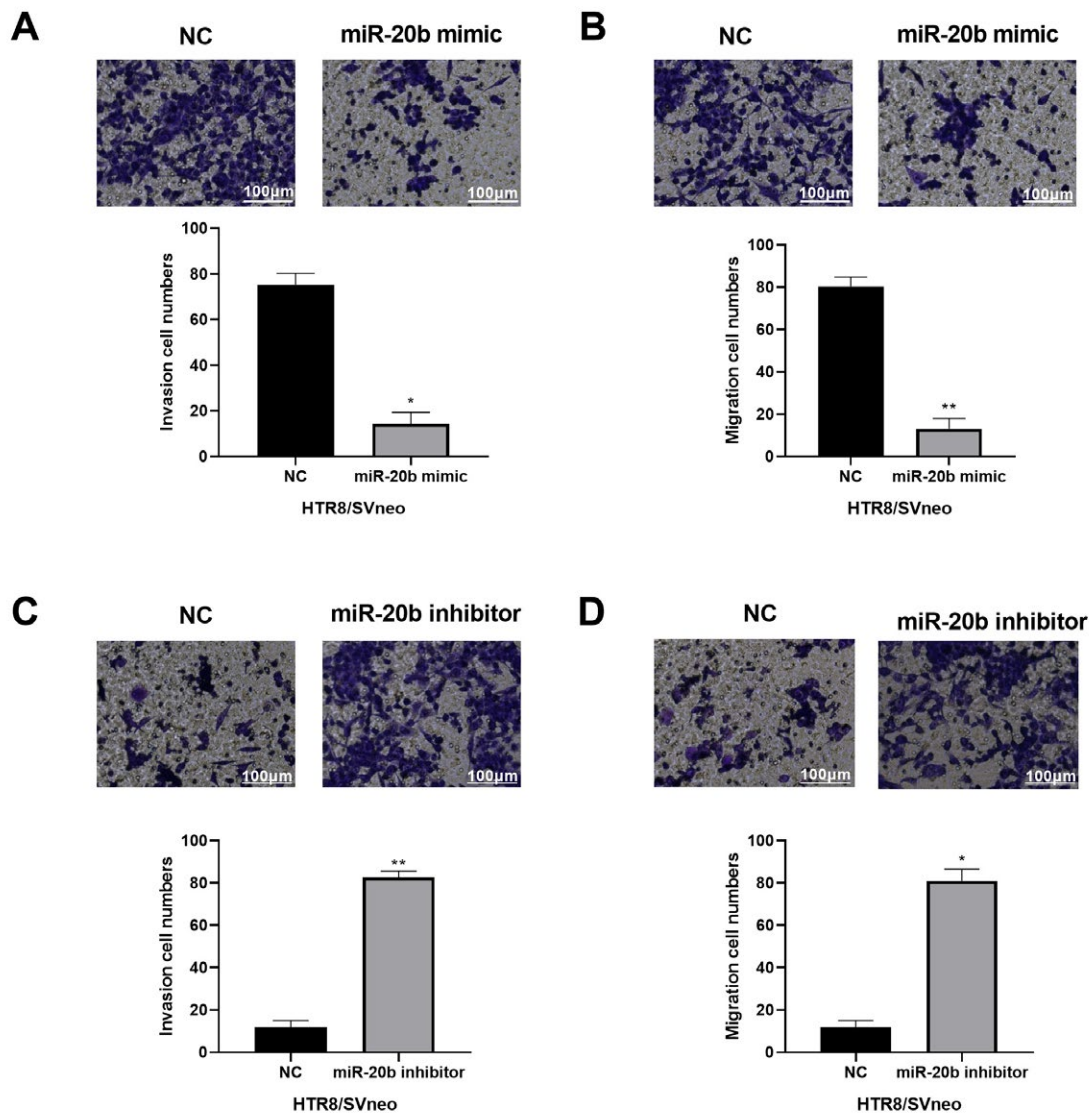


Fig. 3. Overexpression of miR-20b inhibited trophoblast cell invasion and migration. **A, B**) Results of Transwell assays showed that miR-20b overexpression inhibited HTR8/SVneo cell invasion and migration. **C, D**) MiR-20b silence promoted HTR8/SVneo cell invasion and migration. * $p < 0.05$, ** $p < 0.01$.

Overexpression of miR-20b inhibited trophoblast cell invasion and migration

Since miR-20b could inhibit HTR8/SVneo cell proliferation, the Transwell assays were further performed to detect the effects of miR-20b on HTR8/SVneo cell invasion and migration. As expected, the invasion and migration abilities were prominently blocked by accumulation of miR-20b (Fig. 3 A, B). On the other hand, the invasive and migratory abilities of HTR8/SVneo cells were promoted by miR-20b inhibition (Fig. 3 C, D). Taken together, the results indicated that miR-20b overexpression could suppress HTR8/SVneo cell proliferation, invasion and migration abilities.

MCL-1 was a potential target of miR-20b

A putative target gene of miR-20b was next explored. Bioinformatics analysis demonstrated that there were binding sites of miR-20b in MCL-1 3'-UTRs (Fig. 4A), indicating that MCL-1 may be a candidate target for miR-20b. Thereafter, luciferase reporter assay was carried out to validate this correlation. The results indicated that miR-20b mimic obviously decreased the luciferase activity of HTR8/SVneo cells transfected with MCL-1-WT, whereas this reduction was lost in HTR8/SVneo cells transfected with MCL-1-MUT (Fig. 4B). Subsequently, the regulatory effects of miR-20b on MCL-1 expressions were detected. As shown in Fig.

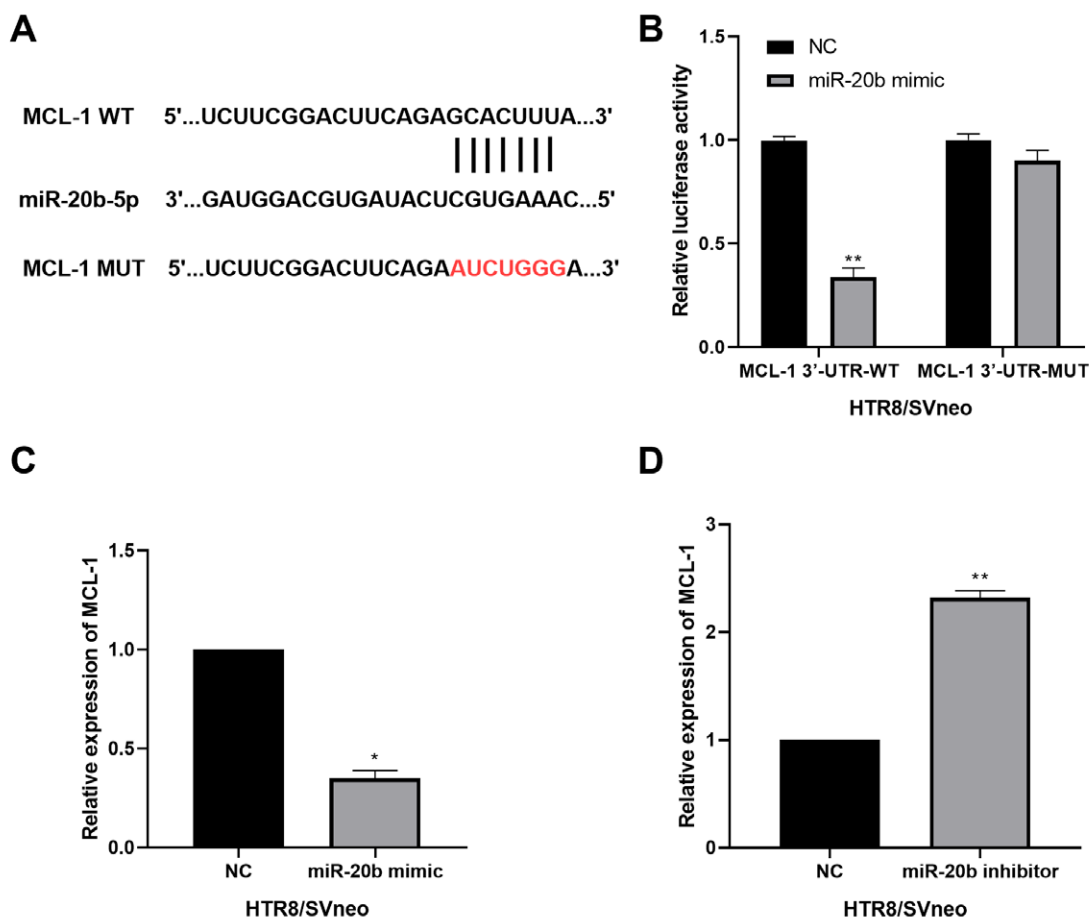


Fig. 4. MCL-1 was a target for miR-20b. **A)** The potential binding sites of miR-20b in MCL-1 3'-UTRs were predicted by bioinformatics analysis. **B)** Luciferase activities in HTR8/SVneo cells transfected with MCL-1-WT vector were significantly decreased while luciferase activities in cells transfected with MCL-1-MUT vector were not obviously influenced. **C, D)** MiR-20b regulated MCL-1 expressions as demonstrated by results of RT-qPCR. * $p < 0.05$, ** $p < 0.01$.

4 C, D, overexpression of miR-20b impaired MCL-1 expressions while knockdown of miR-20b enhanced the MCL-1 expressions. All these data showed that MCL-1 was a direct target of miR-20b.

MCL-1 was required for miR-20b-mediated proliferation, invasion and migration of placental trophoblast cells

As MCL-1 had been confirmed as a target of miR-

20b, we next performed rescue assays to investigate whether MCL-1 participated in the functions of miR-20b in HTR8/SVneo cell proliferation, invasion and migration. Briefly, miR-20b inhibitor and MCL-1 siRNA were co-transfected into HTR8/SVneo cells, and the transfection efficiencies were confirmed by detecting MCL-1 levels in different groups. The results demonstrated that MCL-1 siRNA prominently inhibited MCL-1 expressions and partially reversed

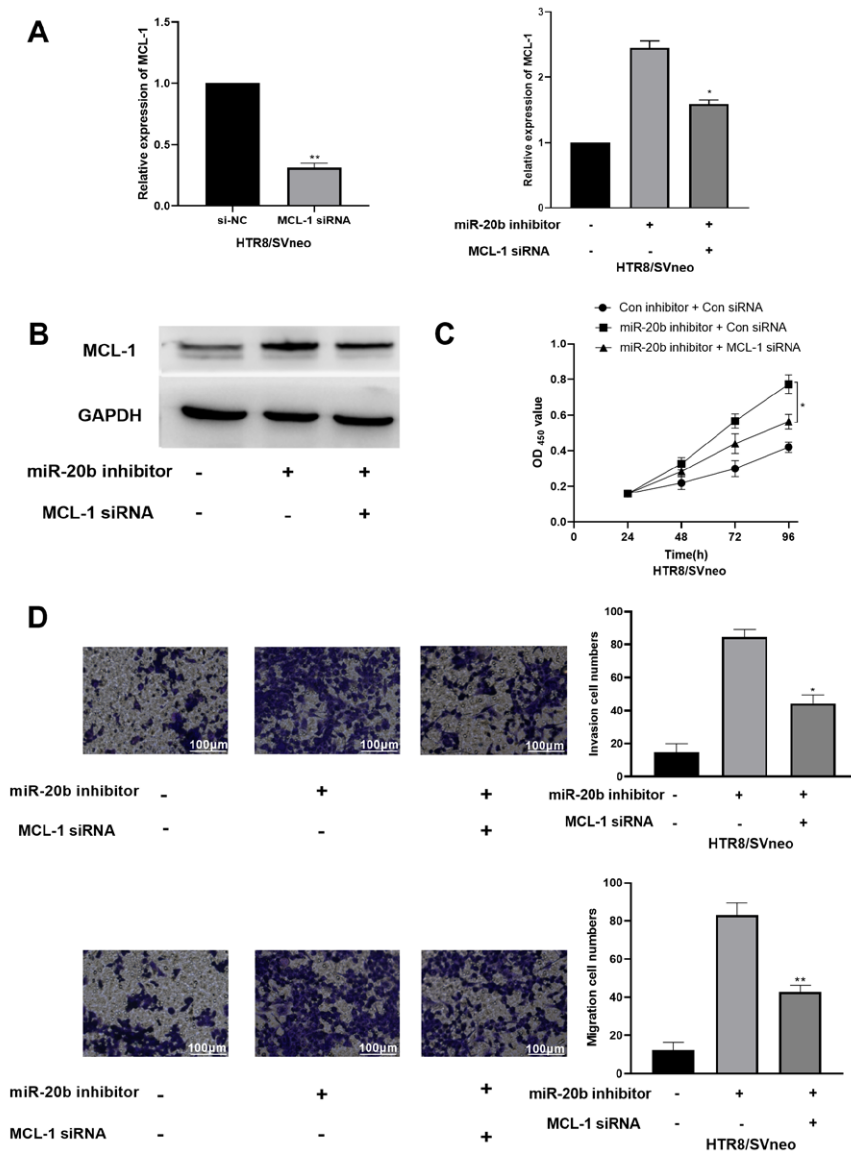


Fig. 5. MCL-1 was required for miR-20b-mediated proliferation, invasion and migration of placental trophoblast cells. **A, B)** MCL-1 siRNA prominently inhibited MCL-1 expressions and the increase in MCL-1 expressions caused by miR-20b inhibitor could be reversed by MCL-1 siRNA. **C, D)** The elevated abilities of HTR8/SVneo cell proliferation, invasion and migration mediated by miR-20b inhibitor were attenuated by MCL-1 knockdown. * $p < 0.05$, ** $p < 0.01$.

the increase in MCL-1 expressions caused by miR-20b inhibitor (Fig. 5A, B). Similarly, downregulation of MCL-1 also reversed the enhanced HTR8/SVneo cell viability caused by miR-20b inhibition (Fig. 5C). In addition, the elevated abilities of HTR8/SVneo cell invasion and migration mediated by miR-20b inhibitor were also attenuated by MCL-1 knockdown (Fig. 5D). Collectively, all these data showed that MCL-1 was essential in the functions of miR-20b in regulating placental trophoblast cell progression.

DISCUSSION

PE is a multi-system disease, which is characterized by elevated blood pressure (systolic/diastolic blood pressure >140/90 mm Hg) and proteinuria (24 h urine protein $\geq$ 0.3 g) after 20 weeks of pregnancy. PE remains one of the main factors for maternal diseases and mortalities. However, the relevant mechanisms underlying PE pathogenesis has not been fully elucidated. A recent research had manifested that the immune system, angiogenesis, trophoblast invasion and proliferation are the basis of placenta formation, being regulated by dysregulated miRNAs in different degrees (16, 17). Another study revealed that a characteristic gene profile in PE was conducive to PE progression and offers novel biomarkers for PE therapies (18). Recently, a multitude of studies have demonstrated that aberrant expression of miRNAs in PE placenta plays crucial roles in PE progression (19). For instance, miR-181a-5p was confirmed to suppress HTR-8/SVneo cell invasion and migration via regulation of IGF2BP2 (20); suppression of miR-18a could inhibit human trophoblast cell invasion and promote apoptosis through regulating the estrogen receptor alpha gene (21); miR-210 upregulation impaired human trophoblast cell proliferation, migration, and invasion by attenuation of Notch1 expressions (22). However, the expression levels and exact functions of miR-20b in PE still remain largely unclear. Therefore, in the current study, we focused on the functions and potential mechanisms of miR-20b in PE progression.

According to previous evidence, miR-20b was

involved in cell behavior. For example, miR-20b promoted Ewing's sarcoma cell proliferation via upregulation of oncogene c-Myc and targeting TGF-beta receptor II (23); miR-20b promoted cervical cancer invasion via targeting tissue inhibitor of metalloproteinase 2 and being regulated by human papilloma virus E6 (24); moreover, miR-20b contributed to high glucose-induced podocyte apoptosis by targeting SIRT7 (25). In our study, the results showed that miR-20b was upregulated in PE placenta tissues. Additionally, we also found that miR-20b overexpression inhibited human trophoblast cell proliferation, migration, and invasion whereas miR-20b silence had the opposite functions. Furthermore, MCL-1 was identified as a direct target of miR-20b in PE. The functions of miR-20b inhibitor in cell viability, invasion and migration were partially counteracted by MCL-1 knockdown.

Collectively, the data in our study demonstrated that expressions of miR-20b in patients with PE were increased. The proliferation, invasion and migration abilities of HTR8/SVneo cells were significantly inhibited by miR-20b overexpression while they were facilitated by miR-20b knockdown. Moreover, MCL-1 was identified as a direct target of miR-20b. MCL-1 knockdown could abrogate the effects of miR-20b inhibition on HTR8/SVneo cell viability, invasion and migration. Therefore, miR-20b may serve as a potential therapeutic target for PE.

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IL-22 relieves sepsis-induced liver injury via activating JAK/STAT3 signaling pathway

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The purpose of this study was to investigate the influence of interleukin(IL)-22 on the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway and sepsis-induced liver injury in rats. A total of 48 Sprague-Dawley rats were randomly divided into sham-operated group (n=12), model group (n=12), low-dose group (n=12) and high-dose group (n=12). Next, rat models of sepsis-induced liver injury were established through cecal ligation and puncture (CLP). At 12 h after surgery, blood was collected by heart puncture to detect liver function of the rats. It was found that the activity of alanine aminotransferase (ALT) and aspartame aminotransferase (AST) and the content of total bilirubin were reduced in low-dose group and high-dose group. Hematoxylin-eosin (HE) staining results revealed that after treatment with IL-22, the liver injury was relieved compared with model group. Moreover, the results of TUNEL staining assay revealed that the apoptosis level of liver cells declined after treatment with IL-22. Enzyme-linked immunosorbent assay (ELISA) results demonstrated that the levels of IL-6 and TNF- α were reduced, while the level of IL-10 was increased after treatment with IL-22. Moreover, it was discovered that the SOD content was overtly elevated in low-dose and high-dose groups compared with that in the model group. Finally, using Western blotting, it was confirmed that in comparison with the model group, the levels of Bcl-2/Bax and JAK/STAT3 signaling pathway-related proteins were markedly raised, while the level of Caspase-3 was decreased in the low-dose and high-dose groups. In conclusion, IL-22 can improve liver function, reduce the apoptosis level of liver cells, the expression of apoptosis-related proteins and the release of inflammatory factors, and alleviate liver injury by activating the JAK/STAT3 signaling pathway.

Sepsis, an organ dysfunction caused by the dysregulated host response to infection, can lead to multiple organ damage in the body, with complex pathogenesis and mortality rate of 35-60% (1). The liver has abundant blood supply and important functions including detoxification, metabolism and immunity, which can be easily damaged by sepsis,

therefore it is one of the most common damaged organs (2). Severe infection can trigger the entry of a large number of Gram-negative bacilli into the body and the release of massive lipopolysaccharides. If the infection is beyond the ability of detoxification and phagocytosis of the liver, endotoxins will accumulate in the body, thereby damaging the liver

Key words: IL-22, sepsis, liver injury, JAK/STAT3 signaling pathway

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and resulting in such clinical symptoms such as septic shock that is life-threatening in severe cases (3). Hung et al. (4) conducted research on 230 patients with sepsis and found that 28.7% of the patients suffered from sepsis-induced liver injury, with a high mortality rate. Interleukin (IL)-22, discovered in 2000, is a member of the IL-10 family and is also a cytokine secreted by T helper 22 (Th22) cells, and it specifically binds to IL-22 receptors (IL-22Rs) in the liver, intestinal canal and kidneys to protect organs (5). A study by Tripathi et al. (6) denoted that the binding of IL-22 to IL-22Rs in the liver can promote the regeneration of liver cells and lead to apoptosis of hepatic stellate cells, thereby protecting the liver tissues. The Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway, a cell transduction pathway activated by cytokines, participates in many biological processes such as cell apoptosis, proliferation, embryonic development, and liver regeneration (7). Zhang et al. (8) revealed in a study that the binding of IL-22 to IL-22Rs can activate the JAK/STAT3 signaling pathway, elevate the levels of phosphorylated (p)-JAK and p-STAT3, reduce the aggregation of white blood cells, and mitigate ischemia-reperfusion injury of liver cells. However, the effects of IL-22 on the JAK/STAT3 signaling pathway and sepsis-induced liver injury in rats have not been studied to date. In this study, therefore, the role of IL-22 in the liver of rats with sepsis-induced liver injury and its molecular mechanism were explored by constructing rat models of sepsis-induced liver injury, in order to discover new therapeutic targets and strategies for sepsis-induced liver injury.

MATERIALS AND METHODS

Study animals and grouping

A total of 48 adult male Sprague-Dawley rats weighing 260–280 g purchased from Shanghai Experimental Animal Resource Center (Shanghai, China) were adaptively fed in the environment with a humidity of $(45\pm5)\%$ at $(23\pm2)^{\circ}\text{C}$ under a circadian rhythm (a light:dark cycle of 12:12 h) for one week, with free access to food and water. Next, they were randomly divided into a sham-operated group ($n=12$), a model group ($n=12$), a low-dose group ($n=12$) and a high-

dose group ($n=12$). Recombinant human IL-22 (Sigma, St. Louis, MO, USA) was intraperitoneally injected into the rats in the low-dose and high-dose groups at the dose of 0.25 mg/kg and 0.50 mg/kg, respectively, while an equal volume of normal saline was intraperitoneally injected into the rats in the sham-operated and model groups. The rats in the four groups were subjected to fasting for 12 h prior to surgery. Thereafter, cecal ligation and puncture (CLP) were adopted to construct rat models of sepsis-induced liver injury. The rats in the sham-operated group received the same treatment as that in the model group, except for cecal ligation.

The experiment in this study was performed according to the relevant provisions of the NIH Guide for the Care and Use of Laboratory Animals, and the experimental protocols were reviewed and approved by the Animal Ethics Committee of Foresea Life Insurance Guangzhou General Hospital Animal Center.

Modeling of sepsis-induced liver injury

Rat models of sepsis-induced liver injury were established by CLP (9). Specifically, the rats were anesthetized with 1% pentobarbital sodium (Sigma, St. Louis, MO, USA) at a dose of 40 mg/kg *via* intraperitoneal injection and fixed in the supine position on an experimental operating table with their limbs bound. Then, the rats underwent depilation on the abdomen with depilatory cream, and the skin of the abdomen was disinfected with iodine tincture and covered with a sterile sheet to expose the abdominal skin. Next, the skin was cut open along the midline of the abdomen using ophthalmic scissors, the fatty tissues were bluntly dissected with a tweezer, and a scalpel was used to cut the muscle layer and peritoneum of the abdomen along the linea alba to expose the cecum. Thereafter, the cecum was pulled out of the abdominal cavity, in which the contents were squeezed to the distal end. Afterwards, arterial branches of the cecum and ileocecum were ligated using a 4# sterile silk thread at 1/4 of the cecum from the end, without ligating the ileum and mesocaecum blood vessels. Subsequently, an indwelling needle (BD 16G) was used to penetrate the distal and proximal ends of the cecal mesentery at the ligation section, some contents were squeezed out, and the cecum was put back into the abdominal cavity. Then, the incision of the abdominal wall was sutured with a silk thread layer by layer. Next, the rats were resuscitated with pre-warmed

sodium lactate Ringer's injection (5 mL/100 g) through intracutaneous injection on the back and returned to the cage, with free access to water and food.

Examination of liver function

At 12 h after the operation, whole blood (5 mL) was drawn from rats by cardiac puncture, and centrifuged at 3000 rpm for 30 min to collect the upper serum for later use. The levels of ALT, AST and total bilirubin in serum were measured using the ELISA kits for ALT, AST and serum total bilirubin and a microplate reader at the wavelength of 450 nm strictly in accordance with the instructions.

Detection of liver injury via hematoxylin-eosin staining assay

The rats in each group were sacrificed 12 h after the operation, and *hematoxylin-eosin* HE staining assay (Boster, Wuhan, China) was conducted to detect liver injury. The liver tissues of the dead rats were separated, rinsed with pre-cooled phosphate-buffered saline (PBS) to remove bloodstains and fixed with 10% formalin for 72 h. Next, the tissues were dehydrated with absolute ethanol and 95%, 90%, 80%, 60%, and 50% ethanol, permeabilized with xylene, and embedded in paraffin to prepare into paraffin-embedded tissue blocks. Thereafter, the tissue blocks were cut into 5 μ m-thick sections using a microtome, deparaffinized with xylene, and dehydrated with anhydrous ethanol, 95% ethanol, 90% ethanol, 80% ethanol, 60% ethanol and 50% ethanol, respectively. Then these blocks were stained with hematoxylin for 10 min, and rinsed with tap water. Afterwards, they were differentiated with hydrochloric acid alcohol for 20 s, rinsed with tap water, and dehydrated with 70% and 80% ethanol for 5 min, respectively. Next, the tissue blocks were stained by eosin for 15 min and sealed with neutral resins after dehydration with graded ethanol and permeabilization with xylene. Lastly, the morphology of liver tissues was observed in the bright field of view under a microscope, and the BI-200 multifunctional pathological image analysis system was employed to observe the pathological changes of rat liver tissues in each group.

Determination of apoptosis level of liver cells through TUNEL staining assay

The level of cell apoptosis in rat liver tissues was examined *via* TUNEL staining strictly *as per* the instructions of the TUNEL kit (Wuhan Beyotime

Biotechnology Co., Ltd., Wuhan, China). Specifically, the rat liver tissue sections were deparaffinized, soaked in 3% H₂O₂ for 10 min, washed with PBS for 3 times, and incubated with proteinase K in a wet box at 37°C for 10 min. Next, they were washed with PBS for 3 times, incubated with the mixture of TdT and DIG-d-UTP solutions in the wet box at 4°C for 2 h, washed with PBS for 3 times, and blocked with blocking solution at room temperature for 30 min. After that, the sections were added dropwise with biotinylated digoxin antibody (diluted at 1:100) for incubation in the wet box at 37°C for 40 min, rinsed by PBS for 3 times, and incubated with SABC-FITC secondary antibody (diluted at 1:100) in the wet box at 37°C for 40 min. Later, they were rinsed by PBS for 3 times and sealed with anti-fluorescence quenching solution. Next, a confocal fluorescence microscope was adopted for observation and photographing, and the number of apoptotic cells (cells with yellow-green fluorescence under the microscope) in rat liver tissues was calculated.

Examination of inflammatory factor content in liver tissues by ELISA

ELISA kits for IL-6, IL-10 and TNF- α (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China) were utilized to detect the content of inflammatory factors in rat liver tissues in each group strictly according to the instructions. The optical density (OD) of samples in each group was read using the microplate reader at the wavelength of 450 nm, standard curves were plotted, and the content of IL-6, IL-10 and TNF- α in each group of samples was calculated.

Determination of SOD activity in liver tissues via hydroxylamine method

The activity of SOD in rat liver tissues in each group was determined *via* hydroxylamine method in strict accordance with the instructions of the kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China). Briefly, tissue samples were mixed in a vortex mixer and incubated at room temperature for 30 min, and the activity of SOD in each group of samples was measured using a microplate reader at the wavelength of 450 nm.

Measurement of expression level of related proteins through Western blotting

The liver tissues of each group of rats were cut into

pieces, weighed, and added with an appropriate volume of radioimmunoprecipitation assay (RIPA) lysis buffer (1 mg/1 mL). Next, they were homogenized until there were no visible tissues, and centrifuged at 12000 rpm and 4°C for 10 min. Thereafter, the supernatant was collected for later use. The concentration of proteins in each group was detected using a bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China). Then, loading buffer was prepared and boiled for 10 min to inactivate the proteins. Next, the same volume of loading buffer was added to the sodium dodecyl sulphate (SDS) polyacrylamide gel for electrophoresis, and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). After that, the membrane was blocked with freshly prepared 5% skimmed milk powder for 1 h, and the target bands were cut, incubated with monoclonal antibodies against Bax, Bcl-2, Caspase-3, p-JAK, JAK, p-STAT3, STAT3, p-p38, p38 and GAPDH (Cell Signaling Technology, Danvers, MA, USA, diluted at 1:1000) at 4°C overnight, washed with Tris-buffered saline with Tween®20 (TBST) for 3 times (5 min/time) and incubated with the secondary antibody (diluted at 1:5000) at room temperature for 1 h, followed by washing with TBST for 3 times. Afterwards, an appropriate volume of electrochemiluminescence (ECL) solution (solution A and solution B were mixed at 1:1) (Thermo Fisher Scientific, Waltham, MA, USA) was added for image development in a visualizer. Lastly, ImageJ software was adopted to calculate the gray value of protein bands, and the expression level of related proteins in rat liver tissues in each group was calculated.

Statistical analysis

The data in this study were expressed as mean $\pm$ standard deviation and analyzed by Statistical Product and Service Solutions (SPSS) 22.0 software (SPSS Inc., Chicago, IL, USA). Survival curves were plotted through Kaplan-Meier. Analysis of variance (ANOVA) was utilized for intergroup comparison, whereas Bonferroni test was adopted for intragroup comparison. Data showing homogeneity of variance were analyzed using ANOVA, while those showing heterogeneity of variance were analyzed by the Welch method. $P < 0.01$ suggested that the difference was statistically significant.

RESULTS

Changes of liver function in rats

Immediately after 12 h of modeling, the rats were sacrificed, and the serum was separated to detect the activity of ALT and AST and the concentration of total bilirubin. The results (Fig. 1) showed that the activity of ALT and AST and the total bilirubin concentration were significantly higher in the model group than those in the sham-operated group, low-dose group and high-dose group ($P < 0.01$).

Damage to liver tissues

The rats were sacrificed 12 h after surgery, and liver tissues were isolated for liver injury detection by HE staining. The results shown in Fig. 2 revealed that in comparison with the sham-operated group, the model group showed obvious denaturation of

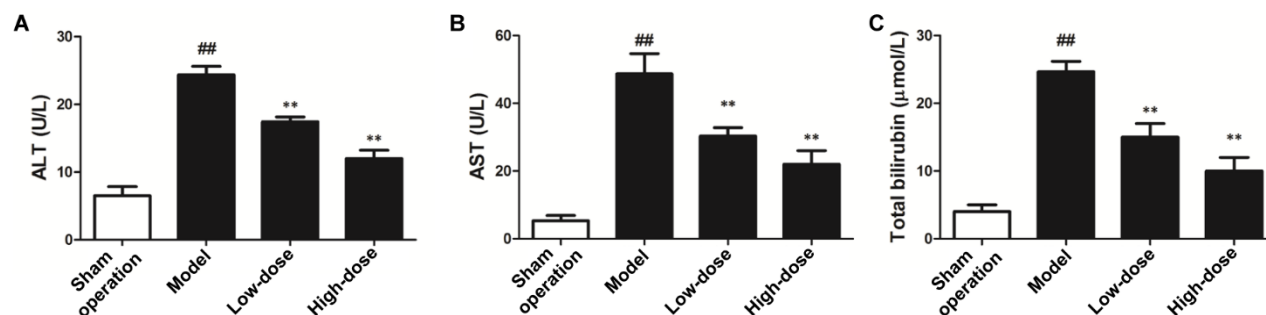


Fig. 1. (A) ALT activity, (B) AST activity, and (C) concentration of total bilirubin. Compared with those in the model group, the serum ALT and AST activity and total bilirubin concentration are remarkably decreased in sham-operated group, low-dose group and high-dose group. ^{##} $P < 0.01$ vs sham-operated group, ^{**} $P < 0.01$ vs model group.

liver cells, significant congestion of hepatic sinusoid, and infiltration of a large number of neutrophils. In comparison with the model group, the denaturation of liver cells, congestion of hepatic sinusoid, infiltration of neutrophils, and liver injury were relieved in the low-dose group and high-dose group after treatment with IL-22.

Apoptosis level of cells in liver tissues

At 12 h after surgery, the rats were sacrificed to separate the liver tissues, and TUNEL staining assay was performed to detect the apoptosis level of cells

in the liver tissues. It was found that the apoptosis level of the cells was markedly higher in the model group than that in the sham-operated group ($P < 0.01$), whereas it clearly declined in the low-dose group and high-dose group after treatment with IL-22 compared with that in the model group ($P < 0.01$) (Fig. 3).

Content of inflammatory factors in liver tissues

After the liver was separated from the rats sacrificed 12 h after surgery, the levels of inflammatory factors therein were measured by ELISA, and the results (Fig. 4) revealed that in comparison with the sham-

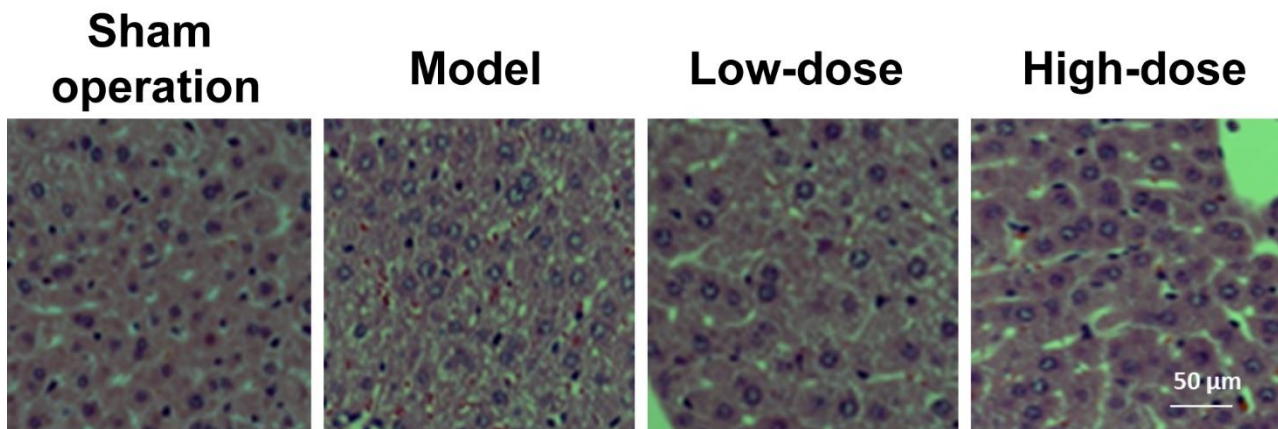


Fig. 2. Liver injury detected via HE staining assay (Scale bar = 50 μm) (magnification: 200 $\times$).

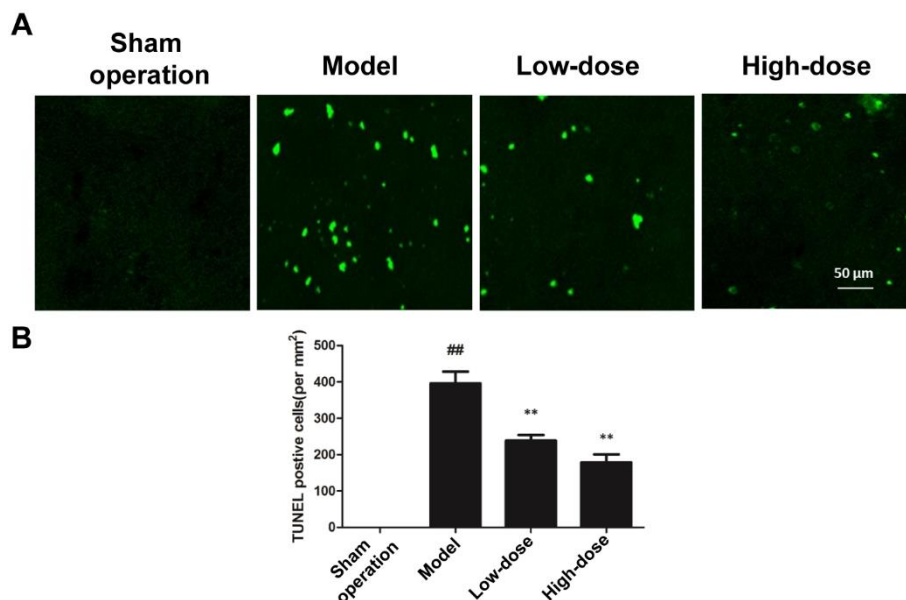


Fig. 3. Apoptosis level of liver cells measured through TUNEL staining assay (magnification: 400 $\times$). The apoptosis level of rat liver cells is notably higher in the model group than that in the sham-operated group, low-dose group and high-dose group. ## $P < 0.01$ vs sham-operated group, ** $P < 0.01$ vs model group.

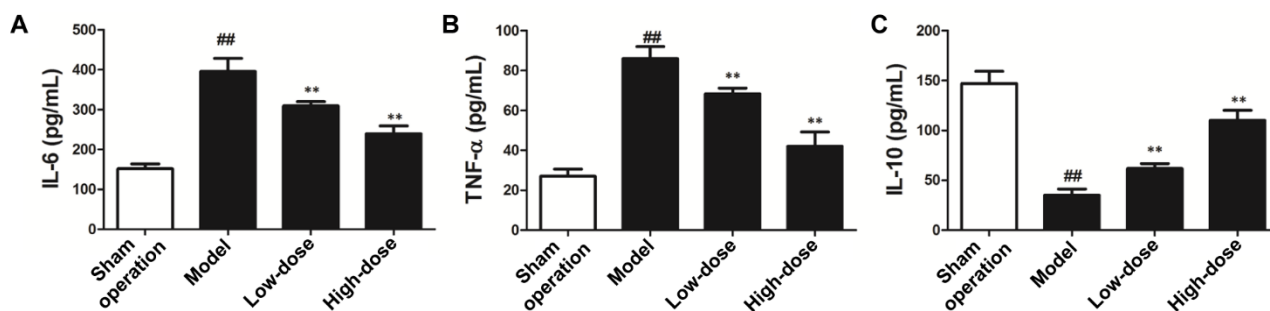


Fig. 4. Content of inflammatory factors in rat liver tissues measured *via* ELISA. **A)** Level of IL-6, **(B)** Level of TNF- α , and **(C)** Level of IL-10. The levels of IL-6 and TNF- α in the rat liver tissues in model group are significantly higher than those in sham-operated group, low-dose group and high-dose group, while the level of IL-10 in model group is significantly lower than that in the other three groups. ^{##} $P < 0.01$ vs sham-operated group, ^{**} $P < 0.01$ vs model group.

operated group, the model group exhibited increased levels of IL-6 and TNF- α ($P < 0.01$) and an obviously reduced level of IL-10 in the liver tissues ($P < 0.01$), while the opposite trends were found in the low-dose group and high-dose group after treatment with IL-22 ($P < 0.01$).

SOD activity in liver tissues

After the rats were sacrificed 12 h post-surgery, the liver tissues were isolated to detect SOD activity therein. It was revealed that the SOD content in liver tissues in the model group was equivalent to that

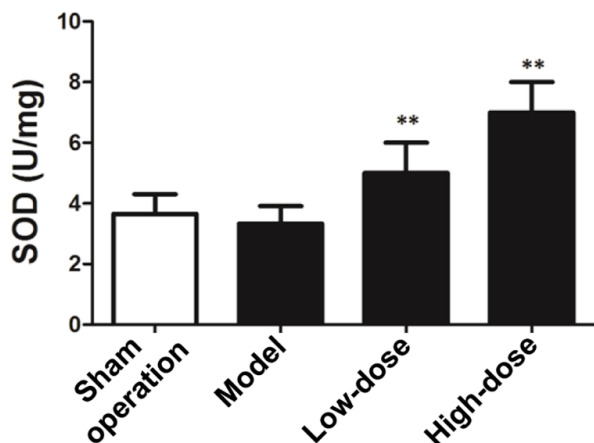


Fig. 5. SOD activity in liver tissues detected *via* ELISA. The SOD activity in model group is equivalent to that in sham-operated group and obviously lower than that in low-dose group and high-dose group. ^{**} $P < 0.01$ vs model group.

in the sham-operated group ($P > 0.05$), while after treatment with IL-22, it overtly elevated in the low-dose and high-dose groups compared with that in the model group ($P < 0.01$) (Fig. 5).

Expression levels of apoptosis-related proteins in liver tissues

After the liver was separated from rats sacrificed 12 h after surgery, Western blotting was employed to measure the expression levels of apoptosis-related proteins. The results (Fig. 6) revealed that compared with those in sham-operated group, the expression level of Caspase-3 was clearly increased ($P < 0.01$), and the Bcl-2/Bax was evidently decreased in the model group ($P < 0.01$). In comparison with the model group, the low-dose and high-dose groups displayed a significantly lowered expression level of Caspase-3 ($P < 0.01$) and a notably raised Bcl-2/Bax ($P < 0.01$).

Effects of IL-22 on the JAK/STAT3 signaling pathway

At 12 h after surgery, the rats were sacrificed to isolate the liver tissues, and then the expression level of JAK/STAT3 signaling pathway-related proteins in the liver tissues was measured. It was found that the expression levels of p-JAK, p-STAT3 and p-p38 were distinctly lower in the model group than those in the sham-operated group ($P < 0.01$). In comparison with the model group, these levels were markedly raised in the low-dose and high-dose groups after treatment with IL-22 ($P < 0.01$) (Fig. 7).

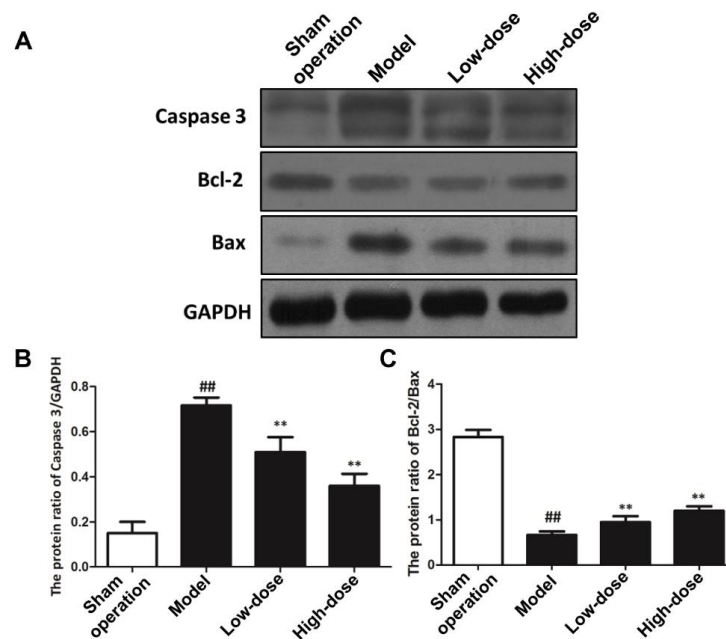


Fig. 6. Expression levels of apoptosis-related proteins in rat liver tissues measured through Western blotting. *A)* Protein bands, *(B)* Statistical graph of Caspase-3, and *(C)* Statistical graph of Bcl-2/Bax. The expression level of Caspase-3 is overtly higher in model group than that in sham-operated group, low-dose group and high-dose group, while the Bcl-2/Bax is significantly lower in model group than that in the other three groups. ## $P < 0.01$ vs sham-operated group, ** $P < 0.01$ vs model group.

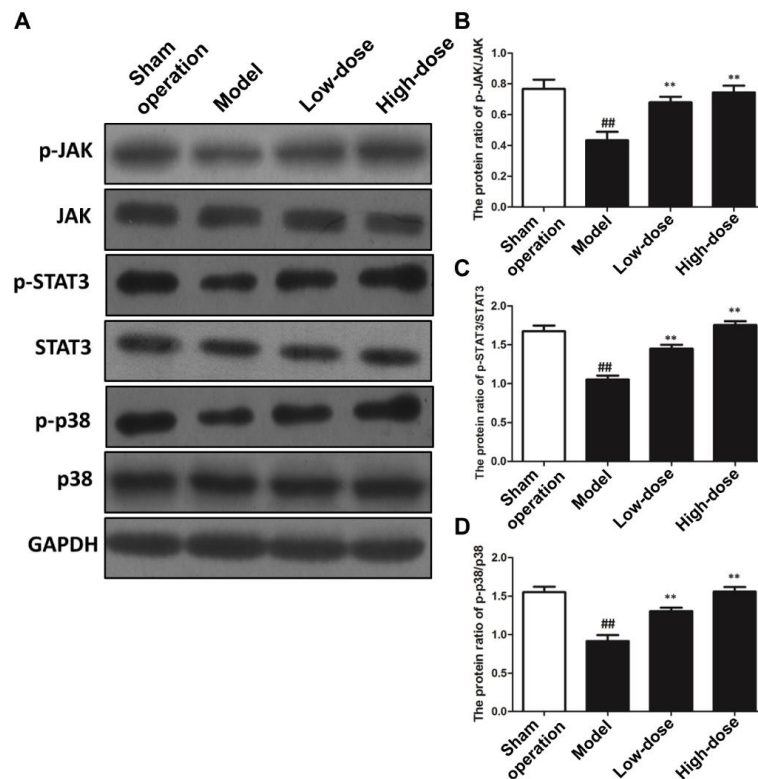


Fig. 7. Expression levels of JAK/STAT3 signaling pathway-related proteins detected through Western blotting. *A)* Protein bands, *(B)* expression level of p-JAK, *(C)* expression level of p-STAT3, and *(D)* Expression level of p-p38. The expression levels of p-JAK, p-STAT3 and p-p38 in liver tissues are clearly lower in model group than those in sham-operated group, low-dose group and high-dose group. ## $P < 0.01$ vs sham-operated group, ** $P < 0.01$ vs model group.

DISCUSSION

As a systemic inflammatory disease caused by infection, sepsis leads to acute liver injury by activating oxidative stress in the body, promoting the release of inflammatory factors, and activating nuclear factor- κ B (10, 11). In this study, rat models of sepsis-induced liver injury were constructed via CLP. The end of the cecum was ligated and perforated, causing the overflow of intestinal microorganisms and bacterial peritonitis, thereby inducing sepsis in rats. One study by Schenz et al. (12) denoted that CLP is the gold standard for the construction of animal models of sepsis. It was found in this study that the activity of ALT and AST, the concentration of total bilirubin and the content of inflammatory factors in peripheral blood were increased, liver tissue injury was serious, and there were a large number of infiltrating inflammatory factors in the rats with sepsis. Moreover, in the liver tissues of rats with sepsis-induced liver injury, there were a large number of apoptotic cells, overtly raised expression levels of apoptotic proteins Caspase-3 and Bax, and a clearly reduced expression level of the tumor suppressor protein Bcl-2. The above results suggest that liver injury arises in rats with advanced sepsis, leading to apoptosis of liver cells. Sepsis-induced liver injury mainly involves Kupffer cells, liver sinusoidal endothelial cells, and liver cells. Activated neutrophils recruited into the liver produce potential oxygen free radicals and destructive enzymes, aggravating acute liver injury (13).

As a member of the IL-10 family, IL-22 is a protein that contains 179 amino acids and is encoded by the human IL-22 coding gene. In the early stage of inflammatory responses, IL-23 can stimulate lymphocytes to secrete a large amount of IL-22. In the advanced stage of inflammatory responses, a large amount of IL-6 is able to activate Th22 cells to secrete IL-22, which binds to receptors to form a transmembrane complex to regulate the inflammatory response in the body (14). In addition, IL-22 exerts a dual effect on the body, i.e., it can alleviate the inflammatory responses in the body and effectively moderate the inflammatory process (15). Li et al. (16) manifested that in the disease process, the regulatory effect of IL-22 on the inflammatory responses depends on the signaling pathway activated by it. Feng et al. (17) showed that IL-22 modulates the

development and progression of liver cancer, and that the aggravated liver inflammation promotes the release of IL-22, enhances the proliferation and repair ability of the liver, and relieves the injury of the liver tissues caused by inflammation. The results of this study revealed that advanced intervention with IL-22 in rats with sepsis-induced liver injury significantly improved liver function, mitigated liver tissue damage, reduced the apoptosis level of cells in liver tissues, lowered the expression of apoptosis-related proteins, decreased the release of inflammatory factors, effectively increased the activity of SOD in liver tissues and alleviated oxidative stress response. Zhao et al. (18) discovered that stimulating the release of IL-22 *in vivo* effectively protects against T cell-mediated toxic liver injury, drug-induced liver injury, and hepatitis. Moreover, Eskiler et al. (19) demonstrated that IL-22 protects liver cells by activating the JAK/STAT3 signaling pathway. After IL-22 activates JAK, STAT3 in cells is rapidly phosphorylated, thus generating dimers entering cell nuclei and binding to DNA of target genes to trigger the phosphorylation of p38, thereby relieving the apoptosis of liver cells. In this study, it was discovered that intervention with IL-22 notably increased the level of p-JAK in liver tissues of rats with sepsis-induced liver injury, and thereby obviously elevated the levels of p-STAT3 and p-p38. Liang et al. (20) noted that IL-22 activates the JAK/STAT3 signaling pathway to up-regulate apoptotic genes and antioxidant genes in cells, protects cells against alcoholic liver injury, and promotes liver cell regeneration and liver reconstruction during liver transplantation.

In conclusion, IL-22 is able to activate the JAK/STAT3 signaling pathway in the liver tissues of rats with sepsis-induced liver injury to improve liver function, lower the apoptosis level of liver cells, the expression of apoptosis-related proteins and the release of inflammatory factors, increase SOD activity and mitigate liver injury, thus providing a theoretical basis for the treatment of sepsis-induced liver injury in clinical practice.

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Component resolved diagnosis by recombinant allergens in patients with allergies to inhalants

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Molecular characterization of IgE reactivity of specific individual components of allergenic extracts is now possible due to the technology of recombinant allergens derived from studies of molecular biology of allergic pathology. The identification of the immunoreactivity to single allergenic components in allergic subjects allows to specifically define her/his allergic profile and obtain the so-termed Component Resolved Diagnosis (CRD). Molecular allergens can be classified into those that induce the respiratory allergic reactivity and those that identify the food-related allergic pathology. It is also essential to identify those molecular allergens whose immunoreactivity is able to connect the two clinical conditions: respiratory symptoms and food allergy symptoms. The present study was conducted on 50 patients with a clinical history of hypersensitivity to pollen and/or allergy and positivity to Skin Prick Test. The sera were analyzed in our laboratories and the panel of recombinant allergens was applied in the case of positivity of the specific IgE. Of the 50 patients enrolled, 31 were selected as positive to 4 main pan-allergen Bet v1, Par j2, Art v1 and Phl p1; among these, 14 subjects showed one allergen-specific IgE towards natural extracts of tested foods even in absence of clinical history. CRD allows for an increased accuracy in allergy diagnosis and prognosis and plays an important role in: a) resolving genuine vs cross-reactive sensitization in polysensitized patients, b) assessing, in selected cases, the risk of severe, systemic vs mild, local reactions in food allergy, and c) identifying patients and triggering allergens for specific immunotherapy (ITS). In light of our results, we believe that the transition from a diagnostic based on the use of allergenic extracts to another one based on the use of single allergenic molecules that is able to define the specific allergenic profile of each patient, seems to be able to revolutionize the allergy diagnosis.

Allergic diseases are atopic pathologies the characteristic of which is the presence of a particular class of specific antibodies, IgE. The term 'atopic' indicates the inherited trend of an individual to produce a high level of IgE reacting to a normally

harmless antigen, called 'allergen' (1). The allergic inflammation is due to a cellular-mediated response, after binding between allergen and complex specific IgE/high affinity receptor on the surface of basophil and mast cells, through degranulation

Key-words: IgE; inhalant allergen; skin testing; type I allergy; component resolved diagnosis; recombinant allergens

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of soluble mediators, such as histamine, serotonin, proteolytic enzyme (pre-formed), leukotrienes and prostaglandins (newly synthesized) (2-4); in addition, there is a secretion of cytokines by T lymphocytes, such as IL-2, IL-4, IL-6, which stimulate proliferation of T cells and B-cell activation in plasma cells to produce specific IgE antibodies (5, 6). The pathogenesis of the allergy is multifactorial for the combination among various genetic, environmental, predisposing and competitor factors.

Allergic disease can manifest as respiratory response (asthma or rhinitis), skin reaction (urticaria or dermatitis), gastrointestinal symptoms, or anaphylactic shock. For example, in susceptible hosts, an allergic response can be evoked by repeated inhalation of spores such as *Aspergillus* (7, 8). These spores become trapped in the thick sputum of asthmatic subjects, causing a cascade of inflammatory reactions in atopic patients, frequently with a consequent pulmonary fibrosis, as in patients with cystic fibrosis (9), asthma (10), chronic diseases of nasal mucosa (11) or some autoimmune diseases (12, 13).

The allergy diagnosis consists of the assessment of clinical forms and of the ability to correlate one case to a pathological condition, through a structured diagnostic process which provides investigations of first level, as *in vivo* investigations, Skin Prick Test, or Patch Test (14); second and third level, as *in vitro* measurement of total and specific IgE on the patient's serum using natural allergenic extracts (15, 16).

The use of recombinant allergens has enriched the *in vitro* diagnostic process, and overcomes many of these difficulties; in fact, they have a defined allergen composition, are quantifiable and standardized (17). The concept of using defined allergenic molecules compared to extract (mixture of allergenic and non-allergenic components) for the diagnosis of type I reactions, is defined Component Resolved Diagnosis (CRD) (18). Thanks to new acquisitions in the field of molecular allergology, allergies could soon be framed as a process of sensitization towards a group of allergen with defined biochemical functions (19). The use of combinations among recombinant allergen, resulting from the sum of relevant antigens of certain allergenic sources, is essential to obtain reliable and reproducible diagnostic tests. A positive reaction to a potential cross-

reactive allergen can allow to predict allergic reaction to all sources containing immunologically-related molecules (20-23). However, some inconvenience is possible; in fact, some recombinant allergens possess a minor binding ability for specific IgE compared to the corresponding natural allergens; this is due to the existence of multiple allergenic isoforms with different binding affinities to the antibodies, and also little variations of the amino-acidic sequence can edit the binding site (24). Some of these isoforms, lacking epitopes, have extremely low allergenicity, but they are able to induce response in the T lymphocytes, essential requirement for the induction of tolerance; and the same effect is obtained by the fragmentation in non-anaphylactic portions (25). The clinical use of these hypo-allergenic recombinant molecules allows to reduce the risk of anaphylaxis during immune-specific treatment (ITS) (26). The WAO-ARIA-GA²LEN consensus document on Molecular-based allergy (MA) diagnostics provides a practical guide for the indication, determination and interpretation of MA diagnostics for clinicians trained in allergology (27).

The aim of this study is to highlight the presence, in serum of patients with allergic manifestations towards inhalant/pollen, of specific IgE to the main molecular component of the most common pollens, such as Bet v1, Par j2, as a deeper, correlating diagnostic allergen extract and respective recombinant allergens (28, 29).

MATERIALS AND METHODS

Study subjects

This study was conducted on 50 patients (31 males and 19 females; mean age 35.6 years) and 20 healthy control subjects (12 males and 8 females; mean age 30.6 years) enrolled in the A.O.U. "Federico II", Naples, Italy. All the subjects enrolled gave informed consent to participate in the study. All the patients presented a clinical history of hypersensitivity to pollen and/or allergy (oral allergy syndrome with symptoms: rhinitis, conjunctivitis, asthma, gastrointestinal symptoms, contact urticaria, dermatitis, anaphylaxis, atopic eczema; reactivity to known allergen sources) and positivity to Skin Prick Test and Spirometry Test. All control subjects underwent the same *in vivo* and *in vitro* determinations as the patients – an open oral

challenge test and rubbing test – and the results of both assays were negative. Spirometry is used to diagnose asthma and other condition that affect breathing. The sera were analyzed in our laboratories.

Skin Prick Test

Skin Prick Test is an essential test procedure to confirm sensitization in IgE-mediated allergic disease in all subjects and was performed on the forearms of all individuals. The recommended method of prick testing includes the appropriate use of specific allergen extracts, positive and negative controls, interpretation of the tests after 15-20 min of application, with a positive result defined as a wheal ≥ 3 mm diameter. It can help to confirm the diagnosis of a suspected type I allergy. The test is minimally invasive, inexpensive, results are immediately available and, when carried out by trained health professionals, reproducible.

Component Resolved Diagnosis

The level of awareness towards the main inhalant allergen was evaluated by specific IgE Assay (ImmunoCAP 250; Phadia, Sweden). The principle of this test is a sandwich immunoassay using a solid phase, “ImmunoCap”, which consists of a cellulose derivative

content in a capsule; this solid phase has an approximately 150-fold higher capacity than a traditional coated tube. The polymer inside the ‘cap’ irreversibly binds the allergens keeping their structure intact, then the allergen reacts with specific IgE of the serum. Fluorescence of eluate is used, by which it derives the concentration (through a standard curve) expressed in kUA/L. For the reliability of the results provided at the end of the reaction, both total IgE and specific IgE control samples were inserted.

Statistical analysis

For the assessment of the significance of the results obtained, Variance Analysis was used for unpaired data with significance $P \leq 0.05$. Fisher’s Exact Test was used to evaluate recombinant allergens vs food natural allergens.

RESULTS

In the sera of patients with suspected allergy to major inhalant allergens, awareness of pollen allergen extract was assessed; subsequently, for testing positive patients, the respective recombinant components were assessed. The panel of recombinant allergens was applied in the case of positivity of the specific IgE (≥ 0.50 kUA/L) according to Table I.

Table I. *Deepening with recombinant allergens as a function of the relative extractive allergens*

Extractive Allergen	Recombinant Allergen
Birch (t3, t4, t7)	Bet v1, Bet v2, Bet v4
Gramineae (g5, g6, g8)	Phl p1, Phl p5b, Phl p7, Phl p12
Pellitory (w19, w21)	Par j2
Composite (w6)	Art a1

Table II. *Cross-reactivity between inhalant allergen and foods*

Inhalant Recombinant Allergen	Cross-reactivity Foods
Birch (Bet v1)	Apple, peach, pear, apricot, plum, walnut, cherry, banana, fennel, carrot, strawberry
Pellitory (Par j2)	Basil, cherry
Gramineae (Phl p1-Phl p5)	Kiwi, wheat, plum, peach, apricot, cherry
Composite (Art a1)	Apple, fennel, carrot, walnut, banana

In some patients sera, we found high concentration of specific IgE for inhalant recombinant allergens, the so-called ‘pan-allergens’, present in numerous extracts, despite their taxonomic distance. In these sera, the possible sensitization to some foods was evaluated. The tested foods are shown in Table II.

A panel of 9 natural and purified allergens and 9 recombinant allergens was used to establish the profiles of molecular sensitization. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive. The molecular allergeo-gram obtained with the recombinant allergen in the 50 enrolled patients highlighted the presence of allergen-specific IgE compared to the chosen recombinants in most samples. The positivity in many patients to pan-allergens, such as Bet v1 and Par j2, suggested us the opportunity to evaluate even the possible sensitization to some foods that express the recombinant molecules under study. We used a panel of 14 natural extracts for foods, and the concentration of the specific IgE ≥ 0.50 kUA/L is considered positive.

Measurement in vitro of specific IgE to inhalant extractive (pollen) and recombinant allergen

Gramineae. Considering the cross-reaction of various grass families, we used three extractive allergens for screening tests: g5, g6, g8 resulted positive in 64%, 62%, 66% of patients, respectively. These patients were tested with recombinant allergens and showed positivity to the major allergen of grass - Phl p1 in 65% of the cases (Fig. 1). Some patients were positive also to profilines and/or

calcium-binding proteins, providing information for therapeutic decisions.

Pellitory. 74% of patients showed sensitization to this allergen, w19 and w21, so concentrated as to

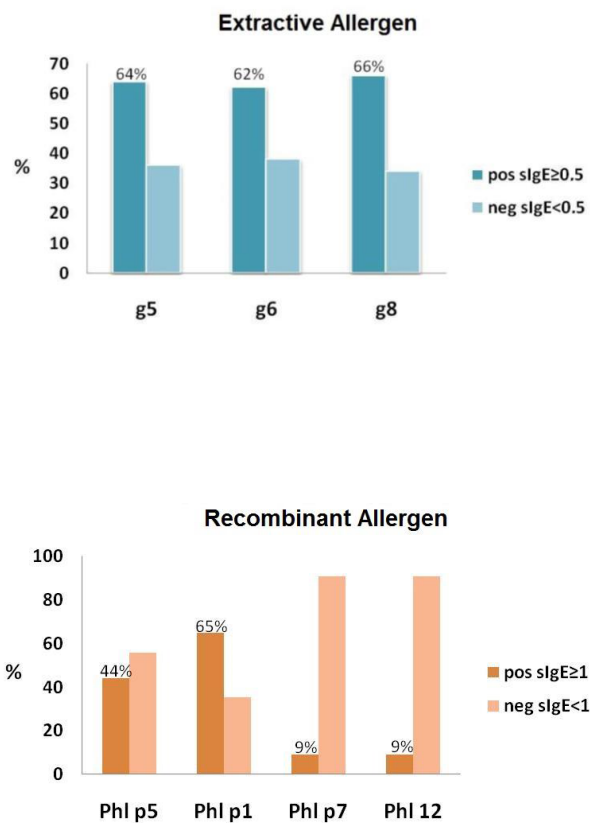


Fig. 1. Reactivity profiles of specific IgE for gramineae: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Gramineae

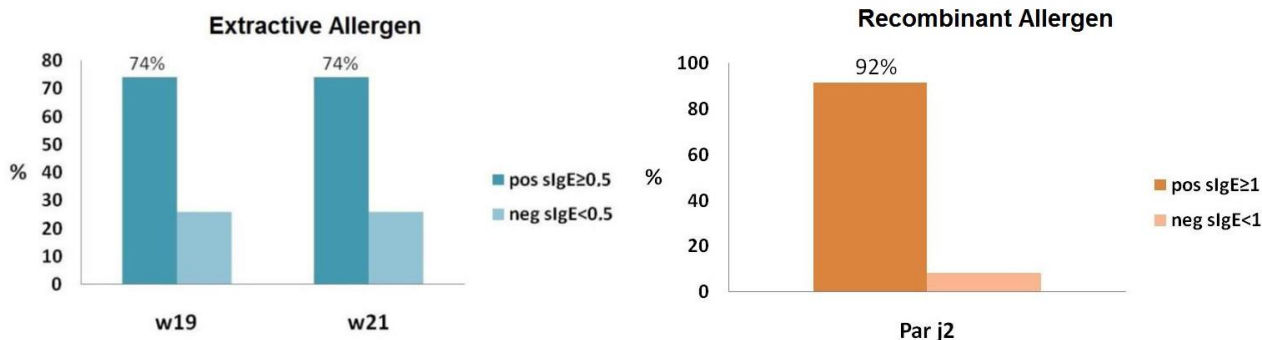


Fig. 2. Reactivity profiles of specific IgE for pellitory: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Pellitory

perform deepening tests according to the molecular recombinant allergen chosen, Par j2, to which 92% of patients resulted positive (Fig. 2).

Composite. The percentage (48%) of patients positive to extractive allergen w6 was lower, and 42% of these resulted positive to the recombinant Art v1 (Fig. 3).

Tree- Birch. Patients positive to extractive allergens t3, t4, t7 were 22%, 22%, 20%, respectively, of whom 19% were positive to recombinant allergen Bet v1 (Fig. 4). Bet v1 was chosen as a marker of specific sensitivity towards birch (5 positive patients) and resulted in potential responsiveness to ITS, and two markers of specific sensitivity: a profiling (Bet v2,

with a positive response in 4 patients) and a calcium-binding protein (Bet v4, positive in 3 patients).

Measurement in vitro of specific IgE of natural allergen for foods: cross-reactivity

Of 50 patients enrolled, 31 (62%) were selected as positive to 4 main pan-allergens Bet v1, Par j2, Art v1 and Phl p1. Among these, 14 (45%) subjects showed one allergen-specific IgE towards natural extracts of tested foods even in absence of clinical history. These data were evaluated by Fisher's Exact Test (Table III). Remarkable significance was noticed, as the subjects resulting positive to the main recombinant

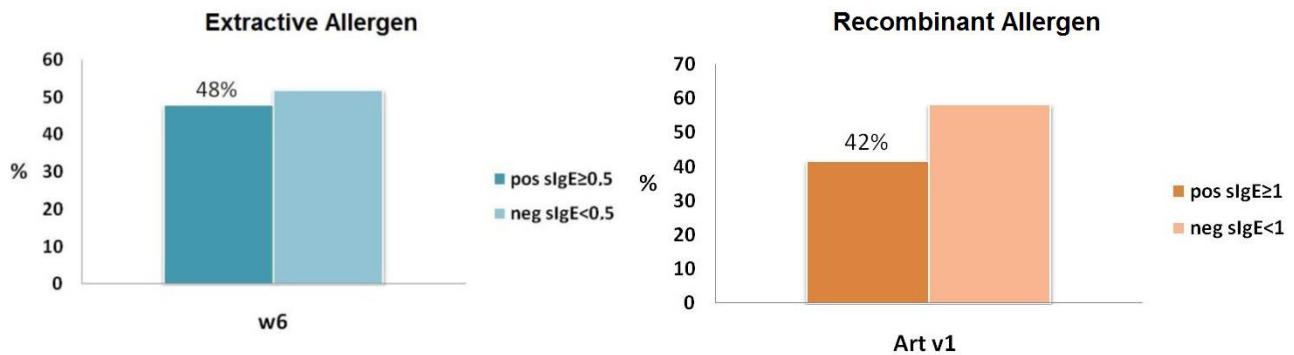


Fig. 3. Reactivity profiles of specific IgE for composites: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Composite

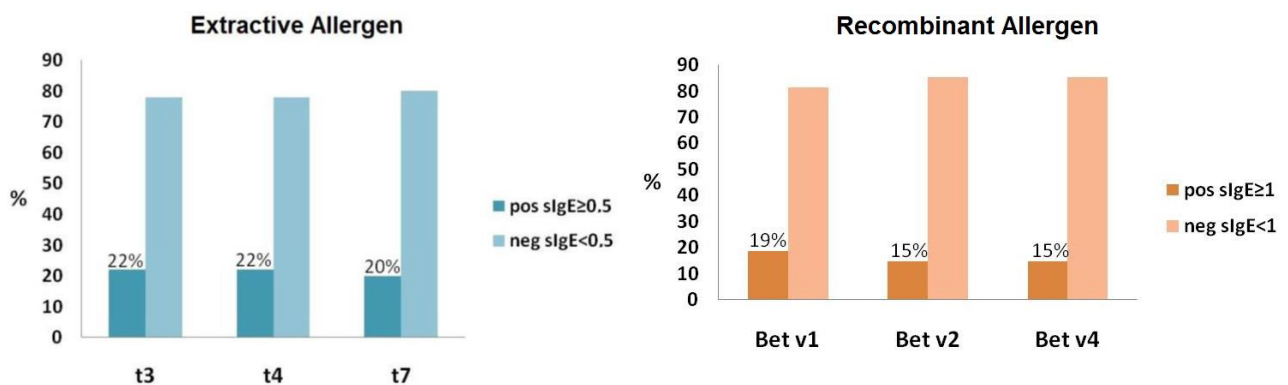


Fig. 4. Reactivity profiles of specific IgE for tree birch: positivity percentage for extractive and recombinant allergens in patients. The levels of the specific IgE ≥ 0.50 kUA/L (natural allergens) or ≥ 1.00 kUA/L (recombinant) were considered positive – Tree birch

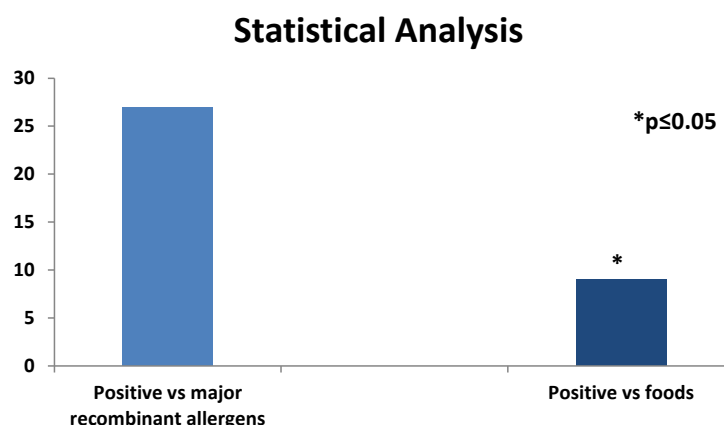


Fig. 5. Difference between recombinant allergens and specific IgE in patients positive to tested foods

Table III. Inhalant recombinant allergen and food natural extractive allergen: positivity in $N=14$ asymptomatic patients.

	Recombinant Allergen					Food Natural Allergen														p
Pz	Betv1	Phlp1	Parj2	Artv1	f95	f94	f237	f255	f242	f92	f256	f276	f31	f84	f269	f44	f49	f4		
2	4.52	0.01	8.74		26.1	18.7	13.8	19.1	15.2	0.95	11.6	5.51	1.27	1.01		8.92	18.2	1.03	0.0023	
9		4.56	10.7		3.02			5.5											0.45	
10			58.3		10.4														Nd	
18		4.61	25.8		20.6			16											Nd	
19			39.3		3.54			3.44											Nd	
20			8.15		12.9												9.83		Nd	
23		1.6	4.67		1.50	0.89	0.77	1.04	1.15		0.6	0.47	0.9			0.54	1.02		0.67	
25		32.00	1.45	1.13	3.50	1.37	0.55	1.08	1.48	0.78	0.95	0.72	9.35			0.98	0.99	0.93	0.31	
26	15.40	9.13	30.60	1.94	3.50	1.36	1.08	0.96	2.15	0.56	1.21		1.33	0.49		1.00	1.25	0.94	0.019	
29		5.69	8.4		16.00				10.8										0.045	
42	6.89	4.84	15.7		0.55	0.93			0.46				0.38				0.56	0.65	0.011	
43		7.52	27.9	9.04										3.1					Nd	
45	3.58			1.03									0.43						Nd	
50		20.50	93.90	3.32	4.76	4.82	3.41	5.12	5.16	3.22	4.75	5.27	5.56	4.63	0.42	4.32	3.48	4.96	>0.001	

pan-allergens resulted positive to natural extracts of the tested foods. The statistical analysis shows a significative difference ($p \leq 0.05$) (Fig. 5).

Evaluation of candidate patients for Specific Immune Therapy

The analysis of molecular allergens resulted useful to study subjects to candidate for immunotherapy. Recent studies on animal models of

molecular allergens of gramineae suggest, in case of positivity, at least one of the major allergens Phlp1 and Phlp5; there is a good possibility of response to ITS, while the response is poor in case of positivity only to Phlp7 and Phlp12 (Fig. 6). In the results outlined, therefore, at least 15% of the examined subjects would be possible candidates for specific immunotherapy. Instead, for a correct prescription of ITS for pellitory, it is necessary to confirm the

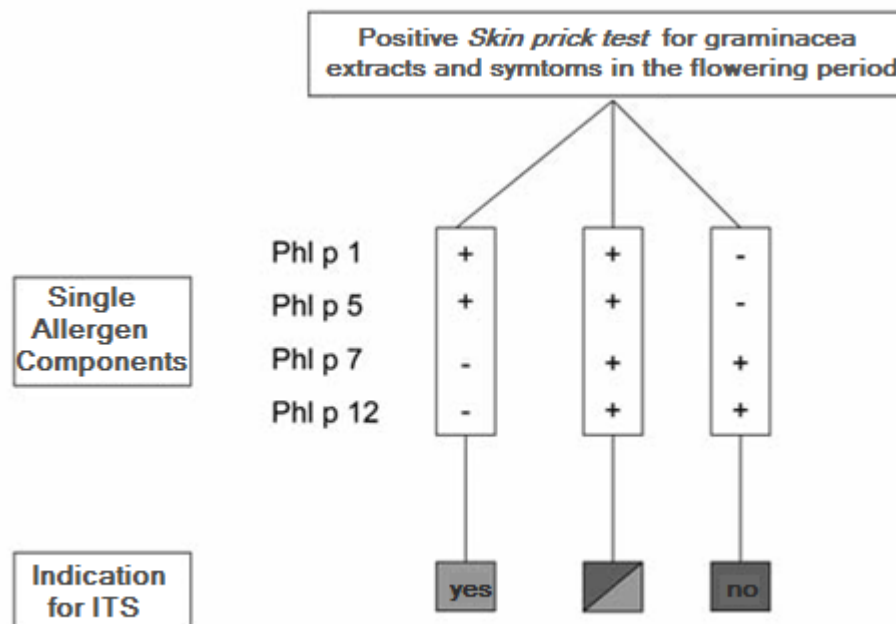


Fig. 6. ITS and allergen recombinant: algorithm

positivity to the specific IgE for major allergen Parj2 (positive to 37% of cases).

DISCUSSION

The analysis of the data obtained indicates a good correlation between clinical history, Skin Prick Test and specific IgE levels, evaluated *in vitro* using a panel of natural allergen extracts. CRD with recombinant allergens shows the presence of specific IgE against some molecular components of the pollen. The diagnostic tests, enriched with recombinant tests, allow to design specific immunologic profile, that constitutes a useful tool to better manage any polysensitized patient and to contribute to a personal appropriate therapeutic treatment.

The identification of Pan-allergen helped us to understand how patients can develop allergy symptoms, often serious, after contact with allergens from different sources and apparently not correlated. A correct diagnosis is difficult with one source containing several different allergens, however recombinant pan-allergen may be predictive of food allergy. Furthermore, the use of a single allergen component allows progress of the appropriateness

of specific immunotherapy ITS. In fact, it is very important to select the right patients for ITS by researching the allergen responsible for the symptomatology.

CRD allows to choose patients for immunotherapy, especially thanks to the detection of those who are allergic to pan-allergen and of the cross-reactions between related species. ITS with extracts often shows low efficacy due to the levels of only 30-40% of antigenic proteins; the recombinant is a purified molecule, so it is 100% efficient for optimal doses, and the use of hypo-allergenic forms of recombinant is possible to reduce adverse reaction. These molecules of new application are missing from certain epitopes recognized by IgE, but they are able to induce response in T-helper lymphocytes, but not to trigger a considerable allergic reaction making ITS safer (30).

Allergies have a common pathogenetic mechanism in which various factors interact by diversifying the clinical manifestation. This mechanism is recallable to release vasoactive amine after interaction with the allergen – immune-globulins of E class and relative receptor activation on basophils/mast cells, with effects on various organs and systems. These

pathological reactions affect children and adults of both sexes in an equivalent way. Therefore, although allergic manifestations have general features regarding how they were triggered, their clinical heterogeneity often poses problems with diagnostic framing.

The transition from a diagnostic based on the use of allergenic extracts to another one based on the use of single allergenic molecules that is able to define the specific allergenic profile of each patient (CRD), seems to be able to revolutionize the allergy diagnosis. The use of recombinant allergen allows to selected candidate patients to specific hypo-sensitization therapy, as well as to make a better diagnostic framing by identifying the allergenic source responsible for the rising awareness by distinguishing between mono-sensitized and poly-sensitized subjects. We can then discriminate whether the poly-awareness is due to co-awareness, that is more primary sensitivity towards major or minor allergens present in the different allergenic sources, or cross-reactivity towards homologue molecules present in different species, sometimes not taxonomically related, which are often clinically meaningless.

Today we no longer have an allergen classification based on distinction among pollen, mold, epithelia, dust mites, etc., but a new classification based on different families of molecules, such as PR-10 proteins (Bet v1 homologues), major allergen of grass and epithelia, lipid-transfer proteins, tropomyosins, storage proteins of dried fruit, calcium-binding proteins, profilins and carbohydrate determinants (CCD).

The present study shows a significative incidence of food allergy in patients without clinical history for these events, but affected by pollen allergy in presence of positivity for a principal pan-allergen such as Bet v1, Par j2, Art v1 and Phl p1.

The laboratory plays the most important role in this revolution and, in close collaboration with the clinical side, should evaluate and validate new diagnostic systems so that they become really important and necessary to the allergic diagnostics, avoiding producing reports of dubious and not immediate interpretation. The task of the laboratory is therefore to provide one allergeo-gram, specific for each patient, accompanied by an interpretative report

that provides the clinician with useful elements for a correct diagnosis and a better therapeutic route to improve the life expectancy of allergic patients.

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LETTER TO THE EDITOR

Blood procalcitonin and c-reactive protein in distinguishing between bacterial and mycoplasma community-acquired pneumonia in childrenF. Qu¹, J. Liu¹, AP. Yang¹ and RL. Tu²¹*Department of Clinical Laboratory, Zhejiang Xiaoshan Hospital, Hangzhou, Zhejiang, P.R. China;*²*Department of Neonatal Paediatrics, Zhejiang Xiaoshan Hospital, Hangzhou, Zhejiang, P.R. China**Received January 30, 2020 – Accepted July 30, 2020*

To the Editor,

Pediatric community-acquired pneumonia (CAP) is a pneumonia in previously healthy children that occurs outside the hospital setting. It is a leading cause of morbidity and mortality, accounting for 16% of all deaths in children under 5 years old and a total of over 920,000 deaths in 2015 (1). CAP in children is most commonly caused by viruses with or without bacteria (2, 3), though mycoplasma infection is not uncommon (4, 5). CAP caused by mycoplasma is treated with antibiotics different from those used for the treatment of bacterial CAP. Early identification of the etiology of CAP is therefore essential to initiate appropriate treatment and reduce mortality.

It is still a challenge to differentiate mycoplasma CAP from bacterial CAP in children. Both diseases have comparable symptoms and signs, and similar non-characteristic radiological findings (4). Routine laboratory tests, such as white blood cell count (WBC) and percentage of neutrophils, have limited sensitivity and specificity. Detection of pathogens from blood or sputum cultures is a lengthy process with false negative and false positive results are common. New biomarkers are therefore needed to ensure timely distinction between mycoplasma CAP and bacterial CAP in children.

Procalcitonin (PCT) is a 12.7KD polypeptide

and a member of the calcitonin gene-related calcitonin gene family. In healthy individuals, serum PCT is low or undetectable, but it increases significantly during inflammation. C-reaction protein (CRP) is produced by the liver. Serum CRP level also increases in response to inflammation. PCT and CRP levels have been used previously to distinguish bacterial infections from viral infections (6-9). Although the use of these measurements to differentiate bacterial CAP from mycoplasma CAP in children has been reported previously, the conclusions remain inconsistent due to small sample sizes of children with mycoplasma CAP in these studies (6, 10, 11).

The objective of the present study was to assess the diagnostic performance of PCT and CRP in distinguishing between bacterial and mycoplasma CAP in children. Children with bacterial or mycoplasma CAP and healthy controls were enrolled, and their serum levels of PCT and CRP, WBC, and percentage of neutrophil were measured.

MATERIALS AND METHODS

Study population

Children with bacterial or mycoplasma CAP who received in-patient treatments in our hospital from July 2015 to September 2017 were included in this study. CAP

Key words: procalcitonin; C-reactive protein; pneumonia; biomarker and diagnosis; children

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was diagnosed based on signs and symptoms of lower respiratory tract infection, and diagnoses were confirmed by chest X-ray findings consistent with pneumonia. Bacterial CAP was diagnosed based on routine sputum culture, whereas mycoplasma CAP was diagnosed based on serologic antibody testing. Patients were excluded if they i) received antibiotics for more than 48 h before admission; ii) suffered from chronic respiratory diseases; iii) were hospitalized for more than 48 h; iv) had negative results for both bacterial culture and serologic antibody testing. Gender-matched healthy children who underwent annual-wellness examinations were recruited in the same period as the controls. The study was approved by the Ethics Committee of Zhejiang Xiaoshan Hospital (approval No. 2016052). Written informed consent was obtained from parents of the participants. All procedures were performed in accordance with the ethical standards of the institutional responsible committee on human experimentation and with the Helsinki Declaration.

Biomarker examination

Blood was drawn within 48 h of admission. WBC and neutrophil counts were obtained by flow cytometry (Beckman coulter, Pasadena, CA, USA). Serum was prepared from each blood sample and stored at -80°C. Serum PCT was measured by electrochemiluminescence using a Cobas 6000 (Roche Diagnostics Ltd, Mannheim, Germany) with a lower detection limit of 0.015 ng/ml. CRP was detected by immunoturbidimetry using an automatic detection system (Beckman Coulter) with a lower detection limit of 0.2 µg/ml.

Statistical analysis

Categorical data were expressed as percentages and continuous variables as mean $\pm$ standard deviation. Differences in PCT, CRP, WBC and percentage of neutrophils were assessed using the Kruskal–Wallis test followed by Dunn's post hoc tests. Correlation among these biomarkers was examined using non-parametric Spearman's test. Diagnostic performance of the biomarkers was evaluated using area under receiver operating characteristic (ROC) curves. The best cut-off values for different biomarkers were obtained based on the highest sensitivity and specificity. A $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

A total of 236 children with bacterial (n=172) or mycoplasma (n=64) CAP and 86 healthy controls were enrolled in this study. Median age was 36, 24 and 24 months for children in the healthy control, mycoplasma CAP and bacterial CAP groups, respectively (Table I). All three groups had a similar gender composition. The distribution of log-transformed PCT and CRP concentrations among the groups is presented in Fig. 1A and 1B, respectively. Children with bacterial CAP had significantly higher serum levels of PCT and CRP, WBC and percentage of neutrophils compared with children with mycoplasma CAP (all $P < 0.0001$). In addition, all biomarkers were significantly higher in children with mycoplasma CAP compared with healthy controls (all $P < 0.0001$). Interestingly, all biomarkers (PCT and CRP, WBC and percentage of neutrophils) were significantly correlated (Table II).

Fig. 1C displays ROC plots for PCT, CRP, and WBC and percentage of neutrophils in distinguishing bacterial CAP from mycoplasma CAP. The area under the ROC curves was 0.84 (95% confidence interval (CI) 0.78-0.90) for PCT, 0.68 (95% CI 0.62-0.75) for CRP, 0.73 (95% CI 0.66-0.79) for WBC and 0.65 (95% CI 0.58-0.73) for percentage of neutrophils (Table II). The area under the ROC curve for PCT was significantly higher compared to that for CRP ($P=0.0001$), WBC ($P=0.0115$) and percentage of neutrophils ($P<0.0001$) in the bacterial CAP group. A cut-off point of 0.201 ng/ml for serum PCT yielded a sensitivity of 64.1% and a specificity of 90.7%. The sensitivity and specificity for CRP, WBC and percentage of neutrophils were 82.8% and 51.7%, 78.1% and 66.2%, 57.8% and 88.0%, respectively (Table III).

The diagnostic values of these markers were analyzed in differentiating children with either bacterial or mycoplasma CAP from healthy children. ROC plots for PCT, CRP, WBC and percentage of neutrophils are shown in Fig. 1D. Our results showed that the area under the ROC curves was 0.96 (95% CI 0.94-0.98) for PCT, 0.89 (95% CI 0.86-0.93) for CRP, 0.87 (95% CI 0.84-0.91) for WBC, and 0.83 (95% CI 0.78-0.87) for percentage of neutrophil. The area under the ROC curve for PCT was significantly

greater compared to that for CRP ($P=0.0014$), WBC ($P<0.0001$), and percentage of neutrophil ($P<0.0001$). The sensitivity and specificity for PCT, CRP, WBC and percentage of neutrophils were 89.8% and 97.6%, 84.7% and 94%, 75% and 91.6%, and 75% and 83.1%, respectively (Table IV).

DISCUSSION

The present study revealed that PCT was superior to other biomarkers (CRP, WBC and percentage of

neutrophils) in discriminating bacterial CAP from mycoplasma CAP, with the area under the ROC curve of 0.84. Inconsistent results have been reported in previous studies. One study reported an area under the ROC curve of 0.88 for PCT in distinguishing children with bacterial CAP from atypical CAP (6), whereas another study found PCT was not useful in distinguishing bacterial CAP from mycoplasma-chlamydia CAP (10). Berg et al. reported that CRP displayed fair diagnostic performance in separating bacterial from viral or atypical CAP in children (11).

Table I. Characteristics of children with CAP and healthy controls.

Variable	Healthy control (n=83)	Mycoplasma (n=64)	Bacterial (n=172)	P value
Age (months)	36 (24-60) ^a	24 (1-180) ^b	24 (1-196) ^b	<0.0001
Male (%)	46 (55.4)	39 (60.9)	108 (62.8)	0.5277
PCT (ng/ml)	0.05 ± 0.02 ^{#, a}	0.31 ± 0.67 ^b	2.96 ± 11.25 ^c	<0.0001
CRP (µg/ml)	1.8 ± 1.5 ^a	15.6 ± 19.5 ^b	39.8 ± 49.0 ^c	<0.0001
WBC (x10 ⁶ /ml)	7.0 ± 1.6 ^a	10.3 ± 5.0 ^b	16.3 ± 8.3 ^c	<0.0001
% neutrophils	40.5 ± 11.0 ^a	55.8 ± 20.8 ^b	66.2 ± 18.9 ^c	<0.0001

data are presented as mean ± SD. Different letters within the same variable indicate significant difference between groups. CAP: community acquired pneumonia; PCT: procalcitonin; CRP: C-reactive protein; WBC: white blood cell count.

Table II. Spearman correlation between biomarkers

	PCT	CRP	WBC	% Neutrophils
PCT				
Rs		0.61628	0.53826	0.47071
P value		<0.0001	<0.0001	<0.0001
CRP				
Rs	0.61628		0.50412	0.5136
P value	<0.0001		<0.0001	<0.0001
WBC				
Rs	0.53826	0.50412		0.49378
P value	<0.0001	<0.0001		<0.0001
% neutrophils				
Rs	0.47071	0.5136	0.49378	
P value	<0.0001	<0.0001	<0.0001	

PCT: procalcitonin; CRP: C-reactive protein; WBC: white blood cell count

Table III. *Diagnostic performance of biomarkers in distinguishing bacterial CAP from mycoplasma CAP*

Biomarkers	Cut-off value	Sensitivity (%)	Specificity (%)	Area under ROC curve	95% CI
PCT	0.2 ng/ml	64.1	90.7	0.84	0.78-0.90
CRP	21.0 µg/ml	82.8	51.7	0.68	0.62-0.75
WBC	12.3 x10 ⁶ /ml	78.1	66.2	0.73	0.66-0.79
% Neutrophils	57.9	57.8	88.0	0.65	0.58-0.73

CAP: community acquired pneumonia; CI: confidence interval; ROC: receiver operating characteristics; PCT: procalcitonin; CRP: C-reactive protein; WBC: white blood cell count

Table IV. *Diagnostic performance of biomarkers in distinguishing children with CAP from healthy controls*

Biomarkers	Cut-off value	Sensitivity (%)	Specificity (%)	Area under ROC curve	95% CI
PCT	0.1 ng/ml	89.8	97.6	0.96	0.94-0.98
CRP	3.1 µg/ml	84.7	94.0	0.89	0.86-0.93
WBC	8.9 x10 ⁶ /ml	75.0	91.6	0.87	0.84-0.91
% neutrophils	49.6	75.0	83.1	0.83	0.78-0.87

CAP: community acquired pneumonia; CI: confidence interval; ROC: receiver operating characteristics; PCT: procalcitonin; CRP: C-reactive protein; WBC: white blood cell count.

An additional study revealed that CRP displayed good diagnostic performance in differentiating bacterial CAP from viral or mycoplasma CAP (12). It is noted that these studies evaluated the diagnostic performance of PCT or CRP in differentiating between children with bacterial CAP and mixed groups (mycoplasma CAP mixed with chlamydia or viral CAP). The sample sizes of patients with mycoplasma CAP were small in these studies. Further studies with larger sample sizes are needed to clarify the diagnostic performance of PCT and CRP in distinguishing between bacterial CAP and mycoplasma CAP.

The present study also showed a significant increase in PCT and CRP in children with bacterial or mycoplasma CAP compared to healthy children. PCT is a better biomarker than CRP, WBC and percentage of neutrophils in distinguishing between children with CAP and healthy children. The area under the ROC curve was significantly greater for PCT compared to CRP, WBC and percentage of neutrophils. Venge et al. measured serum PCT and CRP in 144 healthy controls, 185 patients with bacterial infections, and 54 patients with viral infections. The area under the ROC curve was 0.88 for PCT and 0.99 for CRP in the

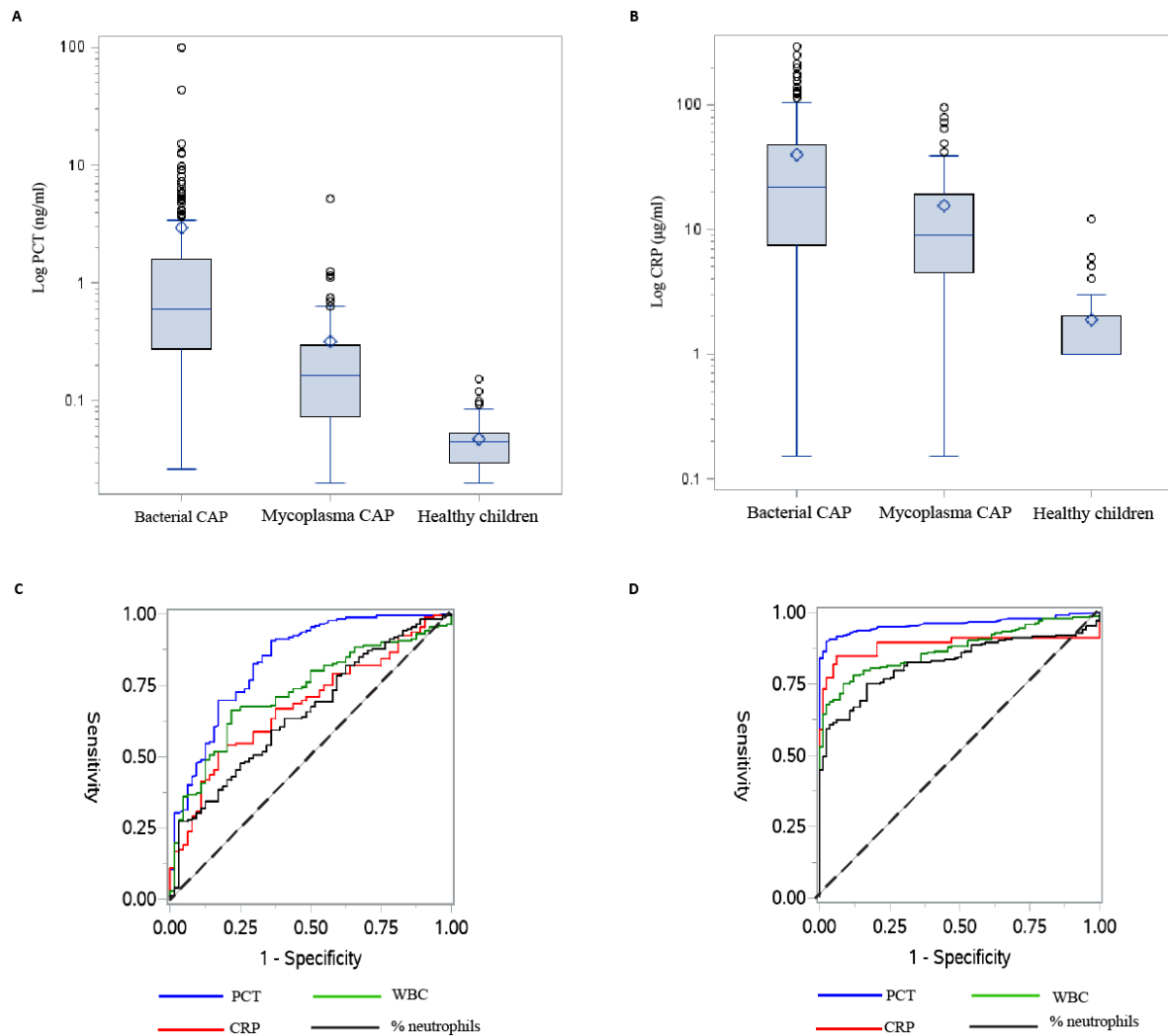


Fig. 1. Characteristics of serum PCT and CRP and their diagnostic performances. **A)** Boxplot of log-transformed PCT concentration (ng/ml). **B)** Boxplot of log transformed CRP concentration (µg/ml). **C)** ROC curves for PCT, CRP, WBC and percentage of neutrophils in differentiating bacterial CAP from mycoplasma CAP. The area under the ROC curve of PCT was significantly higher compared with that of CRP ($P=0.0001$), WBC ($P=0.0115$) and percentage of neutrophil ($P<0.0001$). **D)** ROC curves for PCT, CRP, WBC and percentage of neutrophils in differentiating bacterial CAP from mycoplasma CAP. The area under the ROC curve of PCT was significantly greater compared with that of CRP ($P=0.0014$), WBC ($P<0.0001$), and percentage of neutrophil ($P<0.0001$). PCT: procalcitonin; CRP: C-reactive protein; WBC: white blood cell count.

distinction between healthy controls and patients with bacterial infections (9).

It can be concluded that PCT is a good biomarker, superior to CRP in discriminating bacterial CAP from mycoplasma CAP. PCT performs better than CRP, WBC and percentage of neutrophils in diagnosing CAP in children.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

Relationship between pulmonary function changes and chronic cough in children with cough variant asthmaHX. Shi¹, XF. Sui², DQ. Wang³, QT. Han⁴, FQ. Meng⁵ and XZ. Zhao⁶

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To the Editor

Cough is a frequent symptom of patients in respiratory medicine clinics and has a significant impact on their daily lives (1). In China, more than 30% of patients enter the respiratory consultation room because of chronic cough, which is mainly caused by cough variant asthma (CVA) (2, 3). CVA is a frequent chronic non-specific inflammatory disease of the airways that mostly occurs in childhood (4). Since childhood is a critical period for growth and development, physiological maturity process, drug treatment and physical and mental characteristics are different from adults. Chronic cough is the most common cause of pediatric ailments, and not only affects the daily life of patients, but also brings financial burden to families (5). Cough is a common disease in children, and can develop into chronic cough, mainly including chronic bacterial bronchitis, asthma, and upper respiratory cough syndrome (UACS). The likelihood of chronic cough in CVA is much higher than that in healthy children (6), therefore it is of great significance to find the influencing factors. Studies have indicated that lung function is closely related to the occurrence of

chronic cough, but is related to cough variability (7). The relationship between chronic cough and asthma has not been reported, and pulmonary function tests are clinically common non-invasive methods. The present study aims to explore the relationship between changes in lung function and chronic cough in children with CVA, and to provide a scientific basis for clinical treatment.

MATERIALS AND METHODS

In this study, 86 children with CVA who were treated in our hospital from June 2017 to June 2019 were enrolled. The criteria for inclusion in the study were: under 18 years of age, no family history of asthma, no chronic respiratory disease, no respiratory infection in the previous 2 two weeks, and family members of the patients provided written informed consent. The patients were divided into two groups according to whether the patients had chronic cough. In the control group, there were 39 patients (23 males and 16 females) with an average age of 10.87 years and an average body mass index of 22.14. There were 47 patients (28 males and 19 females) in the chronic cough group, with an average age of 11.09 years, and average

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body mass index of 22.05. There were no significant differences in gender ($P=0.072$), age (0.058) and body mass index (0.065) between the two groups (Table I).

Pulmonary function tests were performed using the Japanese Jester HI-701 pulmonary function meter (Shanghai Yilian Science and Education Equipment Co., Ltd.), which was designed to detect the airway function indicators FVC and FEV1 and the small airway function indicators PEF, FEF25, FEF50, FEF75 in all patients. A total of three tests were conducted, and the final results averaged three times.

Evaluation index

The general data, airway pulmonary function (FVC, FEV1) and small airway pulmonary function (PEF, FEF25, FEF50, FEF75) were compared between the two groups.

Statistical analysis

SPSS 15.0 software was used for data analysis. The measured data were expressed as mean $\pm$ SD, and were compared using the independent sample t -test. The count data was expressed as frequency, and compared using the χ^2 test. Multiple logistic regression analysis was used to count related factors. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison between groups

The gender, age and body mass index of the CVA group and the control group were compared. There were 28 males and 19 females in the CVA group. The average age of patients with CVA was 11.09 ± 1.28 years, and the average body mass index was 22.05 ± 1.09 kg/m². There were 23 males and 16 females in the control group. The average age of the patients was 10.87 ± 1.14 years, and the average body mass index was 22.14 ± 1.13 kg/m². It was found that there was no significant difference in gender ($P = 0.072$), age ($P = 0.058$), and body mass index ($P = 0.065$) between the two groups (Table I).

The pulmonary function levels of the CVA group and the control group were compared. In the CVA group, the FVC was 2.70 ± 1.02 L, the FEV1 was 2.08 ± 1.04 L, the PEF was 4.89 ± 1.82 L, the FEF25 was 3.69 ± 1.47 L/s, the FEF50 was 2.38 ± 1.41 L/s, and the FEF75 was 1.78 ± 0.75 L/s. The FVC of the control group was 3.74 ± 1.17 L, the FEV1 was 3.47 ± 1.22 L, the PEF was 6.21 ± 2.24 L, the FEF25 was 5.06 ± 1.88 L/s, the FEF50 was 3.85 ± 1.24 L/s

Table I. Comparison of general data between patients with chronic cough and control group.

Groups	Male/case	Female/case	Age/year	BMI /kg/m ²
Chronic cough	28	19	11.09 ± 1.28	22.05 ± 1.09
Control	23	16	10.87 ± 1.14	22.14 ± 1.13
t/χ^2		2.917	1.887	1.814
P		0.072	0.058	0.065

BMI: body mass index

Table II. Comparison of pulmonary function levels between patients with chronic cough and control group

Groups	FVC/L	FEV1/L	PEF/L	FEF25 (L/s)	FEF50 (L/s)	FEF75 (L/s)
Chronic cough	2.70 ± 1.02	2.08 ± 1.04	4.89 ± 1.82	3.69 ± 1.47	2.38 ± 1.41	1.78 ± 0.75
Control	3.74 ± 1.17	3.47 ± 1.22	6.21 ± 2.24	5.06 ± 1.88	3.85 ± 1.24	2.55 ± 0.81
t	2.083	2.367	2.191	2.145	2.257	2.319
P	0.041	0.018	0.033	0.037	0.026	0.021

and the FEF75 was 2.55 ± 0.81 L/s. The results demonstrated that the airway function FVC ($P = 0.041$) and FEV1 ($P = 0.018$) of the CVA group were lower than those of the control group, and the results were statistically significant. Similarly, compared with the control group, the levels of PEF ($P = 0.033$), FEF25 ($P = 0.037$), FEF50 ($P = 0.026$), and FEF75 ($P = 0.021$) in the small airway function of the CVA group were reduced, and the difference was statistically significant (Table II).

Multi-factor Logistic regression analysis

Multivariate Logistic regression analysis was used to detect the risk factors of chronic cough in children with CVA. As expected, the results showed that low FVC (OR = 2.651, $P = 0.029$), low FEV1 (OR = 2.012, $P = 0.023$), low PEF (OR = 3.012, $P = 0.019$), low FEF25 (OR = 3.415, $P = 0.025$), low FEF50 (OR = 1.993, $P = 0.043$), and low FEF75 (OR = 3.209, $P = 0.026$) are risk factors for chronic cough in children with CVA (Table III).

DISCUSSION

Compared with asthma, the pathophysiology of CVA is more insidious, lacks specific characteristics, and changes in lung function are not obvious (8). Children with CVA are the main population, and if left treated in time, they will develop into chronic,

recurrent and persistent cough, which will affect their growth and daily life (9).

The level of the big airway indicators FVC and FEV1 in the chronic cough group were lower than those in the control group. Similarly, the level of the small airway indicators PEF, FEF25, FEF50 and FEF75 in the chronic cough group were lower than those in the control group. Especially for the small airway function indicators, low PEF, low FEF25, low FEF50 and low FEF75 were risk factors for chronic cough in children with CVA. In addition, multivariate logistic regression analysis demonstrated that low FVC, low FEV1, low PEF, low FEF25, low FEF50, and low FEF75 were risk factors for chronic cough in children with CVA.

The small airways are more susceptible to invasion by inflammatory mediators, resulting in thickening of the small airway wall, narrowing of the lumen, increased airway reactivity and peripheral resistance, and eventually leading to small airway obstruction (10). However, due to the short study time, small sample size, and many factors that affect cough variability, the chronic cough of asthma cannot be controlled one by one, so further research is needed.

In summary, chronic cough in children with CVA is closely related to changes in pulmonary function. Decreased airway function and reduced small airway function increase the risk of chronic cough, which should be noted in clinical practice.

Table III. *Multi-factor logistic regression analysis.*

Variables	β	SE (β)	Wald- χ^2	P value	OR value	OR lower limit	OR upper limit
Low FVC	0.974	0.446	4.788	0.029	2.651	1.107	6.349
Low FEV1	0.700	0.308	5.166	0.023	2.012	1.101	3.677
Low PEF	1.103	0.468	5.545	0.019	3.012	1.203	7.541
Low FEF25	1.228	0.544	5.083	0.024	3.415	1.174	9.934
Low FEF50	0.690	0.341	4.096	0.043	1.993	1.022	3.887
Low FEF75	1.166	0.524	4.951	0.026	3.209	1.149	8.962

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LETTER TO THE EDITOR

MiR-142 inhibits cell viability, metastasis and EMT through CYR61 in ovarian cancerH. Wang¹, X. Zhou¹, B. Zhang¹ and L. Sun²¹Department of Obstetrics and Gynecology, Lu'an people's Hospital, An'hui, China; ²Laboratory, Lu'an people's Hospital, An'hui, China

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To the Editor,

Ovarian cancer (OC) is a common gynecological malignancy and the leading cause of cancer-related death among all gynecological tumors in China. Ovarian cancer is mostly diagnosed at late stages, because of lack of sensitive biomarkers (1) therefore, it is urgent to identify novel targets for early diagnosis.

MiRNAs are involved in various biological processes that include cell proliferation, metastasis and angiogenesis. For example, miR-142 acted as a tumor suppressor and was involved in the viability and apoptosis of ER-negative breast cancer. Similarly, miR-142 impaired the viability and colony-forming capacities in regulating the expression of CDK4 in colorectal cancer (2). Even in ovarian cancer, miR-142 inhibited the proliferation and chemoresistance by binding to SIRT1 (3). However, as it is still unclear, we explored the role of miR-142 on the metastasis in ovarian cancer.

Cysteine-rich protein 61 (CYR61) is a member of the CCN family that encodes a cysteine-rich secretory protein (4). As a potential biomarker, CYR61 was upregulated in coronary artery disease and acute myocardial injury (5). Habel et al. revealed that CYR61 regulated the expression of related markers of EMT-like processes and triggered the metastatic spreading of osteosarcoma (6).

MATERIALS AND METHODS

Patients and samples

Ovarian cancer patients admitted to Lu'an people's Hospital between January 2016 and June 2018 were enrolled in the study. Fifty-three pairs of ovarian cancer and adjacent non-tumor liver tissue samples were collected from the patients undergoing surgery. The tissues were instantly snap-frozen in liquid nitrogen and then stored at -80°C until use. None of the patients had undergone local or systemic treatment prior to surgery. Written informed consents were obtained from all patients and the study was approved by the Ethics Committee of Lu'an People's Hospital.

Cells and cell culture

Normal human ovarian cancer cells (SKOV3 and HOC1) and normal ovarian cell IOSE80 were purchased from Cobioer (Shanghai, China). The cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemental with 10% foetal bovine serum (FBS, Sigma-Aldrich, Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO₂.

RNA extraction and RT-qPCR

Mir-XTM miRNA qPCR SYBR Kit (Takara Bio) and SYBR Premix Ex Taq II (Takara Bio) were used to carry out the RT-qPCR using a QuantStudio® 6 Flex Real-Time PCR System (Life Technologies, Beijing, China). GAPDH and U6 were used for the standardization for mRNA and

*Key words: miR-142; CYR61; viability; EMT; ovarian cancer**Corresponding Author:*

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miRNA, respectively. The $2^{-\Delta\Delta C_q}$ method was used to analyze the relative expression (7). The primer sequences were: miR-142: 5'-GGCCCATAAAGTAGAAAGC-3' and 5'-TTTGGCACTAGCACATT-3'; U6: 5'-GTGCTCGCTTCGGCAGCACATATAC-3' and 5'-AAAAATATGGAACGCTCACGAATTTG-3'; CYR61: 5'-GGCAGACCCTGTGAATATAA-3' and 5'-CAGGGTTGTCATTGGTAACT-3'; GAPDH: 5'-CTGGGCTACACTGAGCACC-3' and 5'-AAGTGGTCGTTGAGGGCAATG-3'.

Western blot

Total proteins were lysed from tissues and cells by cold RIPA (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was calculated using Bicinchoninic Acid Protein Assay Kit (Pierce; Thermo Fisher Scientific). Equal proteins were separated using 10% SDS-PAGE and subsequently transferred onto a nitrocellulose membrane (Bio-Rad, Beijing, China). The membrane was blocked by 5% non-fat milk and then incubated by using primary antibodies including CYR61 (ab24448, Abcam, Beijing, China), E-cadherin (ab194982, Abcam, Beijing, China), N-cadherin (ab18203, Abcam, Beijing, China) and GAPDH (ab181602, Abcam, Beijing, China) antibody. Next, the membrane was incubated by secondary antibody (Abcam, Beijing, China) and the immunoreactive protein bands were measured using Enhanced Chemiluminescence (ECL) system.

Cell viability assay

SKOV3 cells were seeded in 96-well plates containing 0.1 ml of DMEM at 1,000 cells per well. Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) was employed to calculate cell viability after incubated for 24, 48, 72 and 96 h. After incubating CCK-8 reagent over 3 hours, the absorbance of each well was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

Transwell assay

Transwell insert (Corning, Beijing, China) was utilized to evaluate cell invasive ability. The Transwell insert was placed in a 24-well plate, and SKOV3 cells were cultured in serum without RPMI-1640 medium, trypsinized and then seeded in the upper chamber. In the meantime, the lower chamber was filled with 600 ml 20% FBS RPMI-1640 medium. After 24 h, the non-migrated SKOV3 cells

were removed by a cotton swab. Meanwhile, the invaded SKOV3 cells were fixed for 30 min using 100% methanol. The cells were then stained for 20 min using 0.1% crystal violet. Finally, images of SKOV3 cells were taken under a phase-contrast microscope.

Cell transfection

SKOV3 cells were seeded in 24-well plates and incubated overnight. The miR-142 mimic, miR-142 inhibitor, or inactive control vectors (Life Technologies, Beijing, China) were transfected in SKOV3 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Beijing, China).

Luciferase assay

To verify miR-142 targeted to CYR61, the predicted miR-142 binding sequences on 3'UTR of CYR61 mRNA were mutated, and both the wild type and the mutant sequences were inserted into pGL3 vectors. SKOV3 cells were co-transfected with miR-142 mimic or NC mimic and pGL3-CYR61-WT or pGL3-CYR61-MUT plasmid using Lipofectamine 2000 (Invitrogen, Beijing, China). After culturing for 24 h, the firefly luciferase activity was measured using a dual luciferase assay (Promega, Beijing, China), and Renilla luciferase activity was utilized as the internal control.

Statistical analysis

SPSS software was used to perform the statistical analyses and the data were expressed as means $\pm$ standard deviation (SD). Differences between two groups were compared by *t*-test, while the comparisons between three or more groups were performed through one-way analysis of variance. Pearson's correlation coefficient was employed to estimate the relationship between the expression of miR-142 and CYR61 in ovarian cancer tissues. Statistical analysis was significant when $P < 0.05$. Experiments were repeated at least three times.

RESULTS

Comparison between the expression of miR-142 and prognosis in ovarian cancer

To explore the functions of miR-142 in ovarian cancer, the expression of miR-142 was assessed in ovarian cancer tissues and non-coding tissue samples using RT-qPCR analysis. miR-142 was

low expressed in ovarian cancer tissues compared with non-coding tissue samples ($P<0.05$) (Fig. 1A). The 10-year overall survival of all ovarian cancer was evaluated by Kaplan-Meier method, and it demonstrated that downregulation of miR-142 predicted poor prognosis of ovarian cancer ($P<0.05$) (Fig. 1B).

MiR-142 suppressed the viability, migration and EMT in ovarian cancer cells

The RT-qPCR was conducted to calculate the expression of miR-142 in ovarian cancer cells (SKOV3 and HOC1) and normal ovarian IOSE80 cells. The expression of miR-142 was higher in normal ovarian cell IOSE80 than SKOV3 and HOC1 cells ($P<0.05$) (Fig. 2A). The miR-142 mimic or inhibitor was applied to up- ($P<0.01$) or down-regulate ($P<0.05$) the expression of miR-142 in SKOV3 cells (Fig. 2B). CCK-8 and Transwell assays were performed to assess the functions of miR-142 on the viability and migration in SKOV3 cells. As expected, cell viability was decreased by miR-142 mimic ($P<0.05$), which was enhanced by

miR-142 inhibitor in SKOV3 cells ($P<0.05$) (Fig. 2C). The miR-142 mimic reduced the invasive ability ($P<0.05$), while it was increased by miR-142 inhibitor in SKOV3 cells ($P<0.05$) (Fig. 2D). Moreover, Western blot was utilized to evaluate the expression of EMT-associated proteins, including E-cadherin and N-cadherin. We discovered that E-cadherin was decreased by transfecting miR-142 mimic, while it was increased by miR-142 inhibitor in SKOV3 cells. On the contrary, miR-142 mimic promoted the expression of N-cadherin, which reduced by transfecting miR-142 inhibitor in SKOV3 cells (Fig. 2E). All the results elucidated that miR-142 inhibited the viable, migratory and EMT abilities in ovarian cancer cells.

CYR61 is a target gene of miR-142 in SKOV3 cells

TargetScan (www.targetscan.org), a public database, was employed to predict the potential targets of miR-142 in ovarian cancer cells, and CYR61 was predicted to be a target of miR-142 (Fig. 3A). Luciferase reporter assays were carried out to validate miR-142 targeted to CYR61 mRNA 3'UTR.

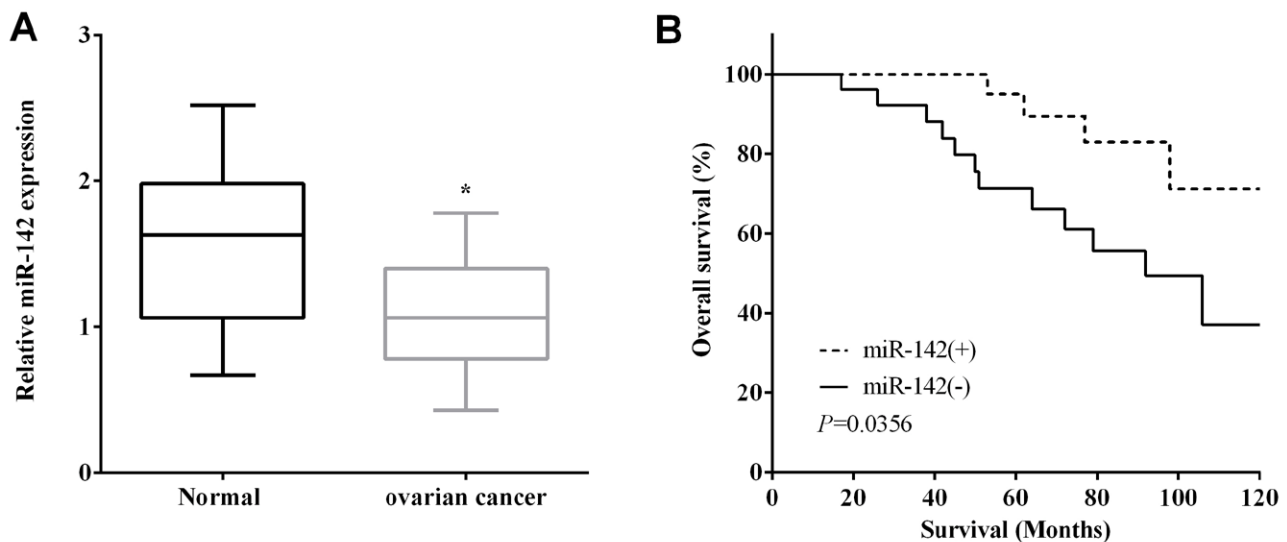


Fig. 1. Comparison between the expression of miR-142 and prognosis in ovarian cancer. **A)** miR-142 was low expressed in ovarian cancer tissues versus non-coding tissue samples. **B)** Downregulation of miR-142 predicted poor prognosis in ovarian cancer.

In comparison with mimic NC, the luciferase activity of CYR61 wild type 3'-UTR was inhibited by miR-142 mimic ($P < 0.05$), but not the mutant CYR61 3'-UTR in SKOV3 cells ($P > 0.05$) (Fig. 3B). The CYR61 expression was evaluated by RT-qPCR after transfecting miR-142 mimic or miR-142 inhibitor in SKOV3 cells. As demonstrated in Fig. 3C, RT-qPCR indicated that compared with NC, miR-142 mimic reduced the expression of CYR61 in SKOV3 cells ($P < 0.05$), whereas miR-142 inhibitor increased the expression of CYR61 ($P < 0.05$) (Fig. 3C). The results revealed that CYR61 is a target gene of miR-142.

To explore the functions of CYR61 in ovarian cancer, RT-qPCR assay was conducted to assess the

expression of CYR61 in tissues and cells. Contrary to the results of miR-142, the expression of CYR61 was higher in ovarian cancer tissues than that of the normal tissues ($P < 0.05$) (Fig. 3D). Moreover, CYR61 was overexpressed in SKOV3 ($P < 0.01$) and HOC1 cells ($P < 0.05$) *versus* ovarian cell IOSE80 (Fig. 3E).

CYR61 mediated by miR-142 to promote cell viability, migration and EMT

Meanwhile, Kaplan-Meier method indicated that overexpression of CYR61 predicted poor overall survival of ovarian cancer patients ($P < 0.05$) (Fig. 4A). To verify the effect of CYR61 on miR-142 functions, miR-142 mimic-transfected SKOV3 cells were

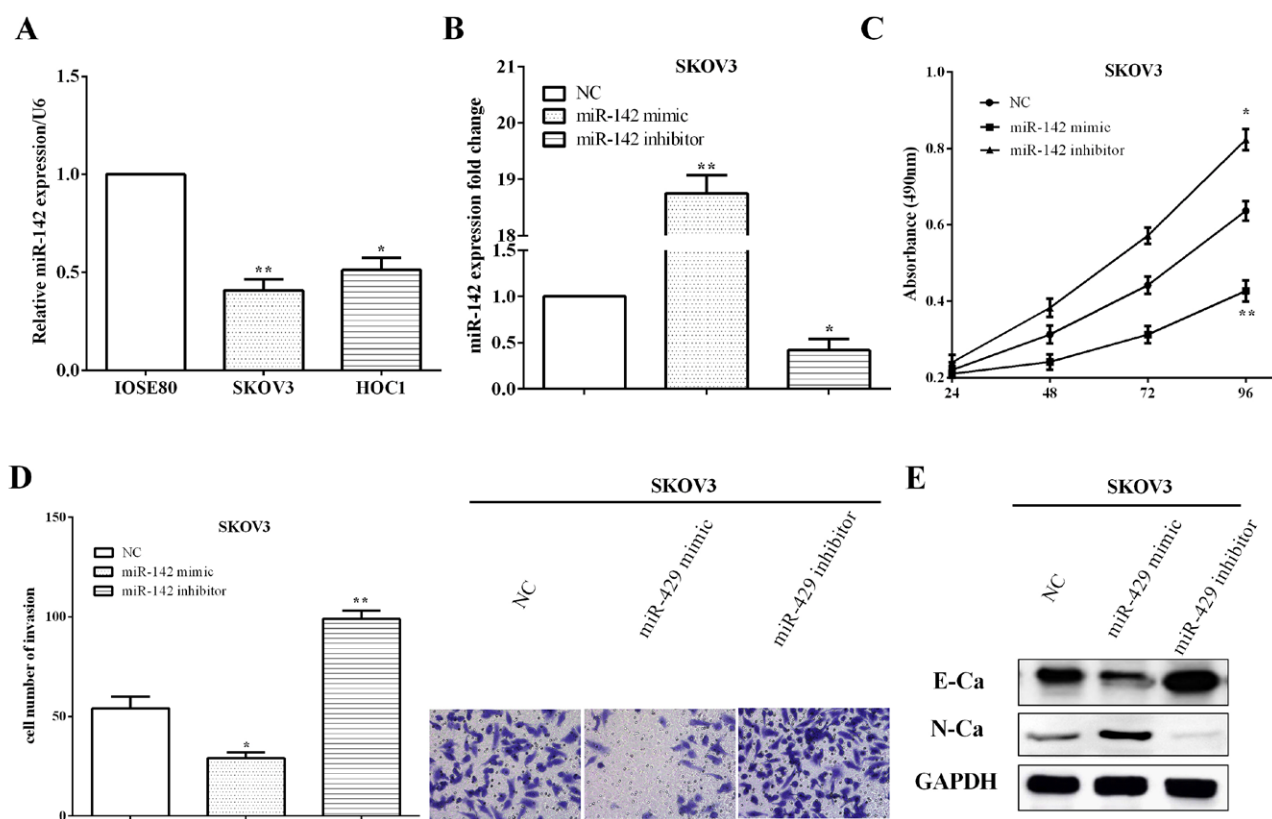


Fig. 2. miR-142 suppressed the viability, migration and EMT in ovarian cancer cells. **A)** The expression of miR-142 was higher in normal ovarian cell IOSE80 than SKOV3 and HOC1 cells. **B)** miR-142 mimic decreased cell viability whereas miR-142 inhibitor promoted that in SKOV3 cells. **C)** miR-142 mimic reduced the invasive ability in SKOV3 cells, while it was increased by miR-142 inhibitor. **D)** miR-142 inhibited the EMT ability in ovarian cancer cells. E-Ca, E-cadherin; N-Ca, N-cadherin; GAPDH, phosphoglycerolaldehyde dehydrogenase.

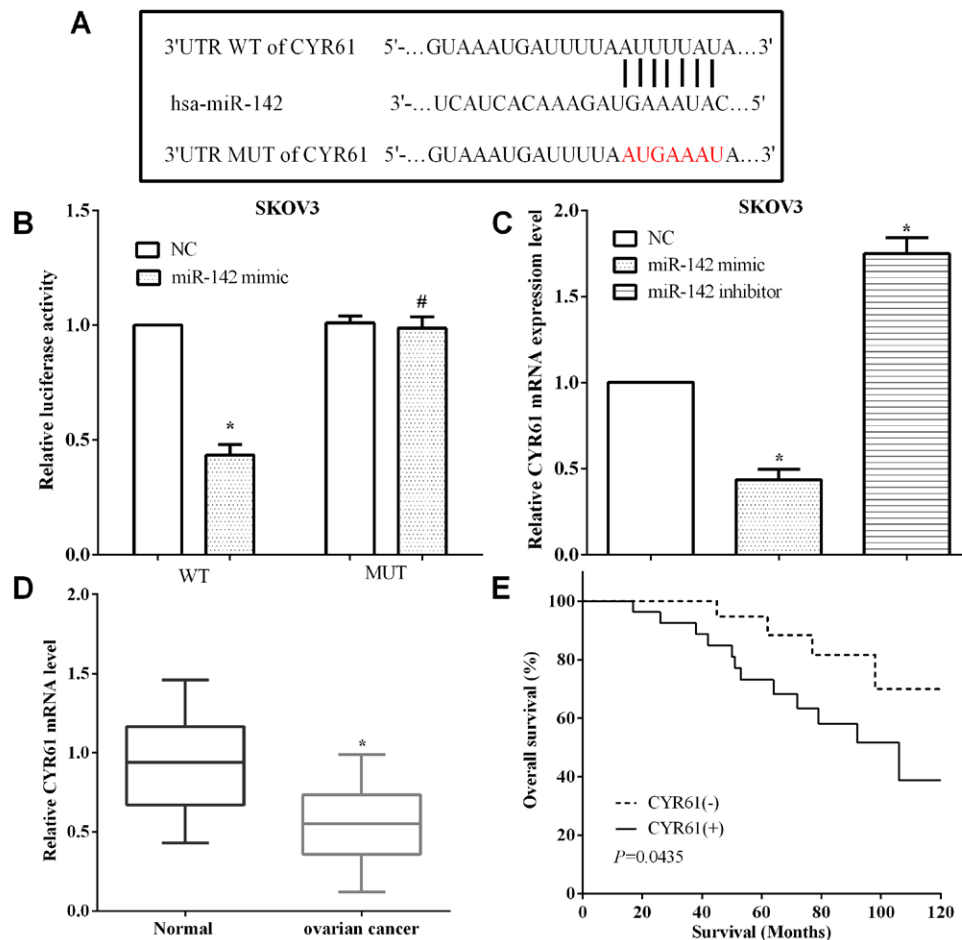


Fig. 3. *CYR61* is a target gene of *miR-142* in SKOV3 cells. **A)** *CYR61* was predicted to be a target of *miR-142* by TargetScan. **B)** The luciferase activity of *CYR61* wild type 3'-UTR was inhibited by *miR-142* mimic, but not the mutant *CYR61* 3'-UTR in SKOV3 cells. **C)** *miR-142* mimic reduced the expression of *CYR61* in SKOV3 cells, whereas *miR-142* inhibitor increased the expression of *CYR61*. WT, wild type; MUT, mutant type.

transfected with *CYR61* overexpression plasmid or control, and the transfection efficiency was evaluated by RT-qPCR as shown in Fig. 4B ($P < 0.05$).

CCK-8 and Transwell assays were performed to assess the viability and migration in SKOV3 cells. As expected, cell viability was enhanced after transfection with *CYR61* overexpression plasmid *versus* transfected negative control in *miR-142* mimic-transfected SKOV3 cells ($P < 0.05$) (Fig. 4C). Similarly, in comparison with negative control, migratory ability was upregulated by *CYR61* overexpression plasmid ($P < 0.05$) (Fig. 4D). In addition, the EMT-associated proteins

were evaluated by Western blot. Overexpression of *CYR61* promoted the expression of E-cadherin while decreasing the expression of N-cadherin, compared with negative control (Fig. 4E). Rescue experiments indicated that *miR-142* regulates cell viability, migration and EMT by targeting *CYR61*, and *CYR61* reversed functions of *miR-142*.

DISCUSSION

MicroRNAs are conserved noncoding RNAs, which has been reported via base-pairing with the 3'-UTR of target gene to mediate target gene

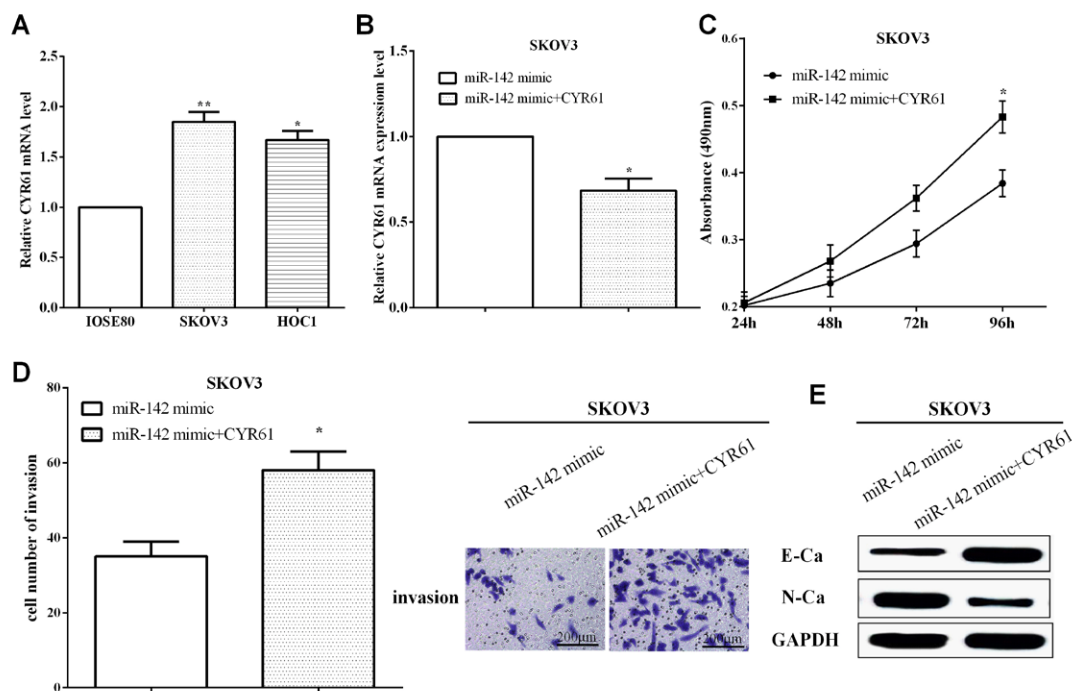


Fig. 4. Upregulation of CYR61 predicted worse outcome of ovarian cancer patients. **A)** The expression of CYR61 was higher in ovarian cancer tissues than that of the normal tissues. **B)** CYR61 was overexpressed in SKOV3 and HOC1 versus ovarian cell IOSE80. **C)** Kaplan-Meier method indicated that overexpression of CYR61 predicted poor overall survival of ovarian cancer patients.

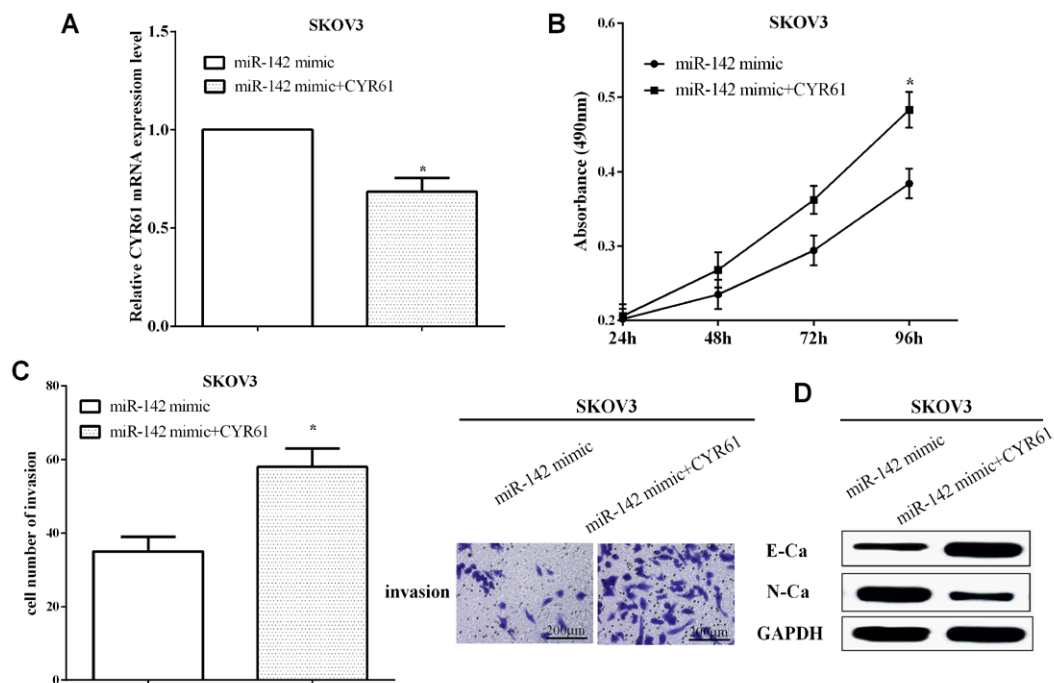


Fig. 5. CYR61 mediated by miR-142 to promote cell viability, migration and EMT. **A)** The transfection efficiency of transfecting CYR61 overexpression plasmid or control in miR-142 mimic-transfected SKOV3 cells. **B)** The overexpression of CYR61 enhanced cell viability. **C)** The migratory ability was upregulated by CYR61 overexpression plasmid. **D)** Overexpression of CYR61 promoted the EMT ability in miR-142 mimic-transfected SKOV3 cells.

expression. Previous studies have elucidated that miR-142 acted as a tumor miRNA in regulation of the metastasis in breast cancer (8). We discovered that low expression of miR-142 was related to the poor prognosis of ovarian cancer patients. Consistent with the findings in hepatocellular carcinoma (9), we discovered that miR-142 suppressed cell viability, migration and EMT.

CYR61 was overexpressed in pulmonary arterial hypertension that enhanced the viability of pulmonary artery smooth muscle cells. Consistent with the findings of Mayer et al. (10), we found that CYR61 overexpression was associated with poor overall survival in ovarian cancer tissues. MiR-142 downregulation enhanced tumor metastasis by targeting CYR61 in gastric cancer (11). Our findings were consistent with those findings, miR-142 regulated CYR61 expression by targeting to CYR61 mRNA 3'-UTR in SKOV3 cells. Similarly to with Li et al. (12), we proposed that CYR61 overexpression improved cell viability, migration and EMT in miR-142 mimic-transfected cells, which suggested that CYR61 reversed the roles of miR-142 in ovarian cancer.

In conclusion, miR-142 regulated cell viability, migration and EMT by directly targeting CYR61 mRNA 3'-UTR in SKOV3 cells. We reported a new type miRNA miR-142 that exhibited significant anti-proliferative, anti-migration/invasive effects and may have therapeutic prospects for the treatment of ovarian cancer.

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LETTER TO THE EDITOR

Relationship between MIF-173G/C polymorphism and cerebral stroke

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To the Editor,

Cerebral infarction (CI) is one of the three major causes of death from human diseases. The morbidity, mortality and disability rates of CI are very high (1). Increasing evidence shows that genetic factors are crucial in the onset of CI, which is manifested as an additive effect of multiple genes. At present, exploring the etiology of CI has become the focus of its prevention. Atherosclerosis is the main pathological basis of vascular disease, while plaque rupture and secondary thrombosis are the main causes of most acute vascular events (2). Previous studies have shown that during the formation of atherosclerosis, the expression of MIF in macrophages and smooth muscle cells begins to increase at the lipid streak phase (3).

The protein encoded by human MIF mRNA is composed of 115 amino acids and has a molecular weight of approximately 12.5 kD. It is now known that the MIF gene has polymorphic sites at -173 (G/C), +254 (T/C) and +656 (C/G) (4). Among these polymorphic sites, MIF-173 (G/C) is closely related to human diseases. It has been found that people with MIF-173 (*C) have significantly higher serum MIF levels than the GG genotype (5). In addition, it was found that the frequency of MIF-173GC genotype in patients with coronary heart disease was significantly higher than that in healthy people (6). The purpose of this study is to detect the distribution of MIF-173 (G/C)

polymorphism and genetic polymorphism in CI, and to explore the relationship between MIF-173 (G/C) polymorphism and blood lipid level or blood pressure.

MATERIALS AND METHODS

The control group (NC) included 105 healthy people (55 males and 50 females) with an average age of 60.35 ± 6.5 years and no blood relationship. Subjects suffering from coronary heart disease, cerebrovascular disease, liver and kidney disease, blood disease, thyroid disease, autoimmune disease, pregnancy, and lipid-lowering therapy were excluded. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of The Third People's Hospital of Qingdao.

The cerebral infarction group (CI) included 132 patients with cerebral infarction (76 males and 56 females) with an average age of 63.82 ± 12.11 years). All patients were clinically diagnosed by CT or MRI (according to the diagnostic criteria of the 4th National Cerebrovascular Disease Academic Conference), and patients with cerebral infarction were examined through the carotid artery color Doppler ultrasound. It does not include strokes caused by cardiogenic, arteritis, trauma, blood diseases, drugs, tumors, cerebrovascular malformations or aneurysms. Patients suffering from severe liver and kidney disease, autoimmune diseases, pregnancy, thyroid disease, and lipid-lowering treatment were excluded.

*Key words: MIF; cerebral stroke; gene polymorphism**Corresponding Author:*

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Clinical biochemical assay

The medical history, physical examination, electrocardiogram, and chest radiograph of all patients were examined. TG and TC were determined by the oxidase method. Fetal bovine serum (FBS) was determined by glucose oxidase method. First, the HDL-C was precipitated, and then the cholesterol in the supernatant was measured by enzymatic method. LDL-C was examined using the formula $(\text{LDL-C}) = \text{TC} - (\text{HDL-C}) \times (\text{TG}/5)$.

Blood sample collection and DNA extraction

After fasting for 12 h, 5 ml of cubital venous blood was obtained from the patients. EDTA was used for anticoagulant. Genomic DNA was extracted by conventional phenol-chloroform method and stored at -20°C .

PCR reaction

The PCR amplification primer sequence was synthesized by Shanghai Yingjun Biotechnology Co., Ltd (Shanghai, China). The primer sequences were as follows: forward: ACTAAGAAAGACCCGAGGC; reverse: GGGGCACGTTGGTGTTCAC. The reaction conditions included: pre-denaturation at 95°C for 5 min, denaturation at 94°C for 30s, annealing at 58.8°C for 30s, extension at 72°C for 30 s, 35 cycles, and full extension at 72°C for 10 min. Finally, the PCR product was placed at 4°C to terminate the reaction.

PCR amplification band detection

First, 2% agarose gel was prepared. In $0.5 \times \text{TBE}$ electrophoresis buffer, 5 μl amplified product was electrophoresed at 100 volts for 40 min. The results were observed under ultraviolet light in the gel imaging system. The amplified product showed a single specific target band (MIF gene-173G/C site is a 366bp fragment).

RELP analysis

The PCR amplification product (10 μl) and restriction enzyme AluI (1 μl) were digested at 37°C for 10 h. 2% agarose gel was then prepared. 8 μl digestion products in $0.5 \times \text{TBE}$ electrophoresis buffer were electrophoresed at 90 volts for 50 min. After the electrophoresis is completed, the results were observed under the ultraviolet rays of the gel imaging system.

Statistical analysis

All data were analyzed by using SPSS 19.0 software. The gene counting method was used to detect the frequency of each genotype in the CI group and the NC group. *Chi*-squared test was used to compare genotypes and alleles between groups. Differences between multiple groups were analyzed by analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

First, the clinical data was compared between the CI group and the control group. The results showed that there were no significant differences between the two groups in terms of age, gender, smoking history and drinking history. In the CI group, patients with a history of hypertension and diabetes were significantly higher than the NC group (Table I). In addition, the BMI level of the CI group was significantly higher than that of the NC group (Table I). Next, the blood lipids, blood pressure and blood glucose were compared between the CI group and the NC group. Compared with the NC group, there were significant differences in blood lipids, blood pressure and blood glucose (except TG) in the CI group (Table II). These results show that CI patients have significant differences in the clinical biochemical indexes compared with healthy people.

The MIF-173G/C polymorphic site is recognized by Alu I endonuclease. The restriction sites are 5' ... AG/CT... 3'. As shown in Fig. 1A, there is a natural cleavage site in the amplified PCR fragment, which can be cut into two fragments of 268bp and 98bp, therefore, the GC type only produces two fragments. When the G-C mutation occurs at -173, another restriction site is created. The PCR product can be digested into three fragments of 62bp, 206bp, and 98bp, which are homozygous CC type. In short, four restriction fragments of 62bp, 98bp, 206bp and 268bp were obtained in MIF-173G/C.

The CI group and NC group had three genotypes: GG, GC and CC. The frequencies of GG, GC and CC in the total population (CI group + NC group) were 61.28%, 33.76%, and 4.96%, respectively. The frequencies of alleles G and C were 78.16% and 21.84%, respectively. The distribution of GG,

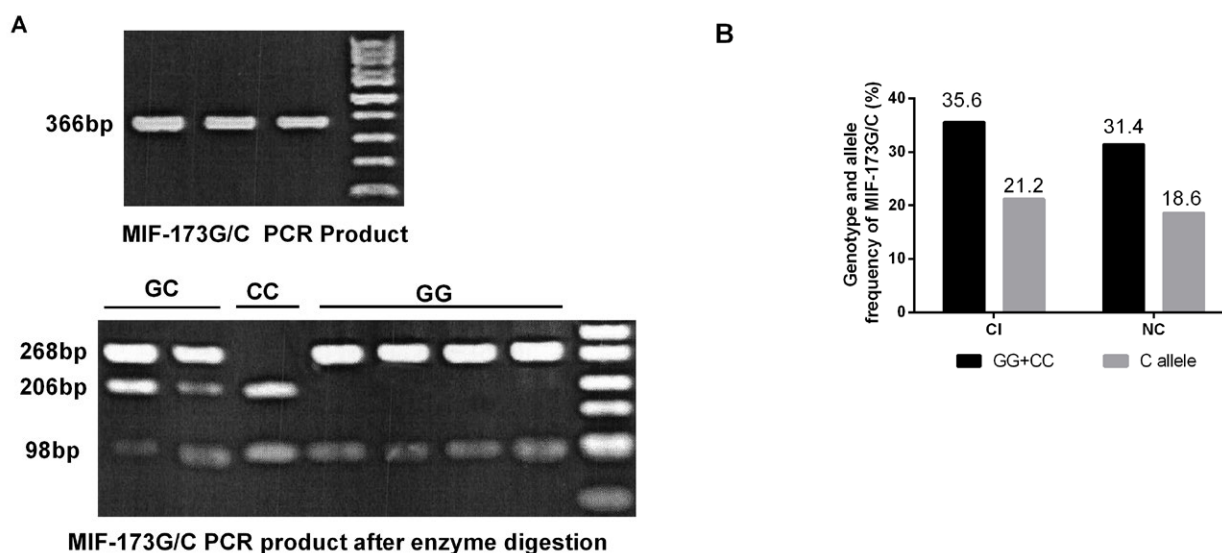


Fig. 1. Polymorphism distribution of MIF-173G/C between the CI group and NC group. **A)** Enzyme digestion analysis of MIF-173G/C polymorphic sites. **B)** Genotype and allele frequency of MIF-173G/C were compared between the CI group and NC group.

GC, CC genotypes in the CI group and NC group is shown in Table III. The results showed that the two groups had the highest frequency of GG genotype and the lowest frequency of CC type. Because there are few cases of CC type, CC type and GC type were combined into the GC + CC group, which is the C allele carrier group. In addition, Fig. 1B shows that there was no significant difference in the frequency distribution of MIF-173G/C genotypes between the CI and NC groups. Nor was there significant difference in the frequency of the two alleles between the two groups (Fig. 1B). Taken together, there is almost no difference in the distribution of MIF-173G/C polymorphism between CI patients and healthy people.

Finally, differences in blood lipid levels, blood pressure, blood glucose, IMT and BMI between different genotypes of MIF-173G/C were analyzed in the CI group. The results showed that the BMI level of the GC + CC group was higher than that of the GG homozygous group. The difference in BMI level of CI group was statistically significant. However, there were no significant differences in other indicators

(Table IV). These results show that the MIF-173G/C polymorphism has little effect on blood lipids, blood pressure and blood glucose of CI patients.

DISCUSSION

MIF is an important pro-inflammatory factor. In recent years, the role of MIF in the development of atherosclerosis has attracted much attention. A large number of studies have found that MIF is closely related to cardiovascular diseases and metabolic diseases such as DM, obesity and hypertension (7). It has been reported that the concentration of MIF in plasma gradually increases from the acute phase to the subacute phase of CI, but it is still lower than in healthy people (8) which indicates that MIF is one of the indicators of CI.

SNP of MIF gene may affect the occurrence of cardiac ischemia-reperfusion injury by influencing the secretion of MIF. It has been found that the frequency of MIF-173GC genotype in patients with cardiac ischemia-reperfusion injury is higher than that in healthy people (9). The C allele may

Table I. Comparison of clinical data between CI group and NC group

Features	CI Group	NC Group
Age	63.82±12.11	60.35±6.5
BMI	25.09±2.11*	22.13±2.65
Sex (male/female)	76/56	55/50
Hypertension (Yes/No)	73/60*	21/84
Diabetes (Yes/No)	40/93*	15/90
Smoking (Yes/No)	58/75	36/69
Drinking (Yes/No)	42/91	33/72

* $P < 0.05$ **Table II.** Comparison of blood lipids, blood pressure, and blood glucose between CI group and NC group

Index	CI Group	NC Group
FBS (nmol/l)	6.35±2.03*	5.22±1.21
TC (nmol/l)	4.78 ±1.12	4.29±1.18
TG (nmol/l)	2.01±0.91*	1.56±0.97
HDL (nmol/l)	1.08±0.26*	1.61±0.38
LDL (nmol/l)	2.78±0.66*	2.39±0.95
SBP (mmHg)	153±21*	119 ±18
DBP (mmHg)	91±15*	77 ±10

* $P < 0.05$ **Table III.** Genotype frequency and allele frequency of MIF-173G/C were compared between CI group and NC group

Group	Cases (n)	Genotypes (%)		Allele frequency	
		GG	GC+CC	G	C
CI	132	85(64.4)	38+9(35.6)	0.788	0.212
NC	105	72(68.6)	27+6(31.4)	0.815	0.185

be a susceptibility marker of cardiac ischemia-reperfusion injury in the Han population. Consistent with the above results, we also found that in the Han population, the MIF-173G/C genotype distribution and allele frequency were not significantly different

between the CI group and the healthy group. It has also been confirmed that the expression of MIF in mature adipocytes is proportional to body mass index, and the secretion of MIF in adipocytes in obese patients is greater than that in normal people (10). In

Table IV. Effects of polymorphic distribution of MIF-173G/C in CI group on blood lipid, blood pressure and blood glucose

Index	CI Group	
	GG (n=85)	GC+CC (n=47)
FBS (nmol/l)	5.98±2.86	5.88±2.21
TC (nmol/l)	4.48±1.26	4.51±1.12
TG (nmol/l)	1.98±0.86	1.88±0.8
HDL (nmol/l)	1.18±0.26	1.11±0.25
LDL (nmol/l)	2.66±0.71	2.88±0.66
SBP (mmHg)	148±18	154±24
DBP (mmHg)	85±10	88±13
BMI (kg/m ²)	22.8±1.76	24.12±2.15*
IMT (mm)	0.89±0.26	0.9±0.24

conclusion, the MIF-173G/C polymorphism may not be related to the occurrence of CI in the Han people. In addition, the MIF-173C allele is associated with higher BMI level in CI patients. This study is the first report on the relationship between MIF-173G/C gene polymorphism and the occurrence of CI.

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LETTER TO THE EDITOR

Mechanism of complement activation on cardiac immune and inflammatory response caused by ischemic postconditioning in acute myocardial infarction

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*These authors contributed equally to this work

To the Editor,

Acute myocardial infarction is caused by acute obstruction of the coronary arteries. The myocardium supplied by the coronary arteries suffers continuous damage, leading to the necrosis of myocardial cells, which produces a series of clinical complications (1). High morbidity and mortality of acute myocardial infarction are the main causes threatening life and health (2, 3).

The complement system is a group of non-specific proteins found in the normal fresh blood of humans and vertebrates and has some involvement in the process of acute myocardial infarction (4). Bavia et al. analyzed complement activation products and serum cytokines (5) and found that complement activation is an early event of acute myocardial infarction and has an impact on inflammatory injury in acute myocardial infarction. Zhong et al. identified blood samples from patients with acute myocardial infarction (6), and it was found that bio-modules are related to complement receptor-mediated signalling pathways and immune processes. Relevant studies have found that the complement components and vascular active substances (C3a, C5a) are characterized by strong directional chemotaxis, which produce oxygen free radicals, proteolytic enzymes, and inflammatory

factors, such as TNF- α , IL-1, IL-6, and NF- κ B, thereby leading to damage, apoptosis, and necrosis of myocardial cells (7). Therefore, the response mechanism of complement system activation in ischemic reperfusion was analyzed.

In summary, there is much research on the complement system of IPTC in acute myocardial infarction, but the mechanism of immune inflammatory responses in the heart is still unclear. Based on this, a rat model of myocardial ischemia-reperfusion injury was established and the effect of complement activation in myocardial ischemia postconditioning on the immune inflammatory response of the heart was comprehensively evaluated using different intervention methods.

MATERIALS AND METHODS

Animals and grouping

Ninety-three SD male rats (purchased from Nanjing Junke Bioengineering Co., Ltd., China) were selected, with a weight range of 200-280g and an age range of 8-12 weeks. A blind selection method was undertaken to randomly divide the rats into three groups (31 rats in each group): ischemia-reperfusion group (S1), ischemia postconditioning group (S2), and sham operation group

Key words: acute myocardial infarction; immune inflammatory response of the heart; complement system; postconditioning of myocardial ischemia; NF- κ B signaling pathway

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(D0). Rats in the S1 group were perfused after coronary ischemia for half an hour, lasting for two h. Rats in the S2 group were treated (ischemia for half a minute, perfusion for half a minute) after half an hour of coronary ischemia and circulated 3 times. The rats in group D0 were only subjected to anterior descending coronary artery threading, but no ligation operation was performed. All procedures in the animal experiments conformed to the International Regulations on the Protection and Management of Animals, and the protocol was approved by the ethics committee of Wuxi Third People's Hospital.

Establishment of myocardial ischemia-reperfusion injury models

The experimental steps for constructing a rat model of myocardial ischemia-reperfusion injury were as follows: the rats were firstly injected intraperitoneally with 6mL/kg 7% chloral hydrate solution (Shanghai Jingke Huaxue Co., Ltd., China), an 8F vascular sheath femoral vein catheter was taken as the intubation tube and, at the same time, the animal ventilator was connected to assist breathing. Then, the pericardium was opened and the heart was exposed. The needle was inserted 1 mm from the sulcus posterior auricularae of the left side of the heart, and taken out from the cone of the pulmonary artery. Finally, a silicone tube was used to compress the anterior descending coronary artery to cause myocardial ischemia, after which the heart of the rat model became gray-white.

Collection and processing of specimens

When the experiment was completed, the left ventricular free wall myocardium was collected in the 2 mm area near the ligation line and cleaned with phosphate buffer (Jiangsu MRC Co., Ltd., China). Then the sample was divided into several parts, and quickly frozen with liquid nitrogen. Half of the tissue samples were stored at -80°C, and the other half were fixed with 4% paraformaldehyde (Nanjing Duly Biotech Co., Ltd., China) for 24 h, embedded in paraffin, and sectioned.

Staining detection of myocardial cell apoptosis

TdT-mediated DUTP nick end labeling (TUNEL) was adopted to stain myocardial tissue cells. After dewaxing and hydration, the myocardial tissue sections were treated with protein kinase K solution (Hefei Dianfeng Biology Co., Ltd., China), and washed three times with phosphate

buffer solution. Then, 50 µL TUNEL reaction mixture (Zhejiang Lanso Biotechnology Co., Ltd., China) was added, and the samples were placed at 30°C for 1 h for reaction. After washing with phosphate buffer solution 3 times, the cardiac cells were observed under an electron microscope. Next, 50 µL converter-POD (Zhejiang Lanso Biotechnology Co., Ltd., China) was added and left to react at 30°C for 30 min, after which the mixture was washed with phosphate buffer solution 3 times. 80 µL diaminobenzidine (DAB) (Shanghai Long Island Antibody Diagnostic Agent Co., Ltd., China) substrate was added, placed at room temperature for 15 min, and cleaned with phosphate buffer solution 3 times. Finally, the sample was stained with hematoxylin (Beijing Makewonderbio Co., Ltd., China), washed for a few seconds after reaction, and sealed after air drying. Phosphoric acid buffer solution was added to the glass slide and the sample was placed under an electron microscope to count cardiac apoptotic cells, calculate the apoptotic rate, and judge the morphological characteristics of the cells.

Expression levels of mRNA of C3a and C5a detected by Real-time PCR

The myocardial tissues of the three groups of rat models were taken, RNA was extracted according to the instructions of the kit, RNA purity and concentrations were determined, and then real-time PCR was undertaken to detect the mRNA expression levels of C3a and C5a in the left ventricular tissue. The primer sequences were C3a: 5'-GACGCCACTATGTCCATCC-3', 5'-TACTCCAGAGGCCAGCAGrITI-3'; C5a: 5'-TGTAAGCAGAATCGCCACAG-3', 5'-AGCTTCCACTGCAGGTTYGT-3'. Reaction conditions: the sample was denatured for 1 min at 94°C, annealed for 1 min at 5°C, and extended for 2 min at 72°C. The operation was repeated 34 times, and the sample was extended at 72°C for 10 min. The mRNA expression levels of C3a and C5a were detected and recorded.

Detection of the expression levels of complement (C3a and C5a), NF-κB, and myocardial immune inflammatory factor proteins by Western blot

Forty µg of equivalent protein were added into the upper sample hole of the prepared rubber plate, and 5 µL markers (Guangzhou Dongsheng Biotech Co., Ltd., China) to be stained were added to both sides of the upper

sample hole. The electrophoresis buffer was added to start electrophoresis until bromophenol blue was about 0.5 cm away from the lower edge of the separation adhesive. The precooled liquid was poured into the sample and the film was transferred at 300 mA for 90 min. After membrane transfer, polyvinylidene membrane (PVDF) was rinsed in double distilled water for 5 min, and then cultured in 5% skim milk solution (Shandong Gushuo Biotechnology Co., Ltd., China) for 2 h. A primary antibody solution (Hubei Condice Chemical Co., Ltd., China) was added to incubate overnight, and a secondary antibody solution (Hubei Condice Chemical Co., Ltd., China) was added to incubate for 2 h. The samples were rinsed with Tween20/Tris-HCl (TBST) solution (Nanjing Senbeijia Shengwukeyi Co., Ltd., China) 3 times. Odyssey appearance was adopted to scan the PVDF film, and Odyssey 3.0 software was used to intercept the target strip and adjust the appropriate background and strip brightness. Finally, image j software was adopted to calculate the gray value of the protein band (equation: the relative expression of the target protein =

the gray value of the target protein/the gray value of the corresponding internal reference protein).

Statistical analysis

The data of this study were analyzed by SPSS19.0 software, and the measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). The counting data were expressed as a percentage (%), and the *t*-test was used for pairwise comparison of data between the three groups. $P < 0.05$ indicated statistical significance.

RESULTS

Comparison of expression levels of complement (C3a and C5a) protein and mRNA in myocardial tissues of three groups of rats

As shown in Fig. 1A, in the S1 group, the expression of C3a protein in rats was 94.27 ± 9.85 , and that of C5a protein was 92.85 ± 11.34 . In the S2 group, the expression levels of C3a protein and C5a protein were

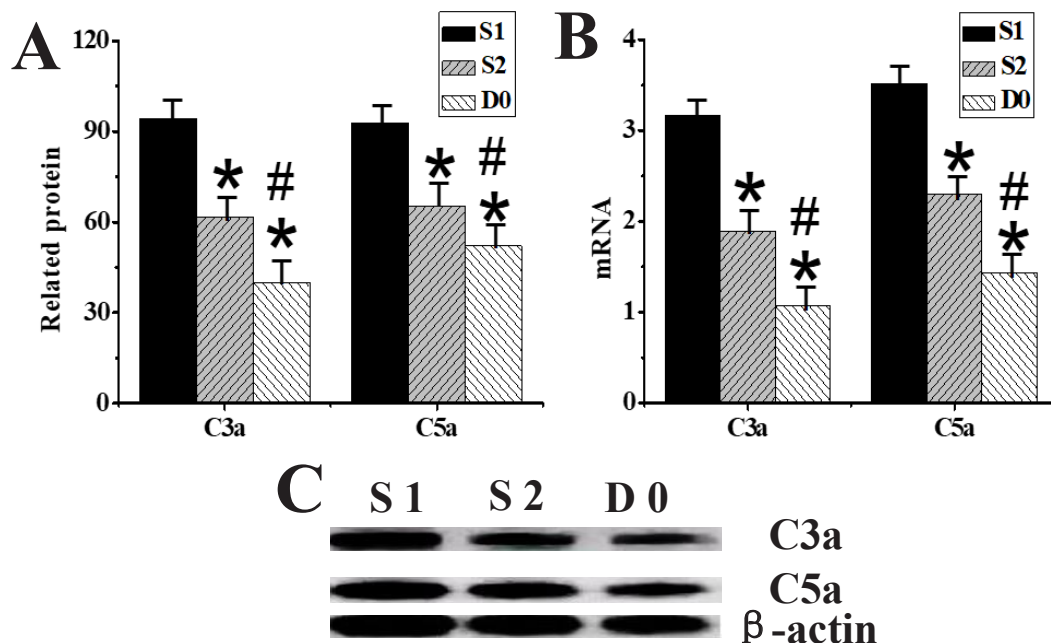


Fig. 1. Expression levels of complement C3a and C5a proteins and mRNA in myocardium of the three groups of rats. **A)** the expression levels of complement C3a and C5a proteins in the three groups; **B)** mRNA expression levels of complement C3a and C5a in the three groups; **C)** C3a and C5a protein bands of rats in the three groups; * statistically significant difference compared with the S1 group ($P < 0.05$); # statistically significant difference compared with the S2 group ($P < 0.05$).

61.55±6.27 and 65.38±6.71, respectively. In the D0 group, the expression levels of C3a protein and C5a protein were 40.12±3.64 and 52.09±4.82, respectively. The expression levels of C3a and C5a proteins in the S1 group were significantly higher than those in group S2 and group D0, and the difference was statistically significant ($P<0.05$). In addition, the expression levels of C3a and C5a proteins in the S2 group were significantly higher than those in the D0 group, and the difference was statistically significant ($P<0.05$). As shown in Fig.1B, the mRNA expression levels of C3a and C5a in the S1 group were 3.16±0.31 and 3.52±0.72, respectively. In the S2 group, the mRNA expression levels of C3a and C5a were 1.89±0.46 and 2.30±0.84, respectively. In the D0 group, the mRNA expression levels of C3a and C5a were 1.07±0.19 and 1.43±0.82, respectively. Accordingly, the mRNA expression levels of C3a and C5a in the S1 group were significantly higher than those in the S2 group and the D0 group, and the difference was statistically significant ($P<0.05$). In the S2 group, the mRNA expression levels of C3a and C5a in rats were significantly higher than those in the D0 group, and

the difference was statistically significant ($P<0.05$). Fig.1C shows the C3a and C5a protein bands of the three groups. It can be clearly observed that the band brightness of the S1 group was the largest, the band brightness of the D0 group was the smallest, and the band brightness of the S2 group was moderate.

Comparison of expression levels of NF-κB protein in myocardial tissues of the three groups

As shown in Fig. 2A, in the S1 group, the expression level of NF-κB was 1.53±0.15. In the S2 group, the expression of NF-κB in rats was 1.02±0.08. In the D0 group, the expression level of NF-κB in rats was 0.76±0.04. Accordingly, the expression level of NF-κB in the S1 group was significantly higher than that in the S2 group and the D0 group, and the difference was statistically significant ($P<0.05$). In the S2 group, the expression level of NF-κB was significantly higher than that in the D0 group, and the difference was statistically significant ($P<0.05$). Fig. 2B shows the NF-κB protein bands in the three groups. It can be clearly seen that the band in the S1 group had the highest brightness, followed by group S2, and group D0 had the smallest band brightness.

Comparison of levels of immunoinflammatory response factor proteins in myocardial tissues of the three groups

As shown in Fig. 3, in the S1 group, the level of IL-1β was 1.35±0.27, the level of IL-1α was 0.97±0.15, the level of IL-6 was 1.43±0.52, and the level of TNF-α was 1.17±0.12. In the S2 group, the level of IL-1β was 0.81±0.33, the IL-1α level was 0.64±0.24, the IL-6 level was 0.62±0.09, and the TNF-α level was 0.58±0.03. In the D0 group, the IL-1β level was 0.57±0.07, the IL-1α level was 0.33±0.02, the IL-6 level was 0.53±0.18, and the TNF-α level was 0.49±0.20. Therefore, levels of IL-1β, IL-1α, IL-6, and TNF-α in rats in the S1 group were significantly higher than those in the S2 group and the D0 group, and the differences were statistically significant ($P<0.05$). In group S2, levels of IL-1β and IL-1α in rats were significantly higher than those in the D0 group, and the difference was statistically significant ($P<0.05$).

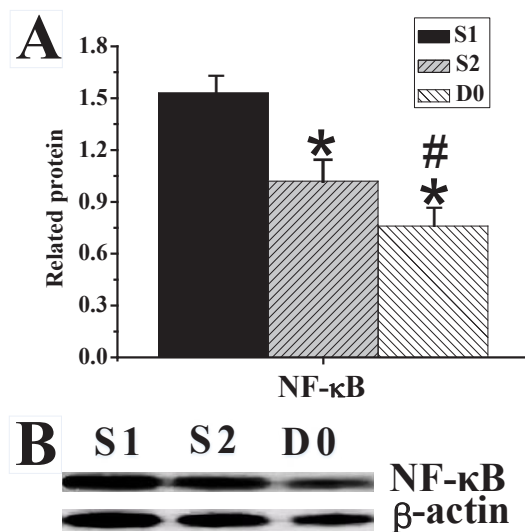


Fig. 2. Expression of complement NF-κB protein in the three groups. A: expression levels of NF-κB protein in myocardial tissues of the three groups; B: NF-κB bands in the three groups. * statistically significant difference compared with the S1 group ($P < 0.05$); # statistically significant difference compared with S2 group ($P < 0.05$).

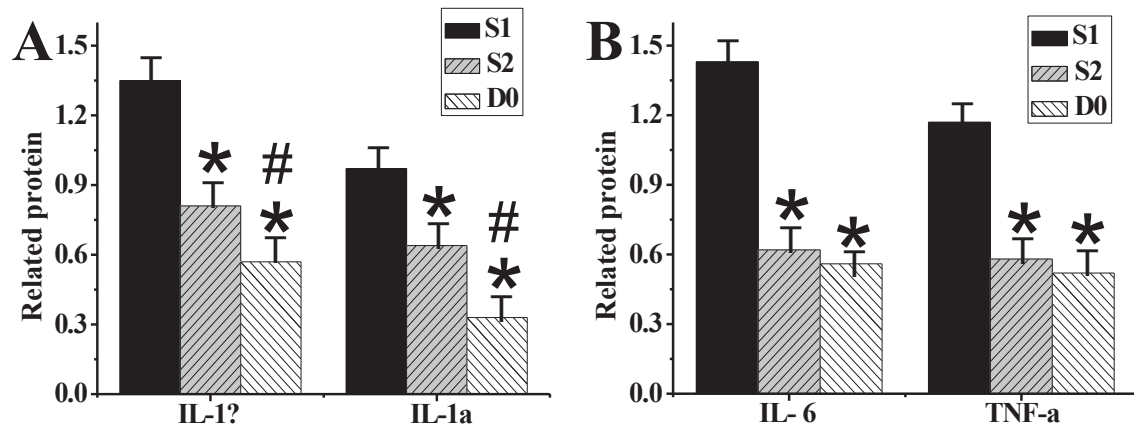


Fig. 3. Levels of IL-1 β , IL-1 α , IL-6, and TNF- α in the three groups. A: levels of IL-1 β and IL-1 α in the three groups; B: levels of IL-6 and TNF- α in the three groups. * statistically significant difference compared with the S1 group ($P < 0.05$); # statistically significant difference compared with the S2 group ($P < 0.05$).

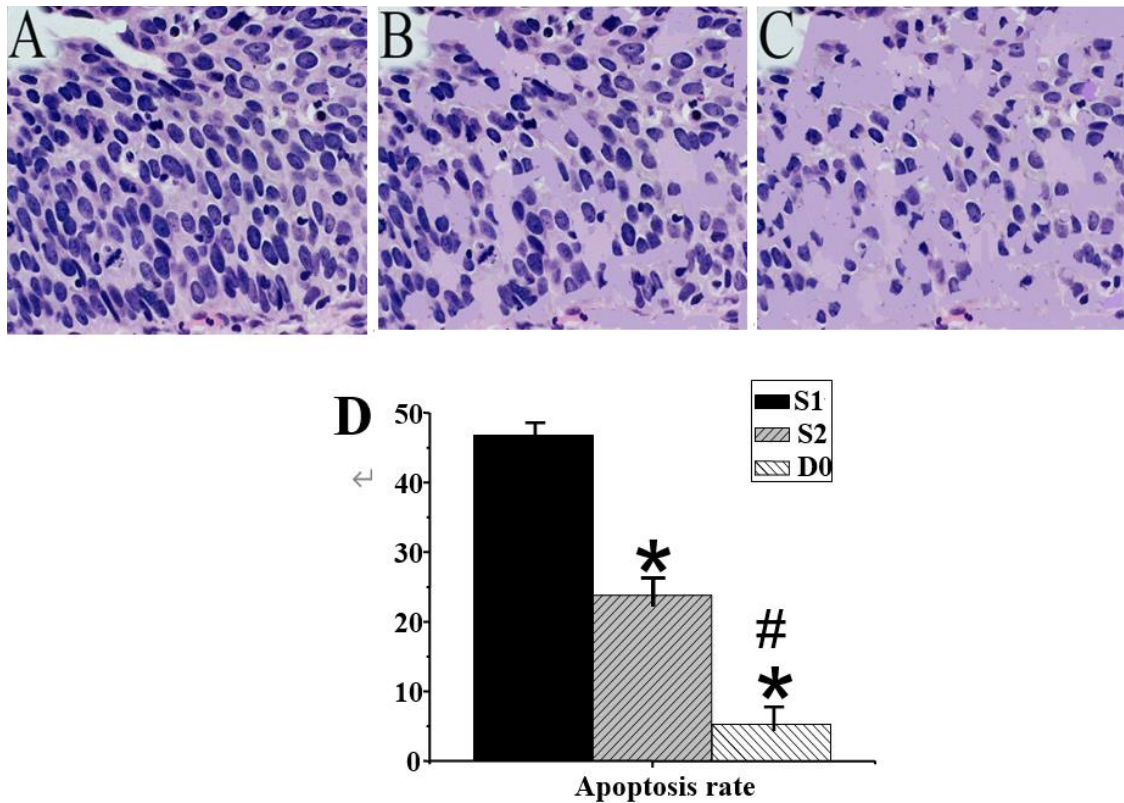


Fig. 4. Apoptosis situations of myocardial cells in the three groups of rats. A: stained cells of rats in the S1 group; B: stained cells of rats in the S2 group; C: stained cells of rats in the D0 group; D: apoptosis rates of cells in the three groups; * the difference was statistically significant compared with the S1 group ($P < 0.05$). # the difference was statistically significant compared with the S2 group ($P < 0.05$).

Comparison of apoptosis rates of myocardial cells in the three groups

Fig. 4 A, B, and C show the cell staining of the three groups of rat models. It can be observed that the staining morphology and size of cardiomyocytes of the rats in the D0 group were normal, which were generally round or oval and blue, and there were basically no apoptotic cells. In the S2 group, the number of cells was significantly reduced, the cell morphology was fragmented, and the size was inconsistent. In the S1 group, the number of cells decreased most substantially, and the apoptotic cells increased significantly, showing dense staining, with few normal cells and more fragmented cells. As shown in Fig. 4D, in the S1 group, the apoptosis rate of rat cells was $46.72 \pm 6.24\%$. In the S2 group, the apoptosis rate of rat cells was $23.81 \pm 3.16\%$. In the S3 group, the apoptosis rate of rat cells was $5.28 \pm 0.47\%$. Accordingly, the apoptosis rate of rats in the S1 group was significantly higher than that in the S2 group and the D0 group, and the difference was statistically significant ($P < 0.05$). The apoptosis rate of rat cells in the S2 group was significantly higher than that in the D0 group, and the difference was statistically significant ($P < 0.05$).

DISCUSSION

In this study, the expression levels of C3a and C5a proteins and mRNA in the S1 group were found to be significantly higher than those in the S2 group and the D0 group ($P < 0.05$), which was consistent with the results of Horwitz et al. (8), which indicated that the complement system regulated the immune inflammatory response through C3a and C5a. In addition, the expressions of C3a and C5a proteins and mRNA in the S2 group were significantly higher than those in the D0 group ($P < 0.05$), which was consistent with the study results of Jin et al. (9) that showed the reduced degree of activation of the complement system of IPTC could significantly reduce the severity of myocardial ischemia-reperfusion injury (10). It was also found that the expression level of NF- κ B in the S1 group was significantly higher than that in the S2 group and the D0 group ($P < 0.05$), and this is consistent with the study results of Zhou et al. (11),

which indicated that the inhibition of the activation of the complement system by IPTC was related to the NF- κ B signaling pathway. The levels of IL-1 β , IL-1 α , IL-6, and TNF- α in the S1 group were significantly higher than those in the other two groups ($P < 0.05$), which was basically consistent with the study results of Takahashi et al. (12). IL-1 β and IL-1 α could counter-stimulate macrophages to produce inflammatory mediators and make the inflammatory mediators have a cascade amplification effect, suggesting that IPTC can effectively reduce the expression of ischemia-reperfusion myocardial inflammatory factors. Overall, IPTC of acute myocardial ischemia infarction can inhibit complement activation in ischemia-reperfusion injury, reduce myocardial cell apoptosis and structural damage, and decrease the expression of the cardiac immune response. In addition, the regulatory mechanism may be related to the NF- κ B signaling pathway. Our study is limited by the small sample sizes of the selected rats. In the future, we plan to increase the sample sizes and to incorporate an analysis of the influence of different variables and genders on the research results.

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LETTER TO THE EDITOR

Hsa_circRNA_104315 increases the malignancy of cervical cancer cells by miR-605-AGO1/RRM2 pathway

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To the Editor,

Cervical cancer is one of the most common, rapidly developing, and high-incidence malignant tumors in gynecology. About 200,000 cervical cancer deaths occur each year (1). In recent decades, due to the continuous advancement of medical technology, cervical cytology screening has been widely used, so that cervical cancer and precancerous lesions can be detected and treated early. Therefore, the incidence and mortality of cervical cancer have decreased significantly. However, early cervical cancer is prone to lymph node metastasis, and the prognosis is relatively poor. It is very important to study in-depth the pathogenesis of cervical cancer (2).

Circular RNA (circular RNA, circRNA) is a non-coding RNA that widely exists in organisms. It is formed by covalently connecting the 3' and 5' ends of reverse splicing. Studies have shown that circular RNA is widely involved in developmental or biological metabolic processes and has diagnostic value during tumorigenesis. circRNA can play a biological role by regulating the combination of microRNA (miRNA) and target genes, such as circular RNA hsa_circ_0012673miR-22 which promotes the proliferation of lung adenocarcinoma (3, 4).

In the present study, the expression of circRNA in cervical cancer cells and tissues was detected to explore the mechanism of action of circRNA in cervical cancer. circRNA_ and its related siRNA and control genes were transfected into human cervical cancer cell line BT-B, and the proliferation, invasion and apoptosis of BT-B cells in each group were analyzed. The target gene of circRNA_104315 was predicted by bioinformatics. circRNA_104315 promotes the development of cervical cancer by regulating the miR-605-AGO1/RRM2 pathway, and may become a molecular target for cervical cancer diagnosis and treatment.

MATERIALS AND METHODS

Clinical sample collection

Twenty cases of matched tumor specimens and normal adjacent cancer specimens were collected from cervical cancer patients in the Hospital of Chengdu University of Traditional Chinese Medicine. Specimens were confirmed by hematoxylin and eosin staining and stored in RNA. All subjects gave written informed consent, and this study was approved by the ethics committee of the Hospital of Chengdu University of Traditional Chinese Medicine.

*Key words: cervical cancer; circRNA_104315; miR-605; AGO1; RRM2**Corresponding Author:*

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Cell culture

Human keratinocyte cell line HaCaT, normal human cervical cell line HcerEpic, human cervical cancer cell lines BT-B, CaSKi, HEC-1-A and Ishikawa were purchased from Tongpai biotechnology Co., Ltd., China. These cells were stored in Dulbecco Modified Eagle's medium (DMEM; Gibco, USA), containing 10% fetal bovine serum (FBS; Gibco, USA), under standard culture conditions (5% CO₂ and 37°C).

Cell transfection

CircRNA_104315 small interfering RNA (siRNA), related si-NC, construct overexpressed circRNA_104315 vector and overexpressed miR-605 vector (Suzhou Biosyntech Co., Ltd., China) were transfected into cells using Lipofectamine 2000 (Invitrogen, USA). Forty-eight h after transfection, real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect the effects of silencing or overexpression. The most effective siRNA was used in subsequent experiments.

RT-qPCR and RNase R treatment

Total RNA was provided by Trizol (Invitrogen, USA) according to the manufacturer's instructions. Ultraviolet spectrophotometer (BD, USA) was used to determine the concentration of RNA. The purity of RNA was determined by A260/ A280 ratio. The ratio of 1.8-2.0 was considered as high purity RNA without protein and DNA doping. The RNA was reverse transcribed into complementary DNA

using a reverse transcription Kit (Takara, Japan). PCR amplification was performed using SYBR premix Ex Taq™ II (Takara). The primer sequences are shown in Table I. The PCR program was: 95°C 3 min, 40 cycles, 95°C 10 s, 59°C 20 s, using the ABI Stepone plus real-time PCR system (Applied Biosystems, USA). Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 as internal parameters, RNA concentration was calculated by 2^{-ΔΔCT} method.

CCK8 assay

The CCK8 method was used to detect the effect on the proliferation of BT-B cells. on the proliferation of BT-B cells. After culturing BT-B cells by the method described above, 3 × 10⁴ cells were seeded in each well of a 96-well plate. After that, CCK8 reagent was added at 0, 12, 24, 36, 48, 60, 72, and 84 hours, and the cell viability was analyzed by a microplate reader. The value represents the average of three replicates of at least two independent experiments in a representative experiment.

Transwell assay

The migration test was performed using a 24-hole Boyden cavity and an uncoated filter with a pore size of 8 μm. The cells were collected from petri dish and washed twice with phosphate buffer saline (PBS, 0.01mol/l, pH 7.4). The cells were suspended in serum-free medium, and 3 × 10⁴ cells were seeded in the insertion chamber. The cells were cultured in a basic chamber (0.6 mL DMEM

Table I. Primer sequences used for RT-qPCR and siRNA

RNA	Primer sequences
circPIP5K1A	F: 5'- CAAGCTTTCTTAAGAAGCCGGGACC-3'
	R: 5'- GTTCGAAAGAATTCTTCGGCCCTGG-3'
GAPDH	F: 5'-AGAAGGCTGGGGCTCATTTG-3'
	R: 5'-AGGGGCCATCCACAGTCTTC-3'
U6	F: 5'-AGGGGCCATCCACAGTCTTC-3'
	R: 5'-AACGCTTCACGAATTTGCGT-3'
circ-PIP5K1A siRNA	5'- UGUUAUAGAAUUUUUGAUGUGUU-3'

medium containing 10% fetal bovine serum) at 37°C (5% CO₂) for 24 h, and then stained with 4,6-diamino-2-phenylindole and counted under a vertical microscope.

Flow cytometry

The osteosarcoma cells were digested and counted and then inoculated into 6-well plates. The cells were cultured in a medium containing 1% fetal bovine serum for 36 h. Serum starvation synchronized the cell cycle to the G1 phase. The cells were collected 48 h after transfection, washed with PBS buffer for 3 times, fixed with 1 ml absolute ethanol, and detected by flow cytometry.

Predicting potential molecules bound to circRNA_104315

Targetsacn 7.2 and RegRNA2.0 was used to further predict potential miRNA and messenger RNA (mRNA) binding to the circRNA_104315 sequence.

Western blotting

Cells were collected and lysed with radio

immunoprecipitation assay (RIPA) Cell Lysis Buffer (Gibco, USA) supplemented with 1 mmol/L benzylsulfonfyl fluoride (Sigma, USA). Protein was extracted by bicinchoninic acid (BCA) Protein Assay Kit (Shanghai Biotechnology Co., Ltd., China). The protein samples were separated on SDS-PAGE gel, electro-transferred to polyvinylidene fluoride membrane, and then blocked in 5% skim milk for 1 h. AP-1, IRF-4, Cdx-2, zic1 and GAPDH primary antibody (Abcam, USA) diluted with 5% bovine serum albumin (BSA) in the ratio of 1:1000 was added and placed on a shaking table at 4°C for 600 rpm overnight; the membrane was washed with TBST (TBS+Tween) for 3 times, each time for 10 min. The second antibody (Goat anti-Mouse IgG (H+L)-HRP; Abcam, USA) diluted with 1:1000 in blocking solution was added and incubated at room temperature for 60 min. After that, TBST was used to wash three times for 10 min each time. The chemiluminescence reagent was mixed with 1:1 (about 100ul) in the dark. The polyvinylidene fluoride (PDVF) membrane was slightly dried with filter

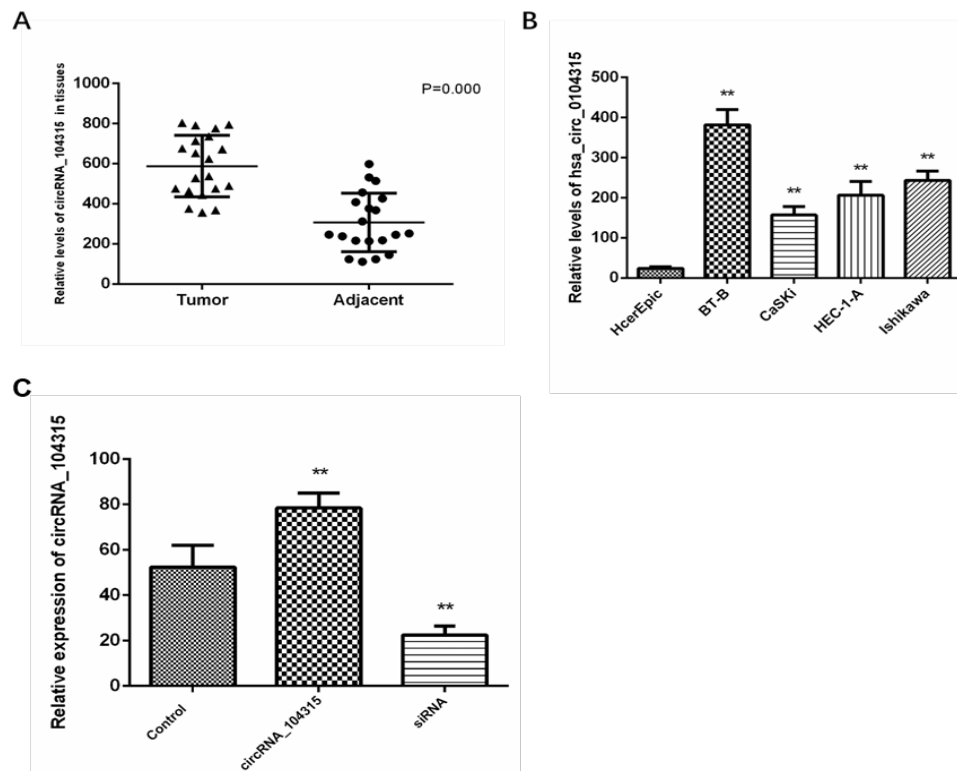


Fig. 1. Expression of circPIP5K1A in cervical cancer. **A)** RT-qPCR detection of circPIP5K1A expression in tumors and their adjacent normal tissues; **B)** The mRNA level of circPIP5K1A in various normal and cancer cell lines; **C)** The expression of circRNA_104315 in different groups * $P < 0.05$, ** $P < 0.01$.

paper and placed on the glass plate. The appropriate amount of developer was added on the protein surface, and the protein expression was detected by enhanced chemiluminescence (Millipore, USA).

Statistical analysis

Data analysis was performed by SPSS Version 19 (SPSS Statistics, Chicago, USA). Descriptive statistics were performed by calculating mean and standard deviation of numerical data, and extracting tables of frequency and percentages for categorical data. The *t*-test was used for comparison between the two groups. One way ANOVA test was used in the comparison of multiple groups. *P* value < 0.05 was considered statistically significant.

RESULTS

Expression level of circRNA_104315 is selectively upregulated in cervical cancer

To assess the status of expression level of circRNA_104315 in the development of cervical cancer, we used RT-qPCR to detect the mRNA level of expression level of circRNA_104315 in clinical samples. As shown in Fig. 1A, expression level of circRNA_104315 is significantly upregulated in cervical cancer tissue relative to adjacent normal tissues. Similar results were detected in BT-B, CaSKi, HEC-1-A and Ishikawa cervical cancer cell lines, while the mRNA level of circRNA_104315 was very low in normal cell line HCoEpiC (Fig. 1B).

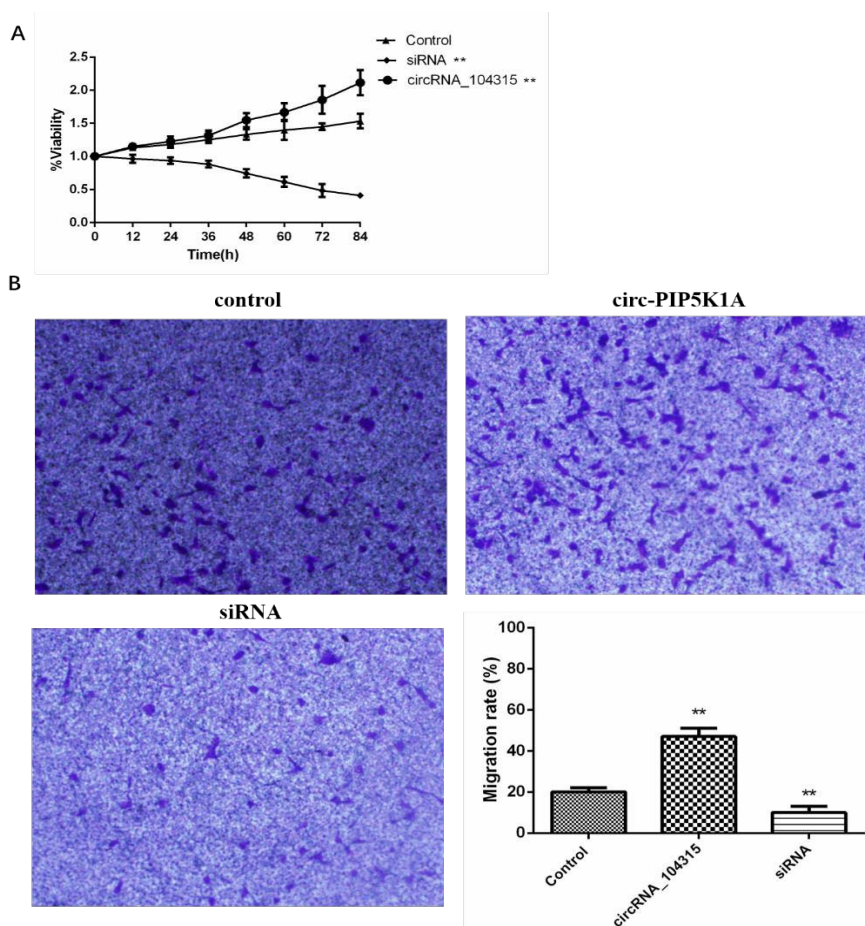


Fig. 2. Proliferation and invasion of circRNA_104315 in BT-B cells. *A)* Detection of cell proliferation using CCK-8 assay; *B)* The invasion of circRNA_104315 in BT-B cells was detected by Transwell assay, **P* < 0.05, ***P* < 0.01.

The expression of circRNA_104315 in different groups

After transfection of circRNA_104315 vector, circ-PIP5K1A siRNA and relative control into BT-B cells, qPCR detection revealed circRNA_104315 was significantly increased with expression level of circRNA_104315 vector, and decreased with siRNA (Fig. 1C).

In order to investigate the biological function of circRNA_104315 in BT-B cells, CCK-8 assay was used to detect the effect of circRNA_104315 on the proliferation of BT-B cells. The results (Fig. 2A) showed that the growth of BT-B cells transfected with expression level of circRNA_104315 was significantly promoted compared with siRNA group and control group, indicating that circRNA_104315 could promote the growth of BT-B cells. Transwell assay showed that the number of BT-B cells increased significantly after expression level of circRNA_104315 was successfully overexpressed ($P < 0.01$) (Fig. 2B).

circRNA_104315 knockdown induces cell death

Flow cytometry was then used to detect the apoptosis of BT-B cells. The results in Fig.3 show that the increase of expression level of circRNA_104315 significantly reduces the apoptosis of cervical cancer cells and promotes the malignancy of the tumor.

circRNA_104315 exerts carcinogenic effects through the miR-605-AGO1/RRM2 pathway

Targetsacn 7.2 and RegRNA2.0 were used to predict the target gene of circRNA_104315. The results showed that there may be a binding site between circRNA_104315 and hsa-miR-605. We successfully constructed a luciferase reporter gene plasmid containing the target gene 3'UTR binding site and co-transfected it with circRNA_104315 to BT-B cells. The luciferase activity was detected 48 h after transfection and compared with the NC simulation. After co-transfection of wild-type 3'UTR with circRNA_104315 and miR-605, luciferase activity (firefly luciferase activity/marine kidney luciferase activity) was significantly reduced ($P < 0.01$) (Fig. 4A).

Since miRNA mainly regulates gene translation at the post-transcription level, we further predicted the target protein of miR-605 and further examined the expression levels of AGO1 and RRM2 proteins by Western blot (Fig. 4B). After overexpressing circRNA_104315 in BT-B cells, the levels of AGO1 and RRM2 protein in the cells increased significantly ($P < 0.01$), but the results were reversed after overexpressing miR-605, and circRNA_104315 and miR-605 had an antagonistic effect.

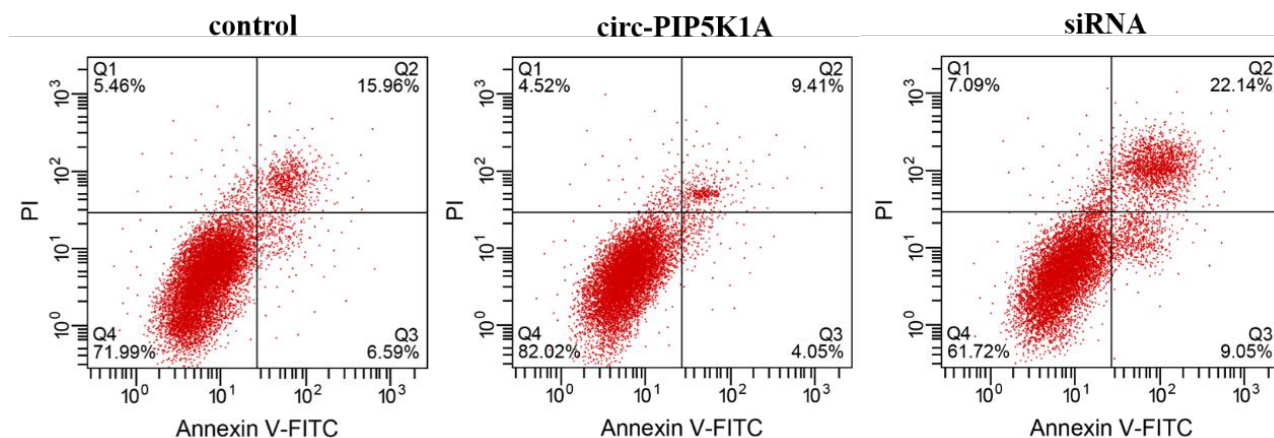


Fig. 3. Apoptosis of circRNA_104315 in BT-B cells.

DISCUSSION

The present research shows that circRNA was significantly up-regulated in cervical cancer tissues and cell lines. circRNA_104315 could reduce the apoptosis of BT-B cells and promote cell migration and proliferation. For example, circ_000467 is highly expressed in osteosarcoma cells (5). RNA circTRIM33 12 as a sponge of microRNA-191 inhibits the progress of hepatocellular carcinoma, hsa_circRNA_0006528 as a competitive endogenous RNA, promotes the progress of human breast cancer through sponge miR-7-5p and activation of MAPK/ERK signaling pathway (6). Overexpressed circ_0067934 promotes cervical cancer progression through the miR5/EIF3C axis as an oncogene (7). In this study, we found that circ_0104315 was up-regulated in BT-B cells, which promoted the proliferation, invasion and migration of

cervical cancer cells. Studies have shown that circular RNA can function as an miRNA sponge, such as CircNT5E as a microRNA-422a sponge to promote the occurrence of glioblastoma (8). The present study found that the target of circ_0004674 is miR-605, circ_0104315 binds to miR-605, and circ_0104315 can partially reverse the effect of miR-605 on cellular protein expression.

miRNA is a small single-stranded endogenous RNA molecule that regulates target gene expression at the post-transcriptional level by degrading target (mRNA) or inhibiting translation. Studies have shown that miRNA can regulate the occurrence, development and growth of tumors (9-11). The biological macromolecule RRM2 is a hot spot in the field of cancer. We have experimentally found that miR-605 and AGO1/RRM2 interact. Studies have suggested that AGO1 may participate in tumor

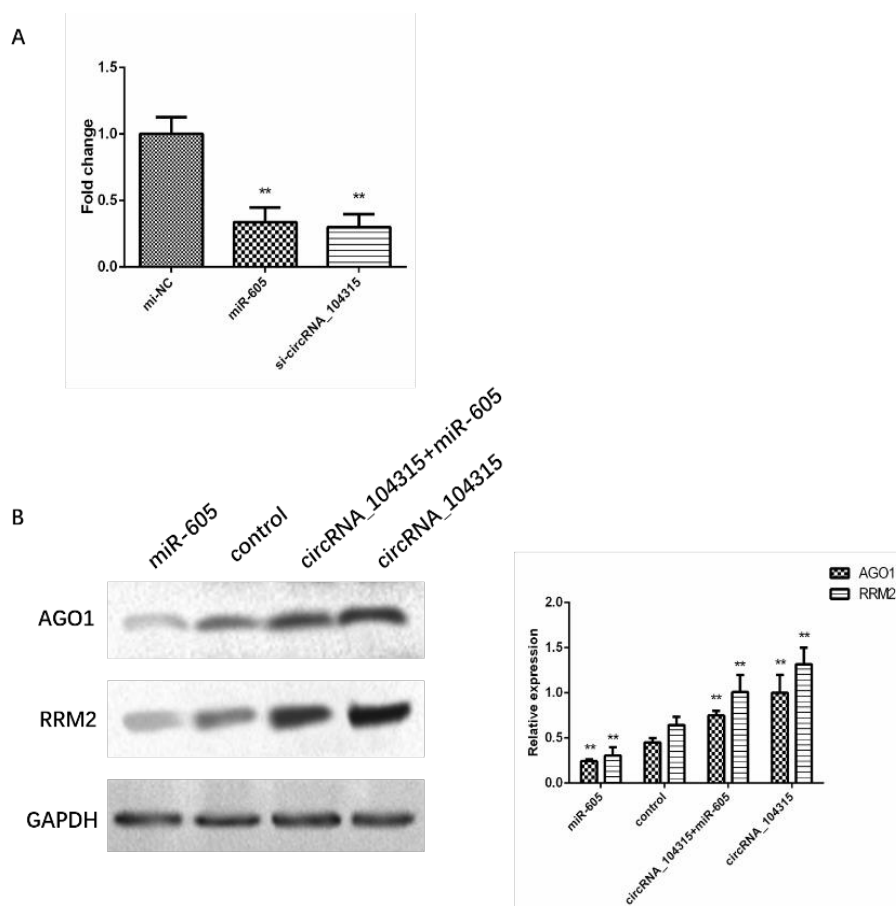


Fig. 4. The target gene of circRNA_104315 in BT-B cells. **A)** Detection of binding ability between circRNA_104315 and hsa-miR-605; **B)** Western blot results of AGO and RRM2.

progression by participating in heterochromatin silencing. AGO1 acts on the DNA promoter region in the nucleus, methylating histones and target genes, thereby suppressing gene expression (12).

In conclusion, circRNA_104315 promotes the development of cervical cancer by regulating the miR-605-AGO1/RRM2 pathway, and may become a molecular target for cervical cancer diagnosis and treatment.

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LETTER TO THE EDITOR

Transthyretin in bladder cancerT. Lei^{1,2}, B. Gong¹, Q. Meng^{1,2} and M. Zhang^{1,2,3}

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To the Editor,

Urothelial carcinomas are the most common malignancy of the urinary tract (1). Bladder cancers are frequently drug-resistant and recur within 5 to 15 years in approximately 85% of cases after initial resection (2-4). Among the current procedures used to diagnose bladder cancer, direct cystoscopic visualization is comparatively invasive. Likewise, cytologic examinations of voided urine samples are not highly sensitive and routinely detect only 35% of true positive cases. It is clear that more suitable and effective diagnostic methods are needed.

Changes in the composition of voided urine as well as measures of quantity and quality can reflect the development of urinary diseases. Urine collection is noninvasive, straightforward, and convenient to perform. Currently, none of the bladder cancer markers are sufficiently sensitive or specific for use in clinical diagnosis. New biological markers for progression and surveillance of bladder cancer are needed.

Transthyretin (TTR) is a 55 kDa homotertrameric transport protein. Varying serum levels of TTR have been associated with specific neuropathologies, including amyotrophic lateral sclerosis (5), and Parkinson's disease (6) among others. Similarly, TTR levels appear to be highly correlated with numerous

cancers. For example, TTR is included among the protein biomarkers that may be used for effective early detection of ovarian cancer. TTR was also associated with pancreatic ductal adenocarcinoma (7), as well as lung (8) and prostate cancer (9), with distinct expression levels identified with individual cancers. However, use of TTR as a potential marker for neoplastic diseases requires further study.

In our previous study, we successfully established two-dimensional (2-D) gels that permitted us to distinguish proteins detected in urine from patients diagnosed with bladder cancer from those in normal controls (10). Interestingly, we found that TTR was lowly expressed in the bladder cancer patients' urinary map. In the present study, the aim is to examine the differential expression of TTR at the mRNA and protein levels. We applied PCR, gene cloning and related methodologies to examine differential detection of TTR and to identify potential mechanisms underlying the role of this protein in the development of bladder cancer.

MATERIALS AND METHODS*Patients and specimens*

This study was approved by the local research

Key words: bladder cancer; transthyretin (TTR); pumc-91; cell line; MALDI-TOF / TOF MS

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ethics boards, including the Scientific Research Ethics Committee, Beijing Shijitan Hospital, Capital Medical University. Urine samples, neoplastic and matched adjacent non-neoplastic tissues, and slides with fixed tissues were collected from five patients with transitional cell carcinoma of the bladder in Xingtai People's Hospital, Xingtai, China. Fresh tissue samples were immediately snap frozen in liquid nitrogen until RNA was performed.

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All participants in the study signed informed consent.

Cloning of the CDS of TTR

Total cDNA was synthesized from 4 µg of total RNA in 12 µl final reaction. The gene encoding TTR was amplified with specific primers, including forward P1: 5'-AGGATGGCTTCTCATCGTCTG-3', reverse P2: 5'-TCATTCCTTGGGATTGGTGA-3'). Restriction sites for the enzymes *Hind III* and *Xho I* were included at the 5' ends of primers P3 and P4, respectively; sequences included, P3, 5'-CAGAAGCTTAGGATGGCTTCTCATC-3' (sense strand), and P4, 5'-TCACTCGAGTCATTCCTTGGGAT-3' (antisense strand). TTR was amplified using the PCR mix (New industry, China), primers P3, P4 and cDNA for a total of 40 cycles. The products were identified on 2% agarose electrophoresis, then recovered and purified according to the manufacturer's instructions (Newbioindustry, China).

Construction of a pcDNA3.1-TTR expression vector

The eukaryotic expression vector pcDNA3.1(+) (Invitrogen, USA) and the TTR gene amplification products were both digested with *Hind III* and *Xho I* (Fermentas, Canada), and ligated with T4 DNA ligase (TakaRa, Japan) for 1h at room temperature. The products of the ligation reaction were used to transform into Trans 109 chemically competent bacterial cells (TransGen Biotech, China). Positive clones were identified by PCR. The pcDNA3.1 (+)/TTR and pcDNA3.1 (+) control constructs were extracted with a non-endotoxin plasmid extraction kit (Vigorous Biotech, China). The gene sequence of pcDNA3.1-TTR was confirmed by Tianyihuiyuan (Beijing Tianyi Huiyuan Sci & Bio, China).

Cell culture and transfection

Human bladder cancer cell line pumc-91 was donated by the Cell Laboratory of Beijing Union Medical College Hospital. Cells were transfected with pcDNA3.1 (+)/TTR and pcDNA3.1 (+) with 0.5 µg DNA and 1.5 µl lipofectamine 2000 (Invitrogen, USA) per well, followed by incubation at 37°C in 5% CO₂ for 48 h and 72 h.

Real-time PCR to detect expression of TTR mRNA

The cells were collected at 48 h after transfection. TTR mRNA was quantitated by real-time PCR Using a standard protocol and primers including forward P5, 5'-CTTACTGGAAGGCACTTGGCA-3' and reverse P6, 5'-CGGAGTCGTTGGCTGTGAA-3'. Expression of GAPDH (primer 5'-TTTGGTATCGTGAAGGACT-3') was used as internal control. All experiments were run in triplicate.

Western blotting and immunohistochemistry

The transfected pumc-91 cells were collected at 72 h after transfection and lysed on ice using a modified immunoprecipitation buffer supplemented with protease inhibitors. Protein concentration was determined by bicinchoninic acid (BCA) Protein Assay Kit (Bio-Rad, Italy).

Western blotting was performed as described previously (3). Briefly, proteins (30 µg) were separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred for 1.5 h to a PVDF membrane (Bio-Rad, USA). The membrane was blocked for 2 h at room temperature and then incubated at 4°C overnight with a primary anti-TTR antibody (Abcam, UK). Horseradish-peroxidase-conjugated goat anti-mouse IgG was used as a secondary antibody. Antibodies bound to target proteins were detected using an enhanced HRP-DAB chromogenic kit (Tiangen, China). Anti-β-actin (Dingguo Biotechnology, China) and anti-GAPDH (Dingguo Biotechnology, China) antibodies were used as internal loading controls.

For immunohistochemistry, fixed and embedded tissues were fully deparaffinized in xylene for 30 min and rehydrated using graded ethanol washes. After washing in PBS, the slides were blocked and antigen retrieval was carried out. The slides were then incubated with anti-TTR (Abcam, USA) at 37°C for 1 h. Slides were then washed in PBS and incubated with streptavidin-biotin conjugated second antibody (DAKO, USA). Immunoreactive

proteins were detected with DAB, and hematoxylin was included as a counterstaining. The slides were washed immediately in running water and dehydrated with graded ethanol washes.

Cell proliferation assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, cells were plated at 5,000 cells per well in 96-well plates and incubated with 10% CCK-8 at 37°C for 3 h. Proliferation rates were determined at 0, 24, 48, 72, 96 h after transfection. All experiments were performed in quadruplicate.

Scratch assay

Once the transfected cells reached 100% confluence, capacity for migration was determined using scratch wound healing assay. Linear scratch wounds were introduced in triplicate with a 200 µl pipette tip. In order

to avoid the impact of cell cycle, cells were maintained in medium with 3% FBS. Cell cultures were washed twice; images were taken at 0, 12, 24, 36, 48, 60 and 72 h after introduction of the linear scratches. Ten areas in each well were selected at random. Cells in three wells from each group were enumerated.

Statistical analysis

Results were analyzed using SPSS 15.0 software and presented as means $\pm$ standard deviation (SD). Statistical analysis was performed using two-tailed Student's *t*-test with *P*-values <0.05 used to determine statistical significance.

RESULTS

Detection of TTR in the 2-D gels of urine proteins from patients with bladder cancer and normal controls

In our previous study (10), we had established a

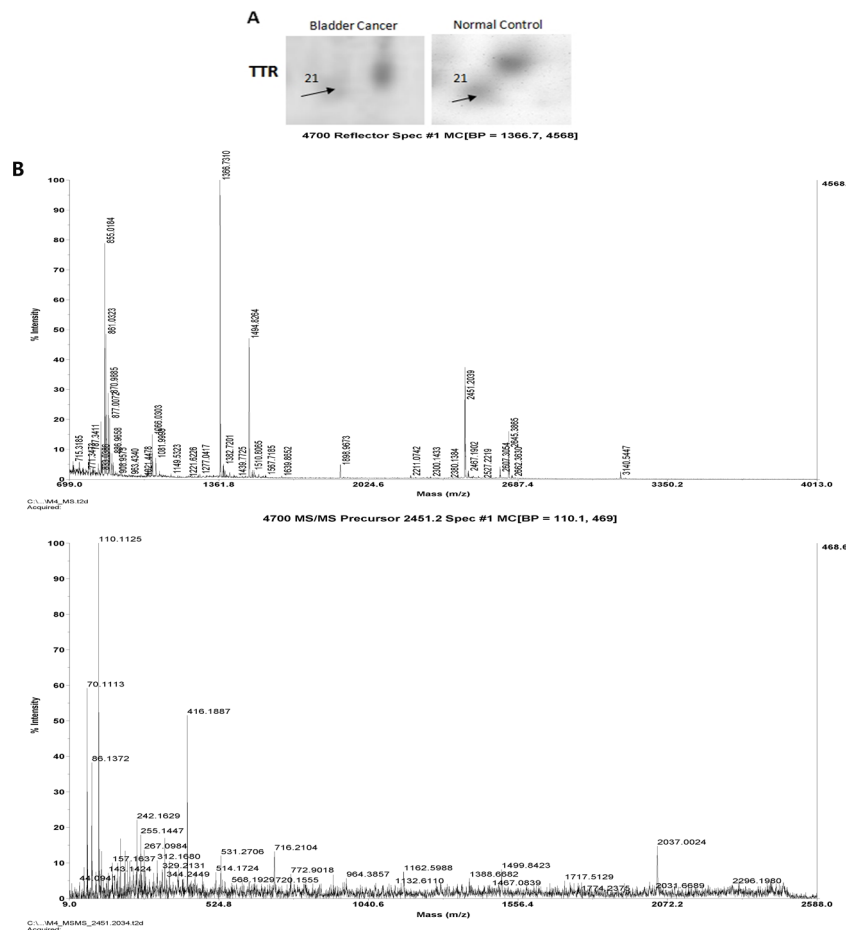


Fig. 1. A) Spot 21 was down-regulated in bladder carcinoma's urinary 2-D gels compared with normal controls. Through MALDI-TOF/TOF analysis, the protein spot 21 was identified as TTR. B) Peptide mass fingerprinting of TTR.

2-D gel system that provided a map of proteins in urine from patients with bladder cancer and normal controls. The density of spot 21 and the associated peptide mass fingerprinting (PMF) are shown in Fig. 1. The results were analyzed by PDQuest software (v.7.1.0). TTR was identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/TOF). Our results indicated lower levels of TTR were detected in urine samples from patients with bladder cancer compared to those observed in samples from normal controls.

Detection of TTR in urine and tissue from patients diagnosed with bladder cancer by qRT-PCR, Western blot and immunohistochemistry

Urine as well as samples of neoplastic and adjacent control (AC) non-neoplastic tissue were collected from transitional bladder cancer patients. The urine samples from five healthy volunteers were pooled to generate the normal control. As shown in Fig. 2A, levels of TTR mRNA were clearly lower in bladder cancer tissues compared to adjacent controls ($P < 0.05$). With respect to the results from

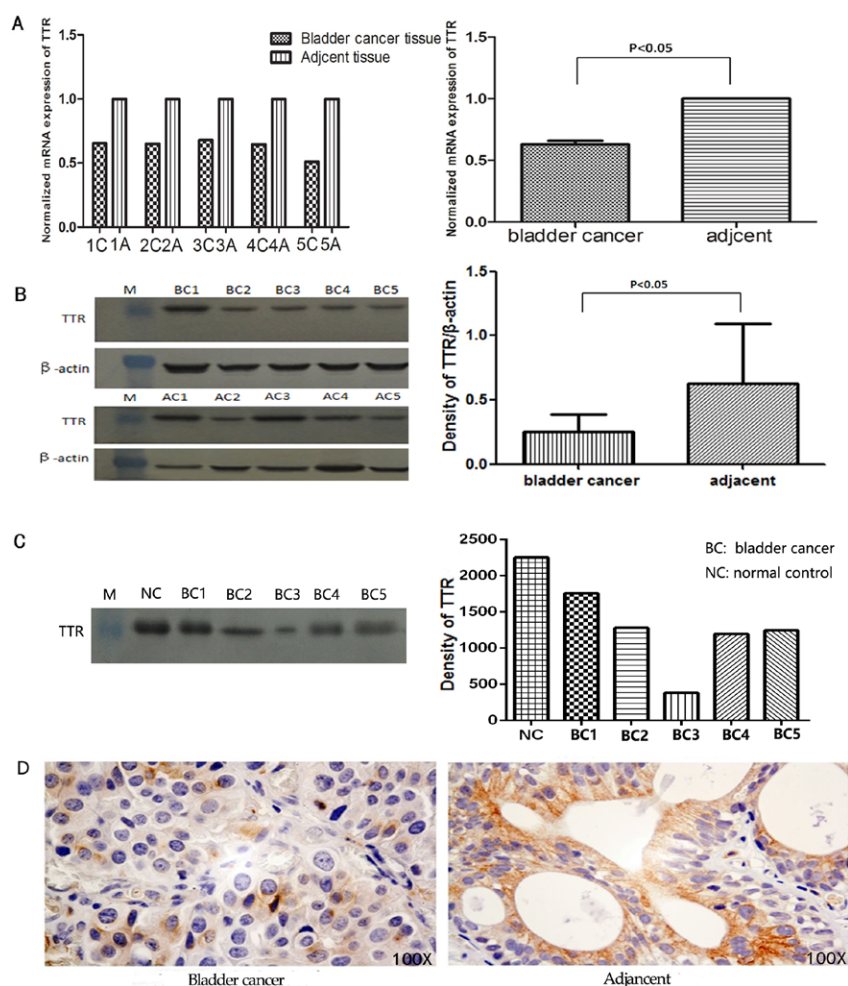


Fig. 2. Verification of TTR in bladder cancer patients and controls at molecular and protein levels, respectively. **A, B)** Expression of TTR mRNA and protein in bladder cancer patients' tissue and adjacent controls. Calculated by *t*-test, both of the differences were significant at mRNA and protein levels ($p < 0.05$). **C)** TTR was lowly expressed in bladder cancers compared to normal controls. **D)** Immunohistochemical analysis of TTR expression in pathologic slides of bladder cancer and adjacent control (X100 magnification).

bladder cancer tissue was decreased distinctly (Fig. 2D). All experiments were repeated three times.

Amplification of TTR from tissue RNA samples yielded a specific 444 bp gene fragment as anticipated (Fig. 3A). TTR expressing plasmid was constructed using the eukaryotic expression vector pcDNA3.1(+)

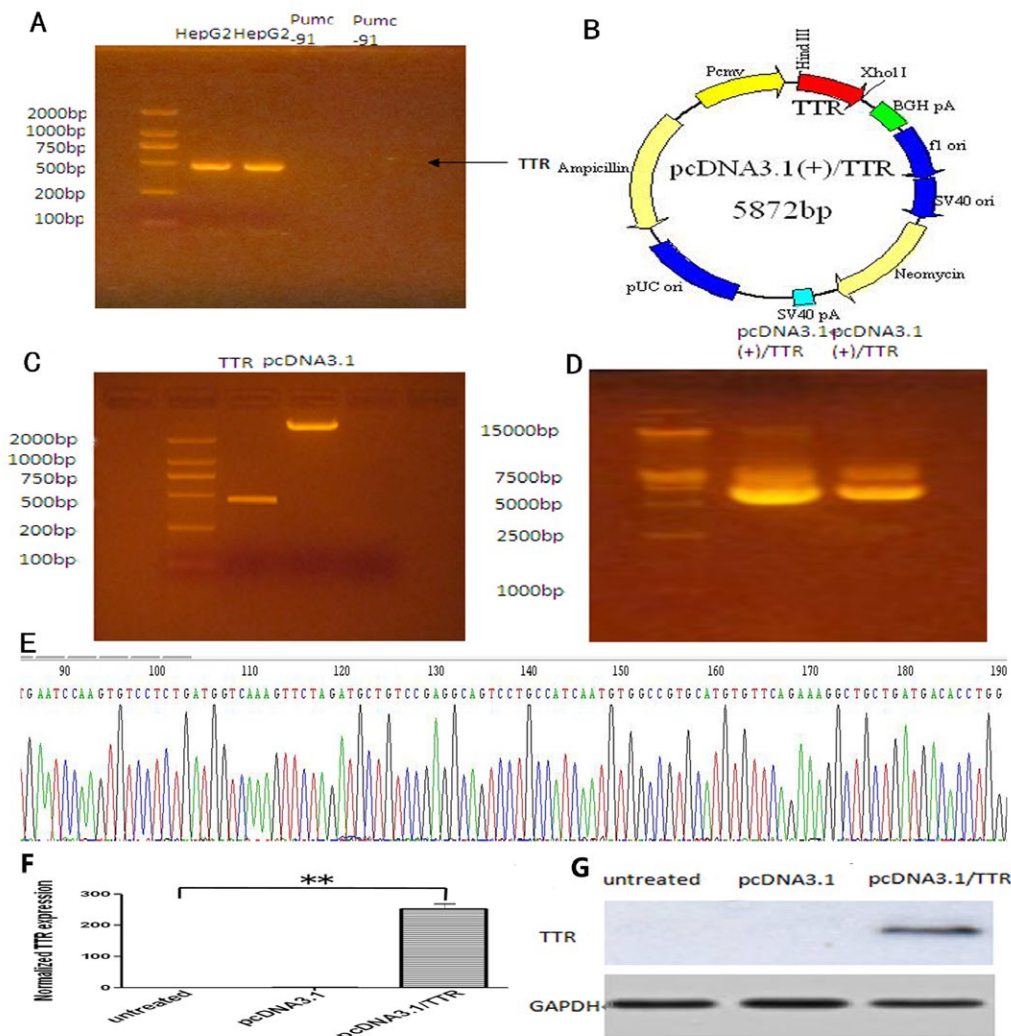


Fig. 3. Construction and identification of pcDNA3.1-TTR plasmid. **A)** Expression of TTR PCR production from hepG2 cells and pumc-91 bladder cancer cells by agarose gel electrophoresis. Lane 1 and 2 were from hepG2, lane 3 and 4 from pumc-91. The size of the amplified TTR fragment was 444bp. **B)** Plasmid construction system. **C)** Enzyme-digested productions by Hind III and XhoI after ligation of TTR and pcDNA3.1(+) expression vector. Lane 1 was TTR and lane 2 was the vector. The sizes were 444bp and 5428bp, respectively, and correct according to the marker. **D)** Extracted recombinant plasmid. The size of the production was between 5000bp and 7500bp. **E)** Sequence of the insert fragment which was 100% homology with the report from GenBank. **F)** TTR mRNA was expressed 250-fold higher than untreated and empty vector groups. Fig. 3G showed the TTR protein was highly expressed in pcDNA3.1-TTR compared with the other two groups. ** $P < 0.001$.

as shown in Fig. 3B. Positive clones were identified by PCR amplification. Agarose gel electrophoresis revealed that both vector and insert fragments were of the correct size and in the correct orientation (Fig. 3, C and D). The sequence of TTR encoded by the plasmid revealed 100% homology with the reference sequence in GenBank (CR456908.1, Fig. 3E). Furthermore, we detected a 250-fold increase in TTR mRNA levels in pcDNA3.1/TTR-transfected pumc-91 cells compared with levels detected in cells transfected with pcDNA3.1 alone (Fig. 3F). Western blot revealed a profound increase in immunoreactive TTR protein in cells transfected with pcDNA3.1/TTR (Fig. 3G).

Overexpression of TTR in pumc-91 bladder cancer cells suppressed cell proliferation and invasion

In order to assess the impact of TTR overexpression on cell proliferation and survival, we measured the number of viable cells in *in vitro* culture at different time points with CCK-8 assay. The rate of cell growth and proliferation was reduced in the pcDNA3.1/TTR-transfected pumc-91 cells compared with those

transfected with pcDNA3.1 alone ($P < 0.01$, Fig. 4A). No significant difference was identified when comparing the proliferation of naïve cells to those transfected with pcDNA3.1 alone.

Scratch assay was performed to analyze the impact of TTR overexpression on the migrating and invasive behavior of pumc-91 bladder cancer cells. Cells overexpressing TTR were significantly less invasive compared with pcDNA3.1 alone (Fig. 4B). This tendency reached statistical significance at 48 h. We detected statistically significant differences in the size of the remaining scratch wound in cultures of cells transfected with pcDNA3.1/TTR compared to those transfected with pcDNA3.1 alone (Fig. 4C).

DISCUSSION

Very few studies have focused on TTR and its impact in respect to neoplastic disease. Young et al. (11) presented an analysis of the urinary proteome in specimens from women with interstitial cystitis/painful bladder syndrome (IC/PBS) using in-depth

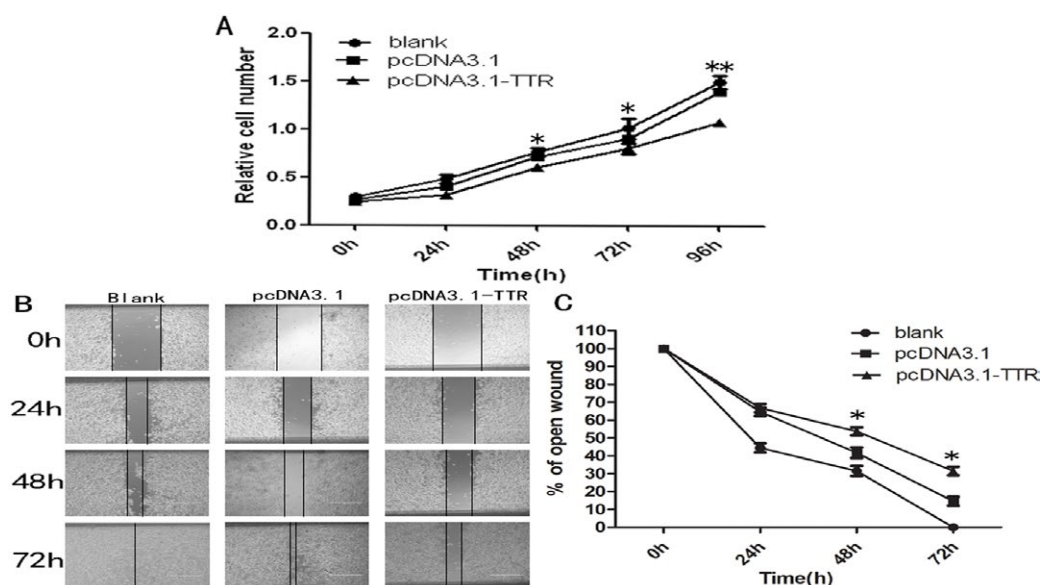


Fig. 4. Overexpression of TTR suppressed Pumc-91 bladder cancer cell proliferation and cell migration. **A)** Growth rate of pcDNA3.1-TTR compared with pcDNA3.1 using a Cell Counting Kit-8 assay. Overexpression of TTR inhibited bladder cancer cell proliferation. From 48h after transfection, the difference has statistical significance between pcDNA3.1 and pcDNA3.1-TTR groups. **B, C)** Effect on cell migration. Wounds after 0h, 24h, 48h, 72h, respectively. The relative ratio of wound closure per field was calculated. From 48h after wounding, there was statistically significant difference. * $P < 0.05$.

shotgun proteomics. In this study, TTR levels were diminished in samples from IC/PBS patients. Likewise, Halder et al. (12) performed 2-D gel electrophoresis and reported a notable increase in TTR in urine specimens from patients with urothelial neoplasms, although no further analysis of this finding was performed. In our previous study (10), we found diminished levels of urinary TTR in specimens from patients diagnosed with bladder cancer compared to those from normal controls. In this study, we explored this finding further by Western blotting, qRT-PCR and immunohistochemistry. We found that expression of TTR was significantly decreased in neoplastic bladder cancer tissue compared to expression levels detected adjacent to non-neoplastic control tissue. In order to understand the impact of TTR expression on cell proliferation and invasiveness, we transfected cells of the pumc-91 human bladder cancer line with a pcDNA3.1/TTR expression plasmid. We found that overexpression of TTR resulted in diminished cell growth and proliferation *in vitro*. Overexpression of TTR also resulted in diminished invasiveness and cell migration in the *in vitro* culture system. Taken together, these observations suggest that TTR may serve to regulate growth and migration/invasion of neoplastic bladder cancer cells.

In conclusion, our results indicated that TTR was down regulated in neoplastic tissue from patients with bladder cancer, and that this protein may play a critical role in promoting disease progression and invasion.

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LETTER TO THE EDITOR

LncRNA GAS5 inhibits cell proliferation and resistance to doxorubicin in anaplastic thyroid carcinoma by regulating miR-96C-Q. Song¹, M. Wang¹, S-M. Zhang¹ and X-Y. Ma²

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To the Editor,

Anaplastic thyroid cancer (ATC) is the most malignant thyroid cancer, and its incidence accounts for about 10-15% of all thyroid cancers. Moreover, ATC develops rapidly, and the infiltration and metastasis can occur early (1). The prognosis of ATC is extremely poor, and the average survival time is about 4-12 months. Most ATC patients die within a few months after diagnosis, and the 5-year survival rate is only 0-15% (2). Therefore, it is of great importance to explore effective ATC treatment.

Long non-coding RNA (lncRNA) refers to an RNA molecule with a length of 200 to 100,000 nt, which does not have protein coding functions. It has been reported that the low expression of lncRNA GAS5 is related to the poor prognosis of patients with thyroid cancer (3). And lncRNA GAS5 has been found to act as a ceRNA to regulate PTEN expression by sponging miR-222-3p in papillary thyroid carcinoma (4). In this study, miR-96 was predicted to be the target of lncRNA GAS5. More importantly, miR-96-5p was also found to promote the proliferation, invasion and metastasis of papillary thyroid carcinoma by down-regulating CCDC67 (5). Doxorubicin (Dox) is one of the most widely used drugs for the treatment of cancer, including ATC.

Docetaxel + Dox combined or not combined with radiotherapy is the treatment choice for ATC.

Abnormal expression of lncRNA GAS5 and miR-96 was detected in ATC. In addition, the effects of lncRNA GAS5 and miR-96 on cell proliferation and Dox resistance was also investigated in ATC.

MATERIALS AND METHODS

Tissue samples

Eighteen patients diagnosed with ATC participated in the study and gave informed consents for participation. These patients did not receive any neoadjuvant chemotherapy before surgery. The experimental tissues were obtained and immediately frozen in liquid nitrogen and stored at -80°C. This study was approved by the Ethics Committee of Qingdao Central Hospital.

Cell culture and establishment of Dox-resistant ATC cells

Human ATC 8305C cells and thyroid follicular Nthy-ori3-1 cells were purchased from JCRB Cell Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, HyClone, Utah, USA) and incubated at 37°C in a humidified incubator with 5% CO₂.

Key words: *LncRNA GAS5; doxorubicin; proliferation; anaplastic thyroid carcinoma; miR-96*

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In order to establish the Dox-resistant ATC cells, 8305C cells were cultured in the 10th passage by selecting cells with gradually increasing Dox concentrations (0.01~1 μ M). Finally, 8305C cells were kept in 1 μ M Dox to maintain resistant characteristics and were named as 8305C/Dox. One week before the experiment, the Dox was removed and replaced with normal medium.

Cell transfection

GAS5 overexpression vector and siRNA or miR-96 mimics and inhibitor were purchased from RiboBio (Guangzhou, China). These plasmids and oligonucleotides were transfected into 8305C or 8305C/Dox cells using lipofectamine 2000 reagent (Invitrogen).

RT-qPCR

Total RNA was extracted with TRIzol (Invitrogen) based on the manufacturer's instructions. Reverse transcription cDNA was synthesized using 500 ng total RNA and miScript reverse transcription kit (Qiagen, Dusseldorf, Germany). RT-qPCR assay was performed

on the QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, USA) using standard SYBR Green Assay protocol. The expression levels of GAS5 and miR-96 were determined by the $2^{-\Delta\Delta C_t}$ method. GAPDH or U6 was used as a standardized endogenous control. The primers used were: GAS5 forward 5'-CTT CTG GGC TCA AGT GAT CCT-3' and reverse 5'-TTG TGC CAT GAG ACT CCA TCA G-3'; miR-96 forward: 5'-ACG ATG CAC CTG TAC GAT CA-3' and reverse, 5'-TCT TTC AAC ACG CAG GAC AG-3'; U6-forward: 5'-GCT TCG GCA GCA CAT ATA CTA AAA T-3' and reverse, 5'-CGC TTC ACG AAT TTG CGT GTC AT-3'; GAPDH forward: 5'-ACA ACT TTG GTA TCG TGG AAG G-3', and reverse, 5'-GCC ATC ACG CCA CAG TTT C-3'.

Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide (MTT) analysis. The treated cells were seeded in 96-well plates. Next, MTT (5 mg/ml) was added to each well and incubated for 4 h. Finally, 150 μ l of dimethyl

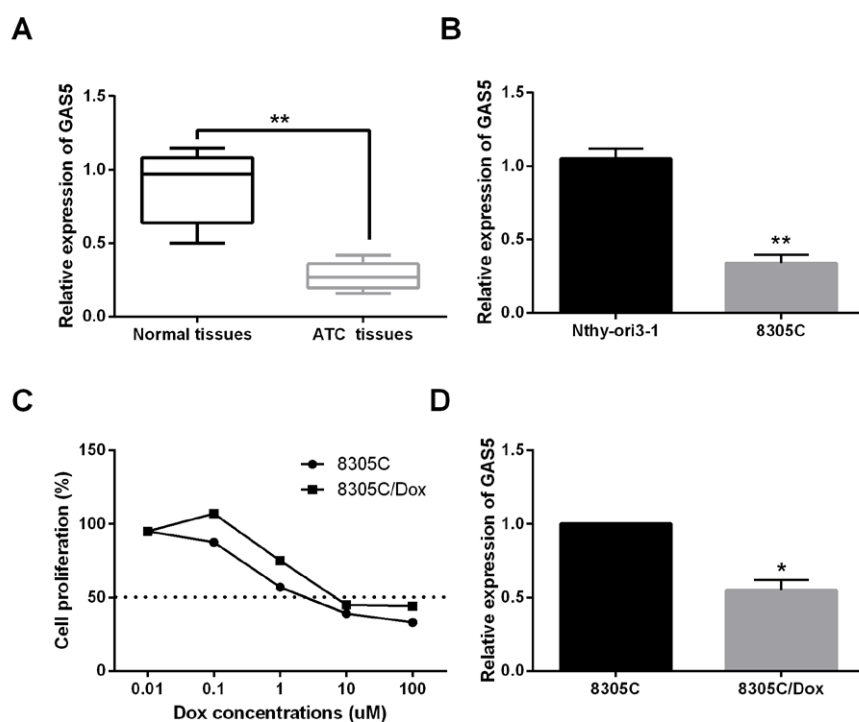


Fig. 1. LncRNA GAS5 is downregulated in Dox-resistant ATC cells. **A)** LncRNA GAS5 expression in ATC tissues and normal tissues. **B)** LncRNA GAS5 expression human ATC cells 8305C and thyroid follicular cells Nthy-ori3-1. **C)** 8305C and 8305C/Dox cells were treated with increasing concentrations of Dox for 48 h. Cell proliferation was measured by MTT assay. **D)** LncRNA GAS5 expression was detected in 8305C and 8305C/Dox cells. * $P < 0.05$, ** $P < 0.01$

sulfoxide (DMSO) was added to dissolve MTT formazan crystals. The OD value was read at 490 nm by an enzyme immunoassay instrument (Bio-Tek, Winooski, VT, USA).

Dual luciferase reporter assay

The pGL3 plasmid with wt-GAS5 or mut-GAS5 was transfected into 8305C cells containing miR-96 mimics. After 48 h of incubation, the luciferase activity was measured by a dual-luciferase reporter gene assay system (Promega, USA).

Statistical analysis

SPSS 19.0 (Chicago, IL, USA) was used to analyze the results. The quantitative variables were analyzed using Student's *t*-test or One-way ANOVA with Tukey test. $P < 0.05$ was considered statistically significant.

RESULTS

The expression level of lncRNA GAS5 was

detected in ATC tissues and cells. Compared with normal tissues, the expression of lncRNA GAS5 in ATC tissues was reduced ($P < 0.05$, Fig. 1A). Compared with thyroid follicular cells Nthy-ori3-1, lncRNA GAS5 was downregulated in ATC cells 8305C ($P < 0.01$, Fig. 1B). Next, Dox-resistant cells were produced by culturing human ATC 8305C cells at an increased Dox concentration. The results showed that the sensitivity of 8305C/Dox cells was higher than that of 8305C cells ($P < 0.01$, Fig. 1C). The IC₅₀ value of Dox to 8305C/Dox (5.4 μ M) was about 3.6-fold higher than that of the parental 8305C (1.5 μ M, Fig. 1C). In addition, RT-qPCR showed that the expression of lncRNA GAS5 in 8305C/Dox cells was reduced compared with the parental 8305C cells ($P < 0.01$, Fig. 1D). These results indicate that lncRNA GAS5 may be involved in tumorigenesis and doxorubicin resistance of ATC.

Next, the GAS5 vector was transfected into 8305C and 8305C/Dox cells. Compared with the control group, GAS5 expression in 8305C and

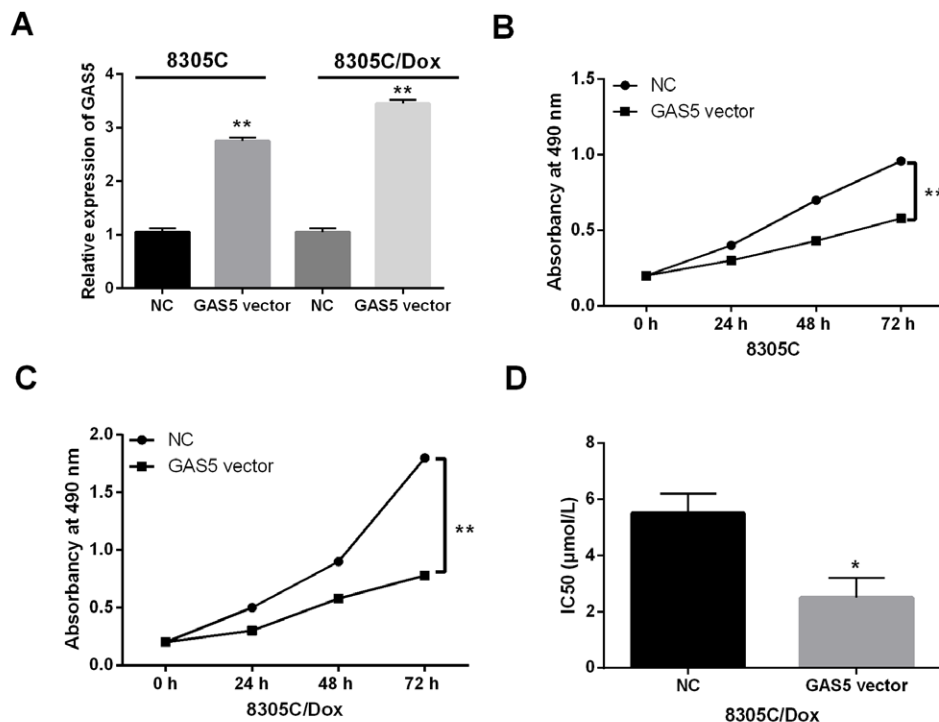


Fig. 2. LncRNA GAS5 is involved in doxorubicin resistance in ATC cells. **A)** LncRNA GAS5 expression in 8305C or 8305C/Dox cells with GAS5 vector. **B)** Cell proliferation in 8305C cells containing GAS5 vector. **C)** Cell proliferation in 8305C/Dox cells containing GAS5 vector. **D)** The effect of GAS5 vector on Dox resistance was assessed by computing IC₅₀ value. * $P < 0.05$, ** $P < 0.01$

8305C/Dox cells was significantly enhanced by its vector ($P < 0.01$, Fig. 2A). Functionally, MTT assay showed that overexpression of GAS5 inhibited cell proliferation in 8305C and 8305C/Dox cells ($P < 0.01$, Fig. 2 B, C). In addition, overexpression of GAS5 resulted in a decrease in Dox resistance in 8305C/Dox cells, which was presented by a lower IC50 value ($P < 0.01$, Fig. 2D). These results indicate that overexpression of lncRNA GAS5 inhibits cell

proliferation and reduces the resistance of ATC cells to doxorubicin.

Then, miR-96 was found to have a binding site with lncRNA GAS5 in starBase version 2.0 (<http://starbase.sysu.edu.cn/>) (Fig. 3A). A dual luciferase reporter assay was performed to confirm the above prediction. The results showed that miR-96 mimics reduced the luciferase activity of wt-GAS5 in 8305C cells, but had no effect on the luciferase activity

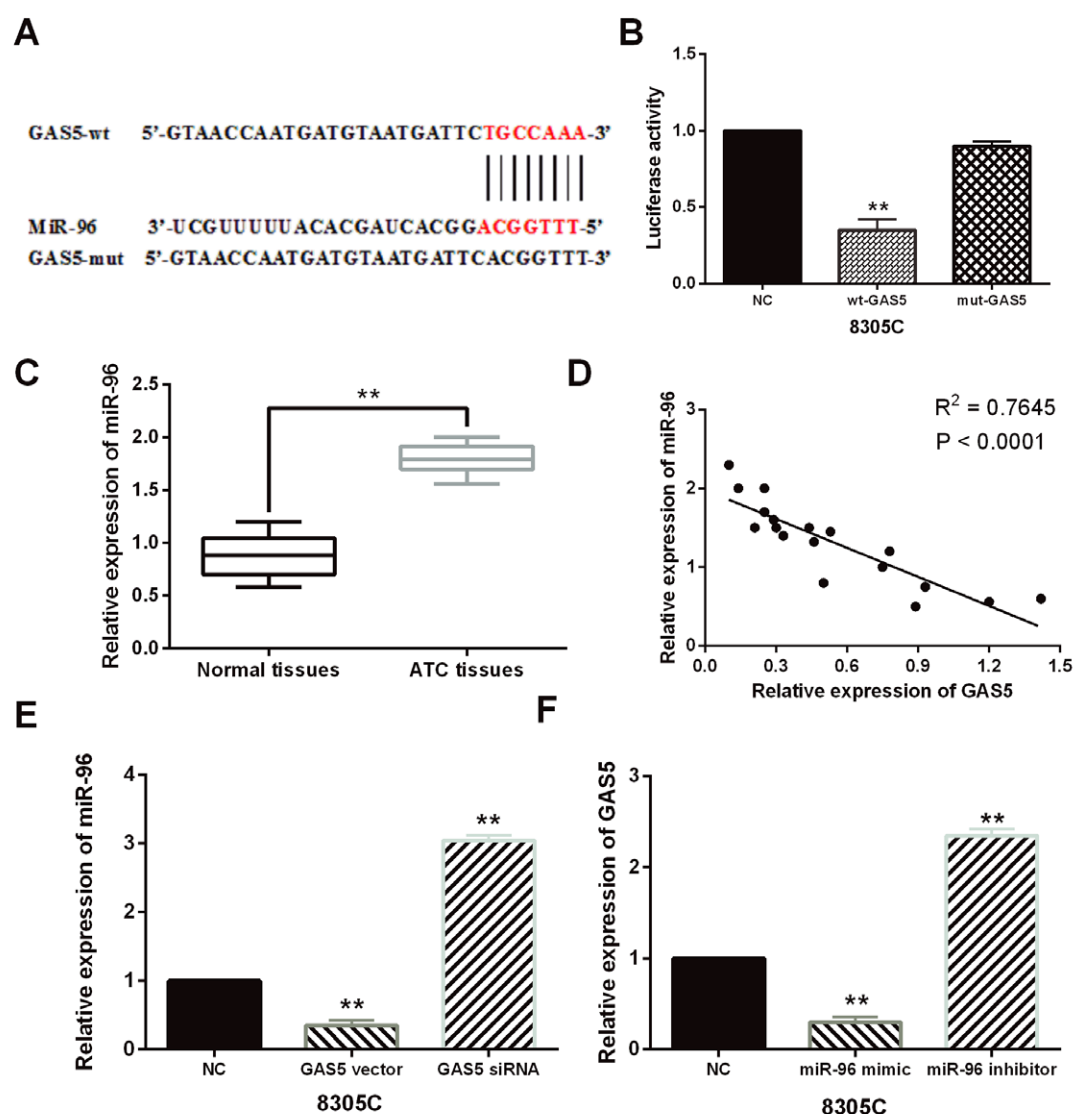


Fig. 3. LncRNA GAS5 acts as a molecular sponge of miR-96. **A)** The binding sites between GAS5 with miR-96. **B)** Luciferase reporter assay. **C)** MiR-96 expression in ATC tissues and normal tissues. **D)** A negative correlation between GAS5 and miR-96 expression in ATC tissues. **E)** MiR-96 expression in 8305C cell with GAS5 siRNA and vector. **F)** GAS5 expression in 8305C cells containing miR-96 mimics or inhibitor. ** $P < 0.01$

of mut-GAS5 ($P < 0.01$, Fig. 3B). This indicates that lncRNA GAS5 can directly target miR-96. To investigate whether miR-96 is involved in ATC, the expression level of miR-96 was detected in ATC tissues and normal tissues. Compared with normal tissues, miR-96 expression in ATC tissues was increased ($P < 0.01$, Fig. 3C). In addition, we found that lncRNA GAS5 was negatively correlated with the expression of miR-96 in ATC tissues ($P < 0.01$, Fig. 3D). At the same time, the overexpression of GAS5 was found to reduce miR-96 expression, while GAS5 downregulation enhanced miR-96 expression in 8305C cells ($P < 0.01$, Fig. 3E). However, it was also found that GAS5 expression was also reduced by miR-96 upregulation and enhanced by miR-96 downregulation in 8305C cells ($P < 0.01$, Fig. 3F). These results indicate that lncRNA GAS5 acts as a

molecular sponge of miR-96 in ATC.

In order to verify the interaction between lncRNA GAS5 and miR-96 in ATC, miR-96 inhibitor or miR-96 inhibitor + GAS5 siRNA was transfected into 8305C and 8305C/Dox cells. RT-qPCR showed that miR-96 expression was reduced by its inhibitor. However, the decreased expression of miR-96 induced by its inhibitor was recovered by GAS5 siRNA in 8305C and 8305C/Dox cells (Fig. 4A). MTT assay showed that downregulation of miR-96 inhibited cell proliferation in 8305C and 8305C/Dox cells. More importantly, down-regulation of GAS5 attenuated the inhibitory effect of miR-96 inhibitor on cell proliferation in 8305C and 8305C/Dox cells (Fig. 4 B, C). In addition, downregulation of miR-96 was found to inhibit the resistance of 8305C/Dox to doxorubicin. And downregulation of GAS5

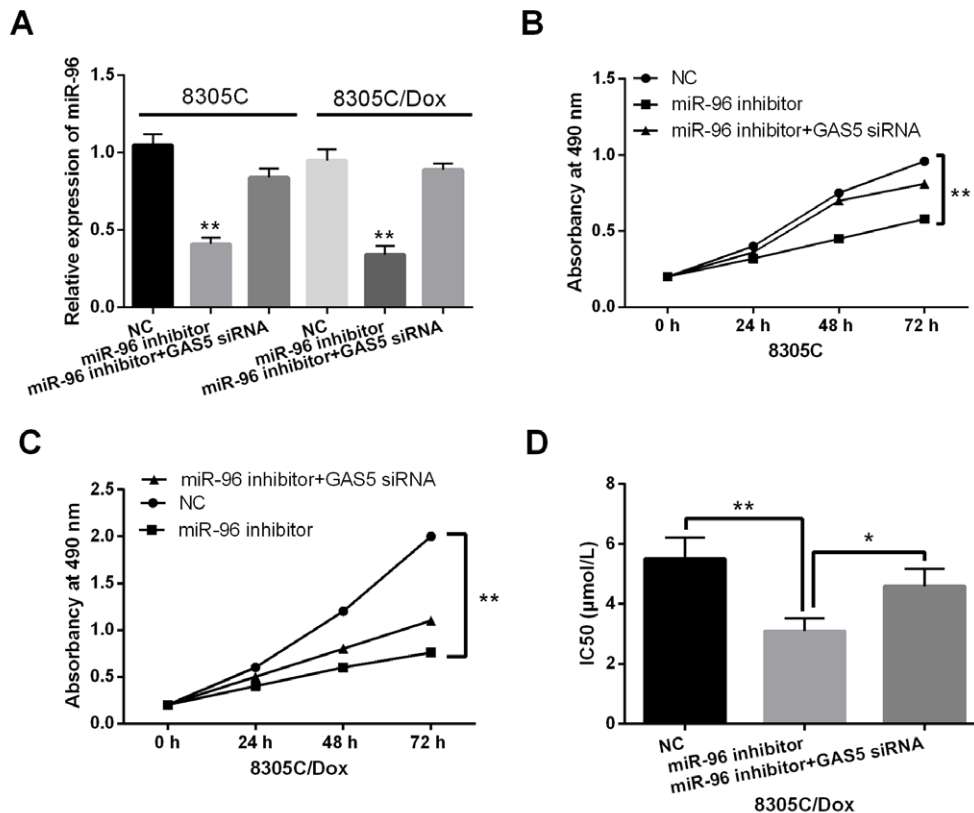


Fig. 4. MiR-96 is involved in GAS5-regulated Dox resistance of ATC cells. **A)** MiR-96 expression in 8305C or 8305C/Dox cells with miR-96 inhibitor or miR-96 inhibitor + GAS5 siRNA. **B)** Cell proliferation in 8305C cells with miR-96 inhibitor or miR-96 inhibitor + GAS5 siRNA. **C)** Cell proliferation in 8305C/Dox cells with miR-96 inhibitor or miR-96 inhibitor + GAS5 siRNA. **D)** The Dox sensitivity was detected by calculating IC₅₀ value in 8305C/Dox cells with miR-96 inhibitor or miR-96 inhibitor + GAS5 siRNA. * $P < 0.05$, ** $P < 0.01$

attenuated the inhibitory effect of miR-96 inhibitor on the resistance of 8305C/Dox to doxorubicin (Fig. 4D). All these results indicate that lncRNA GAS5 can inhibit cell proliferation and resistance to doxorubicin in ATC by downregulating miR-96.

DISCUSSION

In this study, we found that lncRNA GAS5 expression was decreased in ATC tissues, cell lines and doxorubicin-resistant ATC cells. More importantly, overexpression of lncRNA GAS5 inhibited cell proliferation and reduced doxorubicin resistance of ATC cells. These results indicate that lncRNA GAS5 may function as a tumor suppressor in ATC. Consistent with our results, downregulation of lncRNA GAS5 was also found in triple negative breast cancer and liver cancer (6, 7). In addition, lncRNA GAS5 has been reported to inhibit the malignant cell proliferation and chemotherapy resistance to doxorubicin in bladder transitional cell carcinoma (8). Here, it was found that lncRNA GAS5 acts as a molecular sponge of miR-96 in ATC. Similarly, Wang et al. also reported that lncRNA GAS5 acted as a competitive endogenous RNA for miR-96 (9).

At the same time, upregulation of miR-96 was found in ATC. Functionally, upregulation of miR-96 promoted cell proliferation and enhanced doxorubicin resistance of ATC cells. Similarly, miR-96 has been reported to play a carcinogenic effect in papillary thyroid carcinoma (10). These results indicate that miR-96 can act as a tumor promoter in ATC. In the current study, lncRNA GAS5 was found to attenuate the carcinogenic effect of miR-96 in ATC, which indicates that lncRNA GAS5 may inhibit the progression of ATC by inhibiting miR-96 expression.

In conclusion, downregulation of lncRNA GAS5 was found in ATC tissues, cell lines and Dox-resistant ATC cells. Functionally, lncRNA GAS5 can inhibit cell proliferation and resistance to doxorubicin in ATC by downregulating miR-96.

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LETTER TO THE EDITOR

Abatacept relieves kidney injury in diabetic nephropathy rats by inhibiting TGF- β 1/SMAD7 signaling pathwayL. Ye¹, YY. Pan², FH. Jin³, DH. Zhang⁴, QL. Xu⁵ and L. Zhang⁶

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To the Editor,

Diabetic nephropathy (DN) is one of the common chronic microvascular complications of diabetes, which is one of the main causes of end-stage renal failure (1, 2). In Asia and Western countries, the incidence of diabetic nephropathy is increasing yearly (3). In addition, the risk of cardiovascular accidents in patients with diabetes nephropathy is high, and not less than the risk of cardiovascular accidents in patients with coronary heart disease (4). In recent years, with the increase in the average life expectancy of our population and the changes in dietary habits and structure, the prevalence of diabetes has shown a straight upward trend. As treatment methods have improved, leading to longer survival time, the incidence of diabetic nephropathy and other complications has increased. However, the drugs and methods currently used to treat diabetic nephropathy are relatively limited.

Abatacept is a clinically available fusion protein that binds B7-1 to prevent its signal transmission and is used to treat certain autoimmune diseases. Studies have shown that in a diabetic nephropathy rat model, Abatacept can repair the physiological structure

and cellular activity of podocytes, counteract the high glucose environment and reduce proteinuria (5). However, the application of Abatacept in the treatment of diabetic nephropathy requires further basic research and verification. The purpose of this study was to investigate whether Abatacept can effectively prevent or reduce kidney damage in rats with diabetic nephropathy.

MATERIALS AND METHODS

Rat grouping and treatment

Forty-five rats were randomly divided into 3 groups (15 rats in each group), including control group, diabetic nephropathy group and Abatacept group. Rats in the control group were fed with conventional feed, and had free access to water without any intervention. In the diabetic nephropathy group, a rat model was established. After 4 weeks of feeding with high fat and high glucose, 3 days of intraperitoneal injection of STZ in small doses caused fasting blood glucose (FBG) of 16.7 mmol/L or more, which indicated that the model was successfully induced. In the Abatacept group, a rat model of diabetic nephropathy was established. After the successful

Key words: Abatacept; TGF- β 1; SMAD7; diabetic nephropathy

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modeling, the high-sugar and high-fat diet was maintained during the modeling period, and the rats were given intravenous injection of Abatacept for 8 weeks.

The successfully prepared model rats were randomly divided into an experimental group and a diabetic nephropathy group. In the Abatacept group, 50 mg/kg of Abatacept suspension was intragastrically administered. The normal control group and the diabetic nephropathy group were administered with an equal volume of 0.9% sodium chloride solution, administered continuously for 6 weeks, once a day. During the experiment, the blood glucose concentration was measured once a week and the 24-hour urine protein quantification was measured every two weeks.

Specimen collection and testing

At the end of the 6th week of the study, a 24-hour urine sample was taken. After centrifugation, the supernatant was stored. The rats were weighed, and anesthesia was injected intraperitoneally with 10% chloral hydrate. The heart blood was stored in a procoagulant tube (Table I).

Real-time PCR to detect mRNA expression

The total RNA of renal cortical tissue was extracted with Trizol reagent. To synthesize single-stranded cDNA, the reverse transcription reaction was performed using the PrimeScript RT Enzyme Mix Kit according to the specification. The SYBR Premix Ex Taq II Kit was used for real-time quantitative PCR reactions. The reaction conditions were 95°C for 3 min, 95°C for 30s, 60°C for 30s, 72°C for 30s, and 40 cycles. Three replicates were made for each well, and GAPDH was used as an internal reference. The mRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were: Col4 forward 5'-CAAACCACAGCCAAUCCUUCA-3', reverse 5'-AAGAAGGGAAAACCCACUGUAGAGU-3'; FN forward 5'-CAUGGCUUUAGGCGAACCA-3', reverse 5'-CAUCUACAUUCGGCAGGUAUGG-3'; TGF β 1 forward 5'-AGGGCUACCAUGCCAACUUC-3', reverse 5'-CCACGUAGUAGACGAUGGGC-3'; SMAD7 forward 5'-CCAUCAAGGCUUUUGACUAUGAAA-3', reverse 5'-CCAUGGCUGCUGCAUGAAC-3'; GAPDH forward 5'-UGCCACUCAGAAGACUGUGG-3', reverse 5'-UUCAGCUCUGGGAUGACCUU-3'.

Western blot for protein expression

The renal cortical tissue of each group was taken

and ground with protein lysate. The protein lysate was centrifuged and the supernatant was discarded. The BCA method was used to detect the protein concentration. Proteins were separated by 10% SDS-PAGE gel electrophoresis. After transferring the proteins to PVDF membranes, they were blocked with 5% BSA for 3 h. The primary antibody was diluted according to the antibody instructions and incubated at 4°C overnight. The membrane was incubated for 1.5 h with a secondary antibody labeled with horseradish peroxidase, and the band was exposed by ECL chemiluminescence. The primary antibodies against Fibronectin were (FN, BF9010, Yanhui biological, Shanghai, China), TGF β 1 (sc-130348, Santa Cruz Biotechnology, Shanghai, China), SMAD7 (ab90086, Abcam, Beijing, China) and GAPDH (ab181602, Abcam, Beijing, China).

Statistical analysis

SPSS 19.0 software was used to process the data. The data were expressed as mean $\pm$ standard deviation (SD), repeated at least three times in each group. Multiple groups of data were analyzed by one-way ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Effects of Abatacept on renal function

The results showed that compared with the control group, the Fasting blood glucose (FBG), Creatinine clearance rate (Ccr), and 24 h urine albumin excretion rate (UAER) of the diabetic nephropathy group and the Abatacept group were higher than those of the control group. The Ccr and UAER of the Abatacept group were lower than those of the diabetic nephropathy group, and there was no statistical difference in FBG between the Abatacept group and the diabetic nephropathy group. The results showed that early intervention with Abatacept in diabetic rates can effectively protect renal function, including slowing the increase of Ccr and UAER, but it has no obvious positive effect on FBG, which may be related to the site of action of Abatacept (Table II).

Effects of Abatacept on the expression of Col4 and FN in renal cortical tissue

Compared with the control group, the mRNA

levels of Col4 and FN in renal cortex tissues of the diabetic nephropathy group were significantly increased ($P < 0.05$). Compared with the diabetic nephropathy group, the mRNA expression of Col4 and FN in the Abatacept group were significantly

reduced ($P < 0.05$). Compared with the control group, the protein expression of Col4 and FN in the renal cortex of the diabetic nephropathy group were significantly increased ($P < 0.05$). Compared with the diabetic nephropathy group, the protein

Table I. Comparison of general and biochemical indexes of rats among groups

Group	number	FBC(mmol/L)	Ccr(ml/min)	24h UAER(mg/24h)	KW/BW(mg/g)
Control	15	6.82	6.12	0.76	3.75
Diabetic nephropathy	15	20.51	22.14	6.11	5.86
Abatacept	15	21.22	29.31	8.31	7.93

FBG: Fasting blood glucose; Ccr: Creatinine clearance rate; 24 h UAER: 24 h urine albumin excretion rate; KW/BW: Kidney hypertrophy index (kidney weight/body weight).

Table II. Comparison of renal function in each group

group	24h urinary protein(mg/24h)	Ccr(mL/min)	Kidney index	Glomerular area/ μm^2
Control	8.33	3.36	2.46	143
Diabetic nephropathy	40.21*	1.52*	4.98*	186*
Abatacept	22.54 [#]	2.11 [#]	4.22 [#]	156 [#]

* $P < 0.05$ compared with control; [#] $P < 0.05$ compared with diabetic nephropathy group

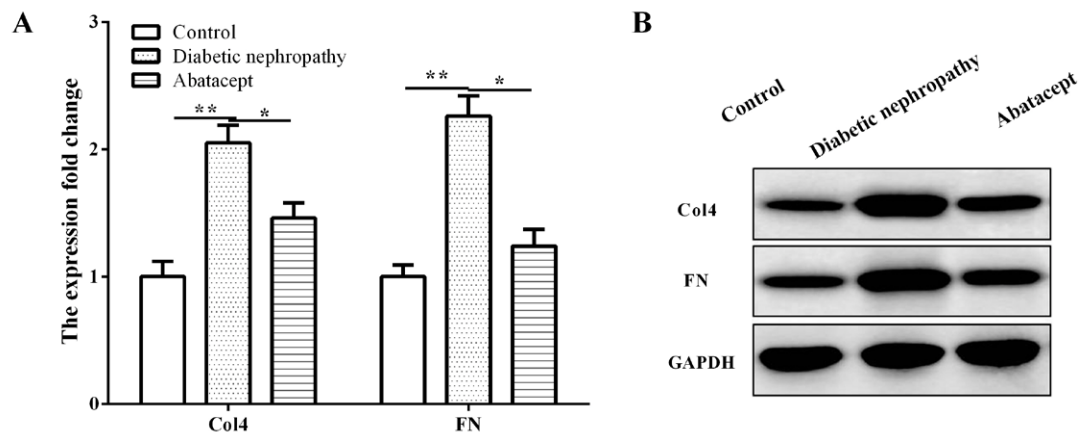


Fig. 1. Effects of Abatacept on the expression of Col4 and FN in renal cortical tissue of rats in various groups. **A)** mRNA levels of Col4 and FN of the diabetic nephropathy group were significantly increased compared with the control. Abatacept significantly reduced the mRNA levels of Col4 and FN in diabetic nephropathy tissues. **B)** Western blot revealed that the protein levels of Col4 and FN were consistent with that of mRNA.

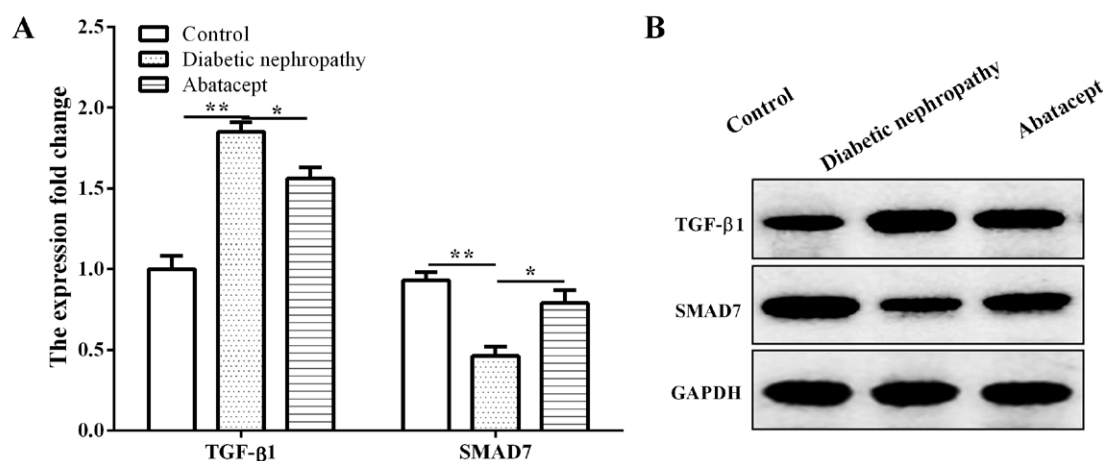


Fig. 2. Effect of Abatacept on TGF- β 1/SMAD7 signaling pathway. **A)** The mRNA of TGF- β 1 was significantly increased, and the expression of SMAD7 was significantly reduced of the diabetic nephropathy group. Abatacept inhibits TGF- β 1/SMAD7 signaling pathway. **B)** The protein levels were consistent with the mRNA levels.

levels of Col4 and FN in the Abatacept group were significantly reduced ($P < 0.05$). The protein of Col4 and FN detected by Western blot was consistent with that of mRNA (Fig. 1).

Effect of Abatacept on TGF- β 1/SMAD7 signaling pathway

Compared with the control group, the mRNA of TGF- β 1 was significantly increased ($P < 0.05$), and the expression of SMAD7 was significantly reduced ($P < 0.05$) in the renal cortex of the diabetic nephropathy group. The mRNA of TGF- β 1 in the Abatacept group was down-regulated ($P < 0.05$), and the expression of SMAD7 was up-regulated ($P < 0.05$). The protein of TGF- β 1 and SMAD7 detected by Western blot was consistent with that of mRNA (Fig. 2).

DISCUSSION

Abatacept can effectively alleviate renal hemodynamic changes in diabetic nephropathy rats (6, 7). In this group of studies, we found that proteinuria symptoms of diabetic nephropathy rats were significantly improved after treatment with Abatacept, and the creatinine clearance rate increased, indicating that Abatacept has a protective

effect on the kidney of diabetic nephropathy rats.

The excessive accumulation of extracellular matrix (ECM) is one of the important causes of early diabetic nephropathy (8). Among them, Col4 is a scaffold that forms the glomerular basement membrane network structure and plays an important role in maintaining the normal structure of glomeruli (9, 10). The results showed that Abatacept may inhibit the synthesis of extracellular matrix and thus exerts renal protection. TGF- β 1/SMAD7 signaling pathway is one of the important signaling pathways regulating the occurrence and development of diabetic nephropathy (11). Studies have reported that Abatacept can reduce the infiltration and metastasis of pancreatic cancer cells by inhibiting the TGF- β 1/SMAD7 signaling pathway (12). Our results suggest that Abatacept may inhibit the synthesis of extracellular matrix by blocking the activation of the TGF- β 1/SMAD7 signaling pathway.

In summary, Abatacept delays diabetic kidney injury and exerts a renal protective effect. The mechanism may be achieved by regulating the expression of TGF- β 1/SMAD7 signaling pathway, alleviating the accumulation of extracellular matrix in the glomerulus, and thereby improving renal function.

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LETTER TO THE EDITOR

Diagnostic value of three-dimensional reconstruction with multislice spiral CT angiography in patients with cerebrovascular diseaseR-D. Sui¹, H-T. Wang¹, X-Y. Yu², X-Q. Zhang³, X-L. Wei¹, D-Y. Xin¹ and D-W. Han⁴

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To the Editor,

At present, cerebrovascular disease is one of the most common diseases in the elderly (1-3). Due to the high incidence, disability and mortality, it seriously endangers the healthy life of elderly patients. Conventional CT angiography (CTA) is mainly used in clinical practice for patients with cerebrovascular disease. However, because the elderly are prone to vascular rupture caused by weak vascular elasticity, the injection rate is reduced during the contrast injection process, resulting in serious bone residual, uneven and discontinuous vascular wall after bone removal on CTA images (4-6). With the continuous development of science and technology, 3D reconstruction with multislice spiral CT angiography (MSCTA) has been gradually applied to clinical treatment (7). In order to explore the diagnostic value of 3D reconstruction with MSCTA in patients with cerebrovascular disease, 60 patients in our hospital from June 2018 to April 2020 were selected, aiming to provide a reference basis for the clinical diagnosis by 3D Reconstruction with MSCTA.

MATERIALS AND METHODS

Sixty patients with cerebrovascular disease who received 3D Reconstruction with MSCTA between June 2018 and April 2020 in our hospital were selected as the study subjects, as shown in Table I.

MSCTA

All 60 patients received MSCTA with a Philips instrument, and relevant parameters were: 250 mA tube current, 120 kv tube voltage, 512×512 matrix, 0.9 mm slice thickness; 100 ml (280 mg/ml) iohexol was bolus injected via the elbow vein at a rate of 3.0ml/s-3.5ml/s with a trigger time of 10-20s. Then bolus tracking technology was used. The aortic arch was the main observation area and the threshold was 100Hu. Scanning site: The baseline was the auditory canthus line, scan starting from the base to the top of the skull. Before scanning, the patient's head was fixed and the patient was not allowed to cough or swallow.

Key words: cerebrovascular disease; application; multislice spiral CT; angiography; three-dimensional reconstruction (3D Reconstruction); diagnostic value

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Table I. Analysis of general clinical data of the patients in two groups

Factors		Indexes (n=60)
Gender (case)	Male	32
	Female	28
Average age (years)		61.08±8.42
Clinical manifestation	Language barrier	10
	Headache	22
	Nausea and vomiting	20
	Other	8
Multi-slice spiral CT plain scan	Cerebral infarction	15
	Simple intracerebral hematoma	17
	Subarachnoid hemorrhage	14
	Malformed vessel mass	6
	No abnormality	8

3D Reconstruction

Sixty patients received 3D reconstruction through maximum intensity projection (MIP), volume rendering (VR), and surface shaded display (SSD).

Statistical analysis

In this study, the research data was processed and analyzed by the data software SPSS20.0. The measurement data were measured by *t*-test, expressed by ($\bar{x} \pm s$), and the count data were tested by χ^2 , expressed by n(%). The difference was statistically significant when $p < 0.05$.

RESULTS

Among the 60 patients, there were 21 cases of aneurysm, 8 cases of cerebral stenosis, 18 cases of

arteriovenous malformation and 13 cases of embryonic posterior cerebral hemangioma, as shown in Fig. 1.

MSCTA results

Through 3D reconstruction with MSCTA, it was found that images of all patients were clearly structured and the specific size, location and shape of the aneurysms could be clearly diagnosed. Twenty-one cases of aneurysm, with lesions of 3.39 mm-22.78 mm in diameter, comprised 2 patients with A2 segment of anterior cerebral artery, 12 with C2 segment venous artery, 2 with basal artery trunk, 3 with posterior communicating artery and 2 with anterior communicating artery according to the lesion locations, as shown in Figs. 2-3. Eighteen cases of arteriovenous malformation included 7 patients with right parietal lobe and 11 patients

with right cerebellar hemisphere, as shown in Figs. 4-5. Eight cases of cerebral stenosis included 3 patients with P1 segment stenosis of posterior cerebral artery and 5 patients with vertebral artery V5 stenosis, as shown in Figs. 6-7. Thirteen cases

of embryonic posterior cerebral hemangioma included 2 patients whose position of segment C2 rises, 3 patients with vascular variation and 8 patients with posterior communicating artery cone, as shown in Fig. 8.

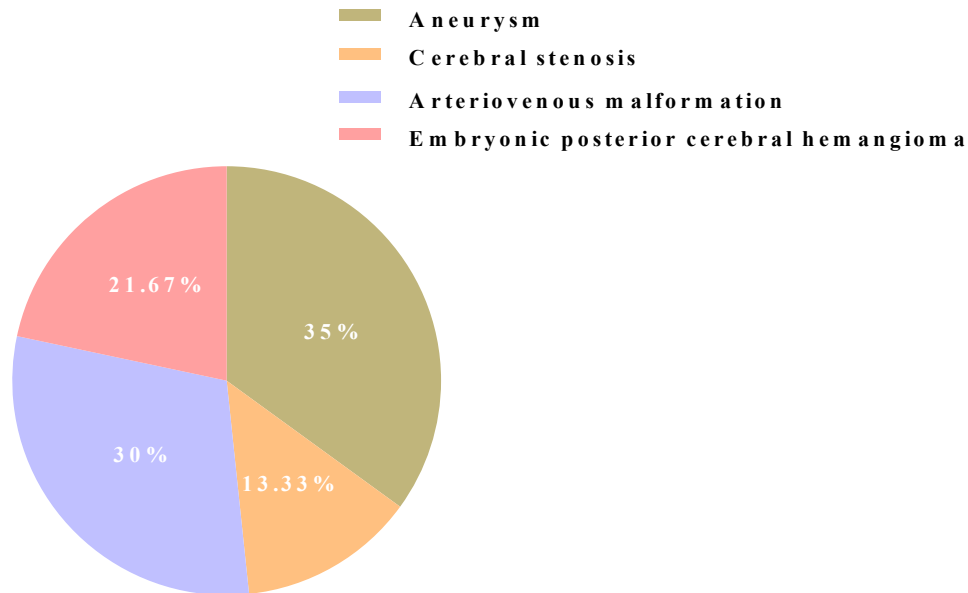


Fig. 1. Analysis of all patient test results. The figure represents the number of patients with aneurysm, cerebral stenosis, arteriovenous malformation and posterior embryonic cerebral hemangioma. The figure shows that the percentage of patients with aneurysms is the highest.



Fig. 2. Results of MSCTA in patients with aneurysm

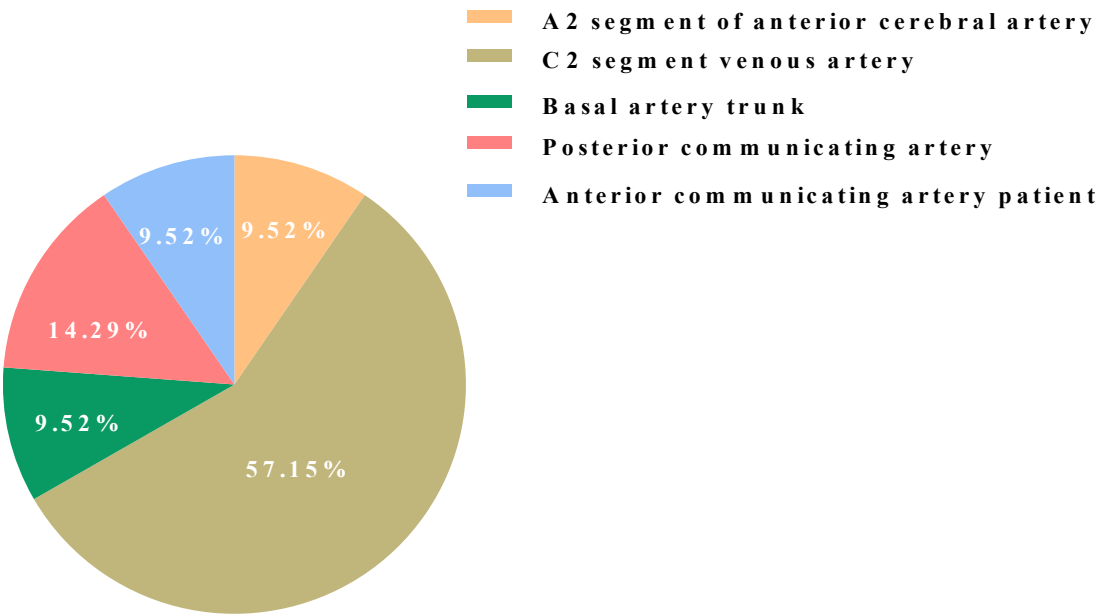


Fig. 3. Distribution of lesions in patients with aneurysm. The figure represents A2 segment of anterior cerebral artery, C2 segment of venous artery, basal artery trunk, posterior communicating artery and anterior communicating artery, respectively. Patients with C2 segment venous artery had the highest proportion.

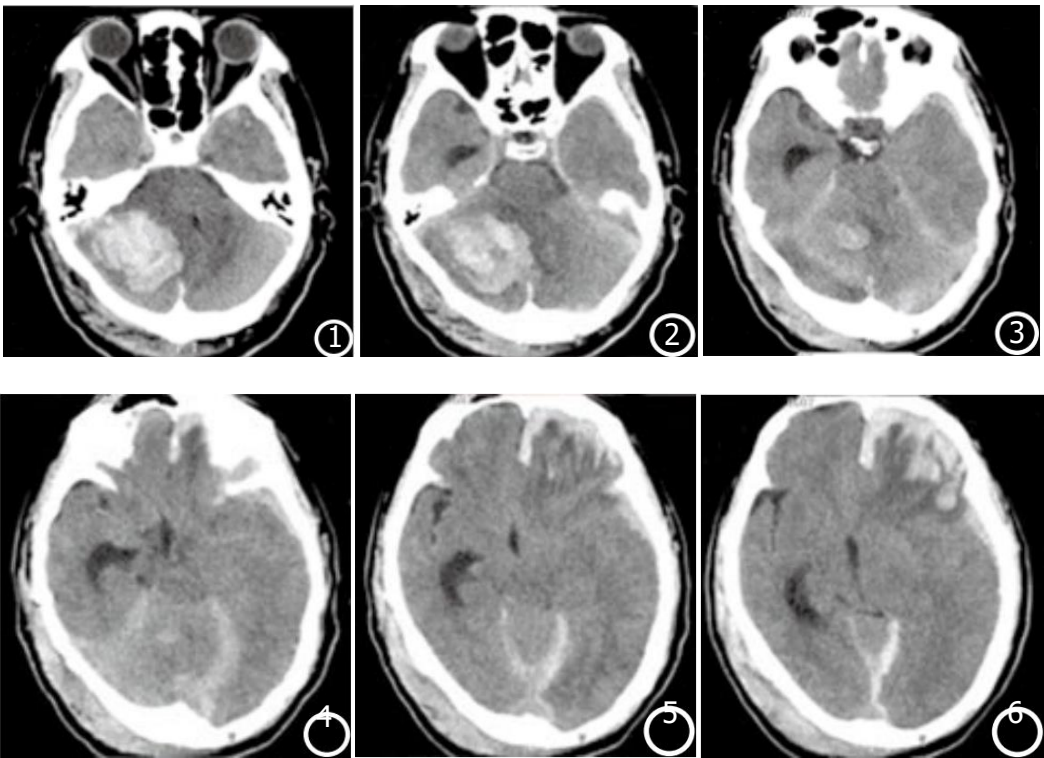


Fig. 4. MSCTA imaging results in patients with arteriovenous malformation

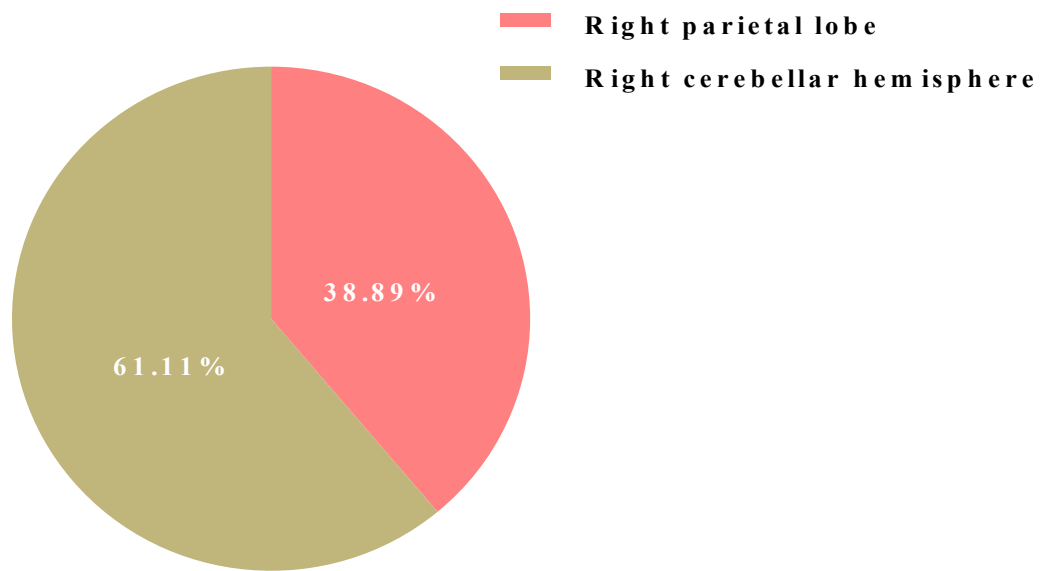


Fig. 5. *Distribution of lesions in patients with arteriovenous malformation. The figure represents patients with right parietal lobe and right cerebellar hemisphere, respectively. Patients with right cerebellar hemisphere had the highest proportion.*

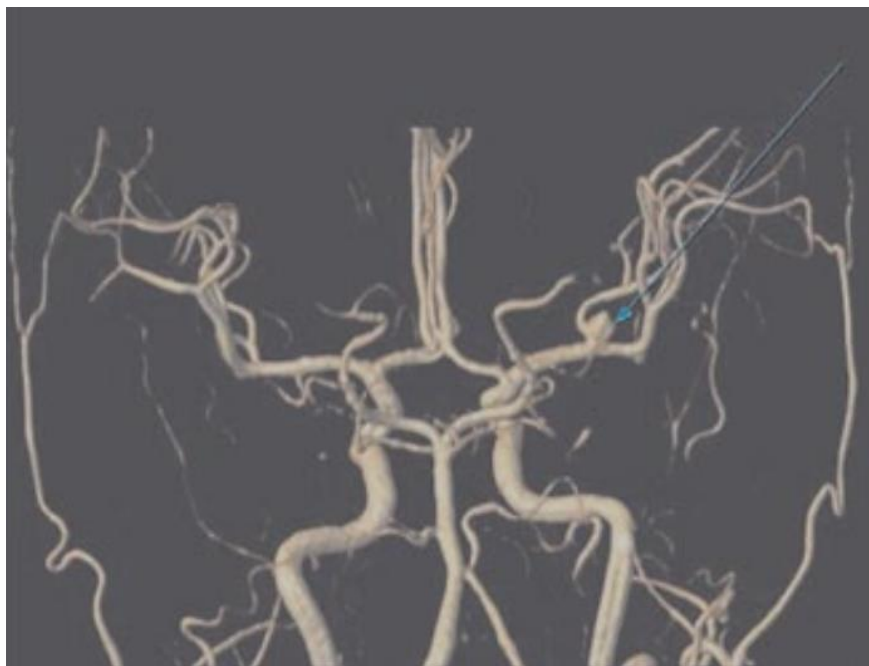


Fig. 6. *MSCTA results in patients with cerebrovascular stenosis.*

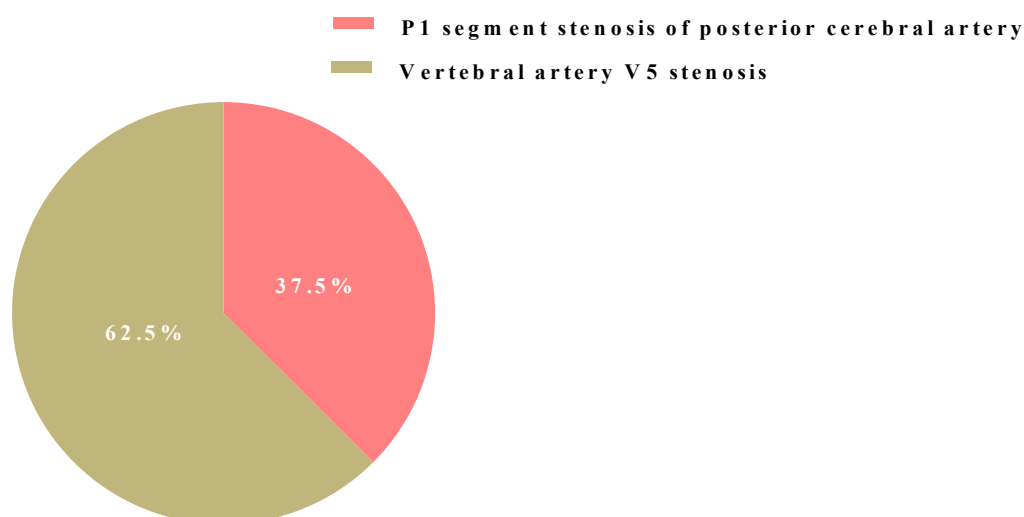


Fig. 7. Distribution of lesions in patients with cerebrovascular stenosis. The figure represents patients with P1 segment stenosis of posterior cerebral artery and vertebral artery V5 stenosis, respectively. Patients with vertebral artery V5 stenosis had the highest proportion.

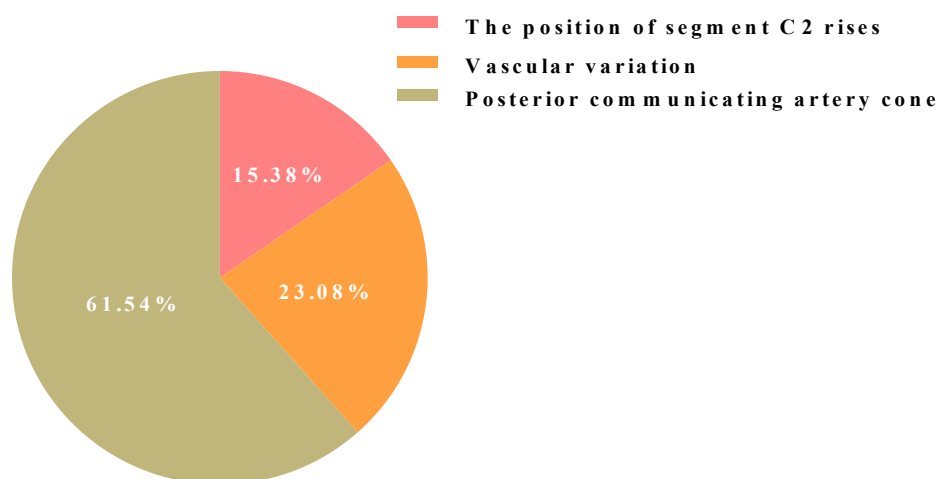


Fig. 8. Distribution of lesions in patients with embryonic posterior cerebral hemangioma. The figure represents patients whose position of segment C2 rises, patients with vascular variation and patients with posterior communicating artery cone, respectively. Patients with posterior communicating artery cone had the highest proportion.

Diagnostic positive rate

Of the 60 patients, 55 were positive, of whom 3 were false positive, accounting for 91.67% and 5% of the total patients, respectively, as shown in Fig. 9.

DISCUSSION

With the continuous development of science and technology, CT angiography technology

continues to develop and has now been widely used in the diagnosis and treatment of cerebrovascular disease (8-10). The majority of the patients are the elderly whose blood vessel elasticity is weak, which will lead to the rupture of the vessel wall. Therefore, in order to avoid leakage of contrast agents, the injection rate is usually reduced during the injection process (11-12). The reduction of the rate leads to serious bone residual, uneven and discontinuous

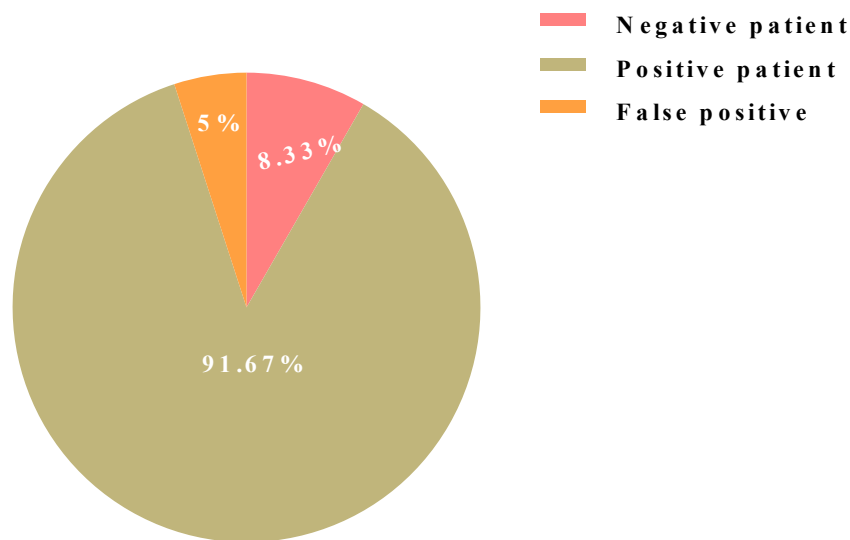


Fig. 9. Analysis of the diagnostic positive rate of patients. The figure represents negative patients, positive patients and false positive, respectively. Positive patients had the highest proportion.

vascular wall, and paracranial vascular artifacts after bone removal on CTA images, which is not conducive to clinical diagnosis.

Experience of application of 3D reconstruction with MSCTA

3D reconstruction with MSCTA is the latest diagnostic method in cerebrovascular disease at present. According to He et al. (13) and this study, it can be concluded that four points should be noted when implementing 3D reconstruction with MSCTA in elderly patients with cerebrovascular disease (1). The patient's head should be fixed before testing to avoid movement during the testing process, which is the key to success of 3D reconstruction with MSCTA (2). Elderly patients with cerebrovascular disease are prone to vascular wall rupture, so high injection rate of contrast agents will cause leakage during the detection process. Therefore, the injection rate should be paid attention to during the injection of contrast agents. The ages of the study subjects were (61.08 ± 8.42) years and the injection rate was controlled at 3.0ml/s-3.5 ml/s. No injection leakage occurred, and the clinical image quality was satisfactory (3). The peak

time of contrast agent varies from patient to patient, as does the trigger time. In this study, trigger time was set to 10-20s. Bolus tracking technology was used and solved the problem that the peak time of contrast agent was different for each patient (4). Cranial artifacts affect the smoothness and continuity of blood vessels, resulting in serrated artifacts which are more serious in the intracranial segments of basal and vertebral arteries. Therefore, the original image of proper thickness should be used in 3D reconstruction with MSCTA, which has a positive effect on reducing serrated artifacts. In this study, 0.9 mm slice thickness was selected to subtract under the premise of ensuring image resolution, which achieved good effects.

Clinical diagnostic significance of 3D reconstruction with MSCTA

According to the research results of Cejna (14) et al., it can be seen that 3D reconstruction with MSCTA has the advantages of thinner scanning layer, high spatial resolution, all-round scanning and no damage. In this study, through 3D reconstruction with MSCTA in 60 patients, it was found that images of all patients were clearly structured, which was

consistent with the conclusion of Lu et al. (15). The display rate of 3D reconstruction with MSCTA was 100% in the diagnosis of cerebrovascular disease. 3D reconstruction with MSCTA is a maximum intensity projection method, which plays an active role in the diagnosis of cerebrovascular disease, especially as the volume rendering technique can adjust the brightness and transparency through different thresholds. The results of this study showed that among the 60 patients, there were 21 cases of aneurysm, 8 cases of cerebral stenosis, 18 cases of arteriovenous malformation and 13 cases of embryonic posterior cerebral hemangioma, indicating that 3D reconstruction with MSCTA can scan all the data and provide accurate 3D images for clinical diagnosis.

In this study, through 3D reconstruction with MSCTA in 60 patients, it was found that images of all patients were clearly structured and the specific size, location and shape of the aneurysms could be clearly diagnosed. Twenty-one cases of aneurysm, with lesion of 3.39mm 22.78mm in diameter, comprised 2 patients with A2 segment of anterior cerebral artery, 12 patients with C2 segment venous artery, 2 patients with basal artery trunk, 3 patients with posterior communicating artery and 2 patients with anterior communicating artery according to the lesion locations. Eighteen cases of arteriovenous malformation included 7 patients with right parietal lobe and 11 patients with right cerebellar hemisphere. Eight cases of cerebral stenosis included 3 patients with P1 segment stenosis of posterior cerebral artery and 5 patients with vertebral artery V5 stenosis. Thirteen cases of embryonic posterior cerebral hemangioma included 2 patients whose position of segment C2 rises, 3 patients with vascular variations and 8 patients with posterior communicating artery cones. The research results proved that 3D reconstruction with MSCTA can comprehensively detect the patients with cerebrovascular disease, and the location and type of lesions can be accurately grasped and displayed in the 3D images, thus providing important and accurate reference data for clinical diagnosis and treatment.

In conclusion, the application of 3D reconstruction technology with MSCTA has a high

diagnostic rate in the clinical diagnosis of patients with cerebrovascular disease, therefore, it is worthy of popularization and application in the clinical diagnosis of cerebrovascular disease.

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LETTER TO THE EDITOR

miR-542 inhibits tumorigenesis and chemoresistance through the AKT/NFκB pathway in cervical cancer

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To the Editor,

Cervical cancer (CC) is one of the most common cancers in the world that affects women (1). The incidence of CC is the second only to breast cancer and ranks second among gynecological malignancies (2). Hence, understanding the molecular mechanism of the genesis and development and metastasis of CC to find potential therapeutic targets is indispensable. BMI1 is a major component of the PRC1, which is involved in DNA damage repair, embryonic development and self-renewal by encoding a ring finger protein (3). Dysregulation of BMI1 is common in numerous cancers and is related to the resistance to certain chemotherapies including Cisplatin (4). Recently, Xu et al. reported that up-regulated BMI1 promoted tumorigenicity *in vivo* and tumor sphere formation *in vitro* through the enhanced regulation of Sox2 genes at transcriptional level, and acted as a latent oncogene that takes part in the carcinogenesis of CC (5). However, the characteristic molecular mechanism and the underlying clinical roles of BMI1 are ill-defined in CC. Herein, we reported that BMI1, located in cytoplasm and nucleoplasm, was up-regulated in CC and the direct target gene of

miR-542, which was significantly positively related with large tumor size, serious TNM stage, distant metastasis and poor prognosis in CC patients. miR-542 inhibited tumor growth *in vitro* and *in vivo* as well as migration, invasion and cell cycle process, and promoted cell apoptosis in HeLa and C33A cells. miR-542 also inhibited chemoresistance to Cisplatin and Tamoxifen. Moreover, overexpression of miR-542 inactivated AKT/NFκB pathway through BMI1 in CC. To sum up, these findings may provide a potential therapeutic target and prognostic biomarker for CC patients.

MATERIALS AND METHODS

This study was approved by the Institutional Ethics Committee of The Second Affiliated Hospital of Shandong First Medical University (No. TMU20180122#01) and conformed to the protocols in the Helsinki declaration. Fifty patients with cervical cancer in The Second Affiliated Hospital of Shandong First Medical University from October 2018 to October 2019 were involved in this study. Patients with cervical cancer did not receive any treatment except surgery. The diagnosis of pathology was

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confirmed by the Department of Pathology in The Second Affiliated Hospital of Shandong First Medical University. The tumor and adjacent normal tissues were obtained from with these patients who provided written informed consents.

Cell culture and transfection

All cell lines used in this report were purchased from ATCC and Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured in 10% FBS (Gibco, Carlsbad, CA, USA) and RPMI 1640 (Invitrogen, Carlsbad, CA, USA), 100 µg/ml streptomycin (Beyotime, Shanghai, China), 100 U/ml penicillin (Beyotime, Shanghai, China) at 37°C in 5% CO₂. Endl/E6E7 was cultured in 10% FBS (Gibco, Carlsbad, CA) and KER-SFM medium (Invitrogen, Carlsbad, CA) in 5% CO₂ at 37°C. Transfection assay was performed using Lipofectamine™ 3000 reagent (Invitrogen) according to the manufacturer's protocol.

RT-qPCR. TRIzol reagent (Invitrogen, USA) was used to extract total RNAs from CC tissues and cells. The cDNA was reverse transcribed using miRNA reverse transcription kit (Takara, Dalian, China). Next, cDNA solution, primers and SYBR Green Master Mix II (Takara) were used to perform RT-qPCR assay on a 7500 Realtime PCR System (Applied Biosystems, Carlsbad, CA, USA). The primer sequences used were as follows: miR-542-3p-qPCR-Forward: 5'-UGUGACAGAUUGAUACUGAAA-3' ; U6 - qPCR - Forward : 5'-TGCGGGTGCTCGCTTCGGCAGC-3'; miRNA-qPCR-Reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'; β-actin - qPCR - Forward : 5'-CGTGACATTAAGGAGAAGCTG-3'; β-actin-qPCR-Reverse: 5'-CTAGAAGCATTTGCGGTGGAC-3'; BMI1 - qPCR - Forward : 5'-CCACCTGATGTGTGTGCTTTG-3'; BMI1-qPCR-Reverse: 5'-TTCAGTAGTGGTCTGGTCTTGT-3'.

CCK8 assay. Briefly, CC cells were plated into 96-well culture plates at a density of 5×10^3 cells in 200 µl of culture medium per well one day prior to transfection. Between 24–72 h of culture, 10 µl CCK-8 reagent (Dojindo, Kumamoto, Japan) was added into each well and incubated at 37°C for 2 h. Relative cell proliferating rate was measured using a microplate reader (Thermo Fisher, Ltd., CA, USA) at absorbance of 490 nm.

Transwell assay. HeLa and C33A cells (5×10^4 cells/well) were added to the upper chamber. The lower

chamber was added with 1640 medium and 10% FBS. The upper chamber with Matrigel (BD Biosciences) was used for cell invasion. Cell migration was detected without Matrigel, and other experimental steps are the same as for cell invasion. The chamber was fixed with 4% paraformaldehyde (Sigma) for 30 min, washed with PBS twice and stained in 0.1% crystal violet for 15 min. The number of cells was counted using a light microscope DM2500 (Olympus Corporation, Tokyo, Japan).

II cycle, the cells were digested using trypsinization (Invitrogen, Carlsbad, CA) and stained with PI (Beyotime, Shanghai, China) using a Cycletest™ Plus DNA Reagent kit (BD, Franklin Lakes, NJ, USA) according to the manufacturer's manual, and were detected by flow cytometry assay. Cell apoptosis was detected by Annexin-V-FITC apoptosis detection kit (Beyotime, Shanghai, China) according to the manufacturer's protocol.

Western blot. Cell extracts were cleaned with cold PBS buffer (Sangon Biotech, Shanghai, China), prepared with RIPA buffer (Solarbio, Beijing, China) supplemented with protease inhibitor Cocktail (MCE, NJ, USA), and protein concentrations were quantified using the BCA protein assay kit (Beyotime) according to the manufacturer's protocols. The protein lysate was separated via 10% SDS-PAGE (Solarbio, Beijing, China). Protein samples were transferred to PVDF membranes (Millipore, USA) and blocked with 5% non-fat milk (Solarbio, Beijing, China). Then, protein samples were incubated overnight at 4°C with BMI1 (CST #5856), E-cadherin (CST #3195), ICAM1 (CST #4915), Vimentin (CST #5741), IKKα (CST #2682), IKKβ (CST #2684), p-AKT (CST #4060), AKT (CST #9272), p-p65 (CST #3033) and GAPDH (CST #5174) primary antibodies (Cell Signaling Technology, Danvers, MA, USA). After washing, protein samples were incubated with secondary antibodies (CST #7076 or #7074, Cell Signaling Technology, Danvers, MA, USA) for 1 h. Finally, ECL kit (Beyotime) was used to assess protein bands.

NFκB luciferase reporter assay. For luciferase reporter assays, cells (10^6 cells) were distributed in 24-well plates and stimulated as indicated. Samples were harvested and prepared for luciferase assays according to the protocol suggested by the manufacturer (Promega). All reporter assays were co-transfected with a pRL-TK reporter plasmid (Promega) to control for intersample variations in transfection efficiency. In the latter case, firefly and pRL-

TK–derived Renilla luciferase activities were measured in each sample with a Dual Luciferase Assay kit (Promega).

Immunofluorescence. CC cells transfected with pcDNA3+pcDNA3, pcDNA3+miR-542 and miR-542+BMI1 were seeded on glass bottom culture dishes in a 24-well plate, fixed with 4% paraformaldehyde (Sigma) for 10 min, and perforated with 0.2% TritonX100 for 5 min. After blocking with 10% donkey serum (Solarbio, Beijing, China) for 20 min and washing with cold PBS (Sangon Biotech, Shanghai, China), the cells were incubated with p65 (Abcam, MA, USA) at 4 overnight. The culture dishes were incubated with AlexaFluor488-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 2 h. The nuclei of cells were stained with DAPI (Solarbio, Beijing, China) for 10 min. Confocal fluorescence microscopy (Zeiss Germany, Germany) was used to capture fluorescence confocal images.

Immunohistochemical assay. Immunohistochemical staining of the tumor tissue was performed according to the kit manufacturer's protocol (Zhongshan Golden Bridge, Beijing, China) on 4% paraformaldehyde fixed, paraffin embedded 4 μ m tissue sections. Antibody against BMI1 (CST #6964) and KI67 (CST #9449) was used at the dilution range of 1:200. The stained sections were evaluated by two different specialists.

In-vivo experiments

Six 6-week-old female BALB/c-nude mice were purchased from Beijing Vital River Laboratory Animal Technologies Co. Ltd, Beijing, China. The mice were randomly divided into 2 groups: Lenti-Con and Lenti-miR-542. Lentiviral-transduced HeLa cells were subcutaneously injected into the ventral forearm of the mice. During 1-4 week of *in-vivo* explant assay, the subcutaneous lengths and widths of tumors were measured weekly. Mice were sacrificed 4 weeks post-injection, the tumors were extracted and visually compared. The study was approved by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Shandong First Medical University.

Statistical analysis

Statistical Product and Service Solutions (SPSS19.0, IBM Co., Armonk, NY, USA) statistical software was used for data analysis and GraphPad Prism (Version V,

La Jolla, CA, USA) was introduced for image editing. Measurement data were expressed as mean $\pm$ standard deviation and compared using the t-test. $p < 0.05$ was considered to be statistically significant.

RESULTS

BMI1, located in cytoplasm and nucleoplasm, was aberrantly elevated in CC

The expression level of BMI1 was aberrantly raised in cervical cancer tissues compared with the adjacent normal tissues using TCGA database (Fig. 1A). The results of RT-qPCR showed that the BMI1mRNA level was increased in 42 cervical cancer patients (Fig. 1B). The patients of cervical cancer with high BMI1 level had a poor prognosis according to Starbase software (<http://starbase.sysu.edu.cn/>) (Fig. 1C). In addition, the higher protein levels of BMI1 in cervical cancer tissues were verified through the immunohistochemical staining (Fig. 1D). Meanwhile, BMI1 expression was found to be lower in human cervix epithelial cell End1/E6E7 than in cervical cancer cells, including SiHa, Caski, HT-3, C33A and HeLa (Fig. 1 E, F). Higher expression at mRNA and protein levels of BMI1 in HeLa cells than in C33A cells may indicate the correlation with HPV infection. Immunofluorescence assay showed the distribution of BMI1 in HeLa and C33A cells, which located in cytoplasm and nucleoplasm (Fig. 1G). Furthermore, the expression level of BMI1 was significantly positively associated with large tumor size, serious distant metastasis, and TNM stage in CC patients (Table I).

BMI1 was a direct target gene of miR-542

BMI1 was found to have a binding site with miR-542 in StarBase. Highly conserved predicted binding sites and the mutant sites were shown in the 3'UTR of BMI1 (Fig. 2A). EGFP reporter assay indicated that the EGFP activity of BMI1 was decreased by miR-542 overexpression and increased by miR-542 knockdown in HeLa and C33A cells, indicating that miR-542 directly targets BMI1 (Fig. 2B). In addition, altered miR-542 failed to affect the EGFP activity of BMI1 3'UTR MUT in C33A and HeLa cells (Fig. 2C). Moreover, BMI1 mRNA and protein expression

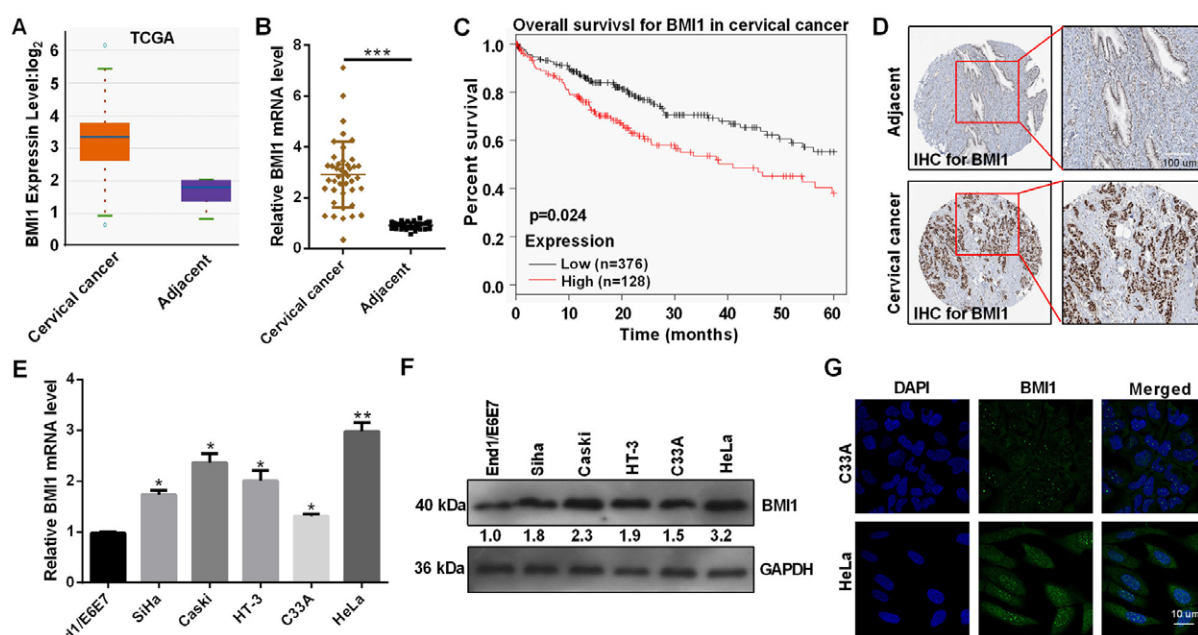


Fig. 1. BMI1 is up-regulated in cervical cancer. **A)** TCGA database showed the relative mRNA level of BMI1 in CC. **B)** RT-qPCR showed the BMI1 level in CC tissues. **C)** Starbase software showed the overall survival with high or low expression of BMI1 in CC patients. **D)** IHC showed the protein level of BMI1 in CC and the adjacent normal tissues. **E)** RT-qPCR showed the mRNA level of BMI1 in CC cells and End1/E6E7 cell. **F)** Western blot showed the protein level of BMI1 in CC cells. **G)** IF showed the distribution of BMI1 in HeLa and C33A cells. Data are presented as means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant.

Table I. Association of BMI1 with the clinicopathological features of CC patients

Variables	No. cases (50)	BMI expression		<i>p</i> -value
		Low (n)	High (n)	
Age				
< 50 years	20	9	11	0.883
$\geq$ 50 years	28	12	16	
Tumor size				
$\geq$ 5 cm	27	8	19	0.025 ^a
< 5 cm	21	13	8	
TNM stage				
I-II	19	13	6	0.005 ^a
III-IV	29	8	21	
Distant metastasis				
No	17	12	5	0.006 ^a
Yes	31	9	22	
Lymph node metastasis				
Absent	16	7	9	1.00
Present	32	14	18	
Histological grade				
Well	22	11	11	0.422
Moderately/Poorly	26	10	16	

^a χ^2 test. *P*-values in bold print indicate statistically significant differences.

levels were decreased by up-regulated miR-542, and enhanced by down-regulated miR-542 in HeLa and C33A cells (Fig. 2 D, E). Moreover, miR-542 was inversely correlated with BMI1 expression in StarBase database (Fig. 2F) and in 42 cervical cancer tissues (Fig. 2G).

miR-542 was down-regulated in cervical cancer

To detect whether miR-542 is involved in cervical cancer progression, its expression level was measured in cervical cancer tissues and cells. Downregulation of miR-542 was identified in cervical cancer tissues compared with the adjacent normal tissues through

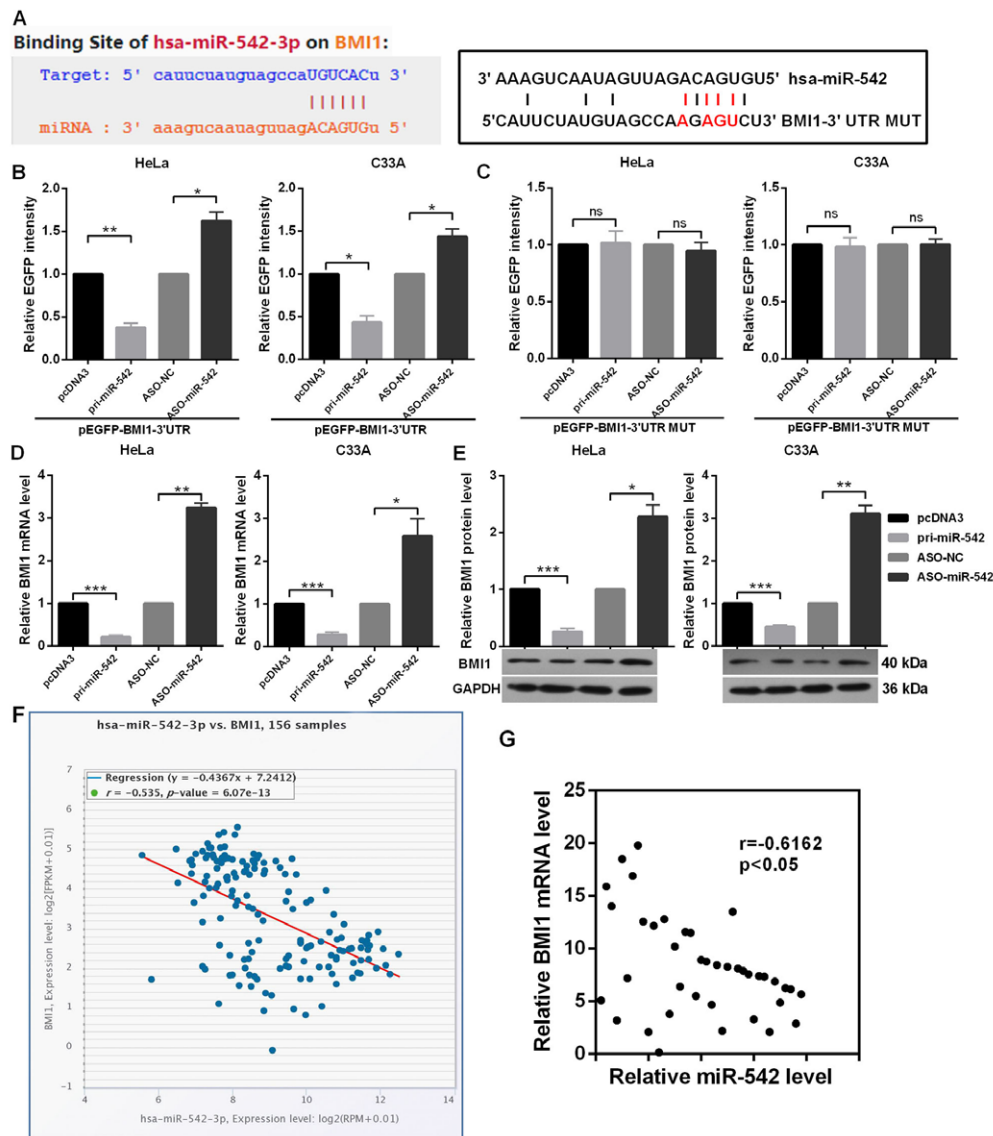


Fig. 2. miR-542 directly targeted BMI1. **A)** Predicted miR-542-3p binding sites in BMI1 mRNA WT and MUT shown through StarBase. **B, C)** Co-transfected with pri-miR-542 or ASO-miR-542 and the wild type or mutated 3'UTR of BMI1 regulated the EGFP intensity in C33A and HeLa cells. **D)** BMI1 mRNA levels with the indicated transfection were measured by RT-qPCR assay in C33A and HeLa cells. **E)** BMI1 protein expression levels transfected with pri-miR-542 or ASO-miR-542 and respective controls were determined by Western blot in C33A and HeLa cells. **F)** BMI1 mRNA level was negatively correlated to the miR-542 level in CC tissues using StarBase. **G)** BMI1 mRNA level was negatively correlated to the miR-542-3p level in CC tissues by RT-qPCR assay. Data are presented as means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant.

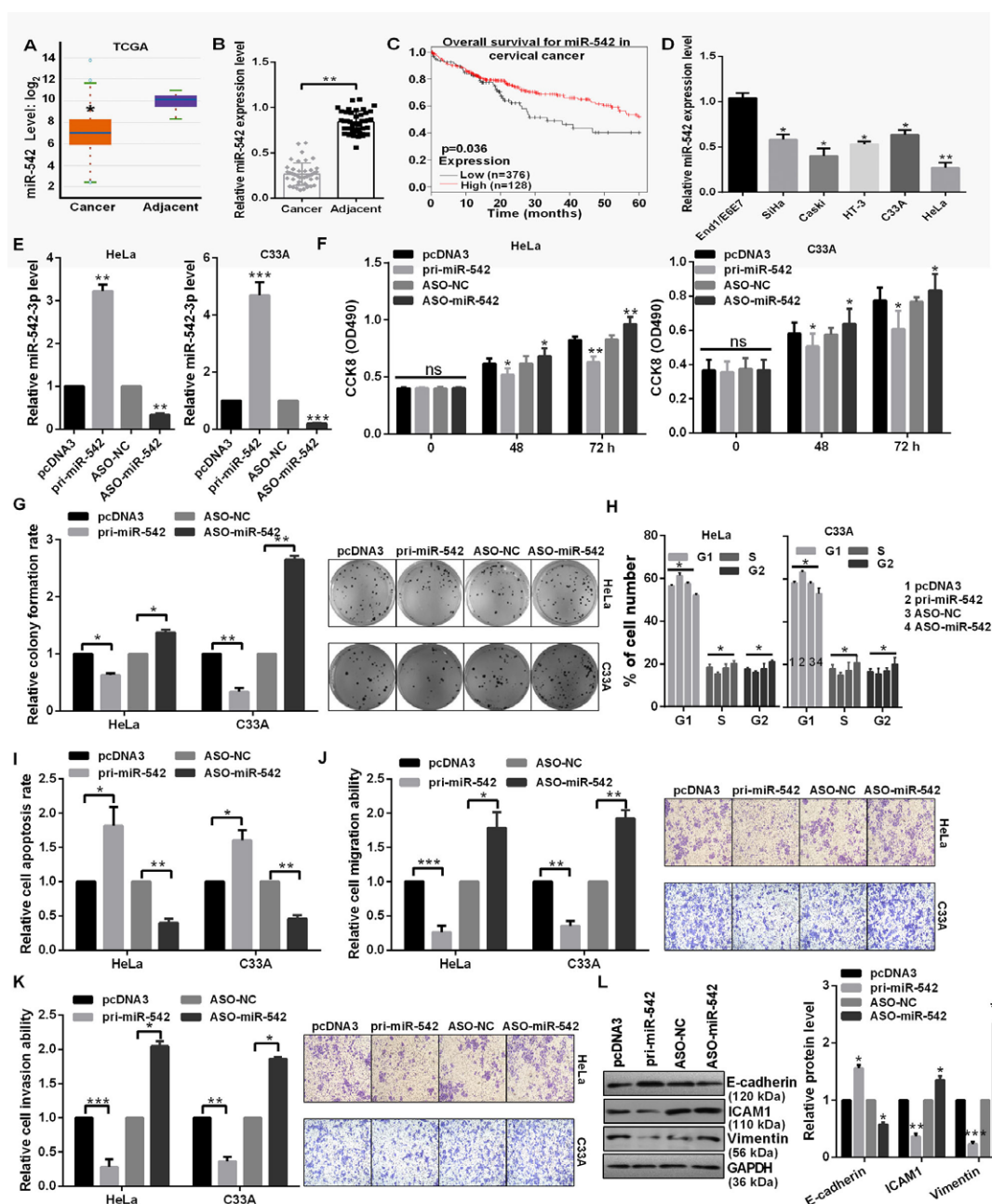


Fig. 3. The role of miR-542 in CC cells. **A)** TCGA database showed the expression level of miR-542 in CC. **B)** RT-qPCR showed the miR-542 level in CC and the adjacent normal tissues. **C)** Kaplan Meier plotter software showed the overall survival of CC patients with high or low expression of miR-542. **D)** RT-qPCR showed the level of miR-542 in CC cells and End1/E6E7 cell. **E)** The efficiency of pri-miR-542 or ASO-miR-542 was detected by RT-qPCR assay in HeLa and C33A cells. **F)** The role of altered miR-542 expression on cellular viabilities of C33A and HeLa were detected by CCK8 assay. **G)** Colony formation assay showed the proliferation ability transfected with pri-miR-542 or ASO-miR-542 in HeLa and C33A cells. **H)** Flow cytometric analysis showed that miR-542 overexpression in HeLa and C33A results in an increase of the G0/G1 phase and a decrease of the S and G2 phase. **I)** Flow cytometric assay showed miR-542 overexpression promoted cell apoptosis and ASO-miR-542 inhibited cell apoptosis in HeLa and C33A cells. **J, K)** Transwell assays showed that miR-542 overexpression inhibited invasion and migration ability in HeLa and C33A cells. **L)** Western blot assay showed the indicated protein expression levels treated with pri-miR-542 or ASO-miR-542 and the control groups in HeLa cells. Data are presented as means $\pm$ SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant.

the TCGA database and RT-qPCR assay (Fig. 3 A, B). Starbase software showed that the patients of cervical cancer with high miR-542 level had a preferable prognosis (Fig. 3C). Meanwhile, miR-542 expression was found to be lower in cervical cancer

cells (SiHa, Caski, HT-3, C33A and HeLa) than in human cervix epithelial End1/E6E7 cells (Fig. 3D).

miR-542 inhibited proliferation ability and EMT process in CC cells

The plasmids of miR-542 overexpression or knockdown were transfected into C33A and HeLa cells. pri-miR-542 transfection enhanced the miR-542 level and ASO-miR-542 treatment inhibited the miR-542 level in CC cells (Fig. 3E). CCK8 assay showed that pri-miR-542 restrained cell activity, while ASO-miR-542 accelerated cell activity in HeLa and C33A cells (Fig. 3F). Colony formation assay showed that pri-miR-542 exhibited an obvious decrease of cell growth and ASO-miR-542 treatment promoted cell growth in HeLa and C33A cells (Fig. 3G). Overexpression of miR-542 could induce the cell cycle arrest in G1 phase and ASO-miR-542 accelerates G1 to S and G2 transition in CC cells (Fig. 3H). In addition, transfected pri-miR-542 significantly increased the apoptotic rate in cells but was decreased in cells treated with ASO-miR-542 (Fig. 3I). Cell migration and invasion were found to be restrained by miR-542 overexpression and facilitated by miR-542 knockdown in CC cells by Transwell assay (Fig. 3 J, K). In addition, overexpression of miR-542 decreased the expression level of E-cadherin and increased the expression level of the molecular marker of mesenchymal cells (ICAM-1, Vimentin) using Western blot assay in HeLa cells (Fig. 3L). miR-542 knockdown in HeLa cells has the opposite effects. Taken together, these results indicate that miR-542 inhibited CC cell proliferation, migration and invasion *in vitro*.

miR-542 inhibited chemoresistance in vitro and tumor growth in vivo

The effect of miR-542 was assessed on the regulation of chemoresistance to Tamoxifen and Cisplatin. Cisplatin chemoresistance was decreased when treated with pri-miR-542 in HeLa and C33A cells compared to cells transduced with pcDNA3 in a dose-dependent manner among 0 to 4 ug/ml by CCK8 assay (Fig. 4A). CCK8 assay showed that Tamoxifen chemoresistance was significantly decreased in HeLa and C33A cells transduced with

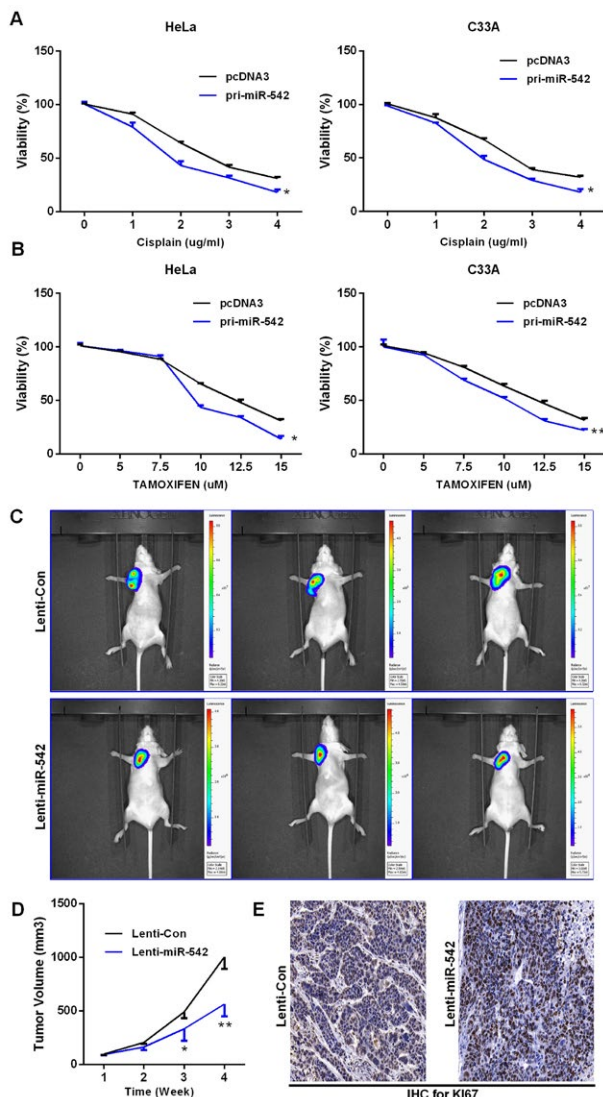


Fig. 4. miR-542 promoted drug-sensitivity and inhibited tumor growth. **A)** Transfected HeLa and C33A cells were treated with 0–4 µg/ml cisplatin in 96-well plates for 24 h. CCK8 assay was then used to investigate cell viability. **B)** Transfected HeLa and C33A cells were treated with 0–15 µM Tamoxifen in 96-well plates for 24 h. CCK8 assay was then used to investigate cell viability. **C–E)** Lenti-miR-542 inhibited tumor size and tumor growth rate following the down-regulation of Ki67 using IHC compared with the Lenti-Con group. Data are presented as means ± SD. * $P < 0.05$; ** $P < 0.01$; ns: not significant.

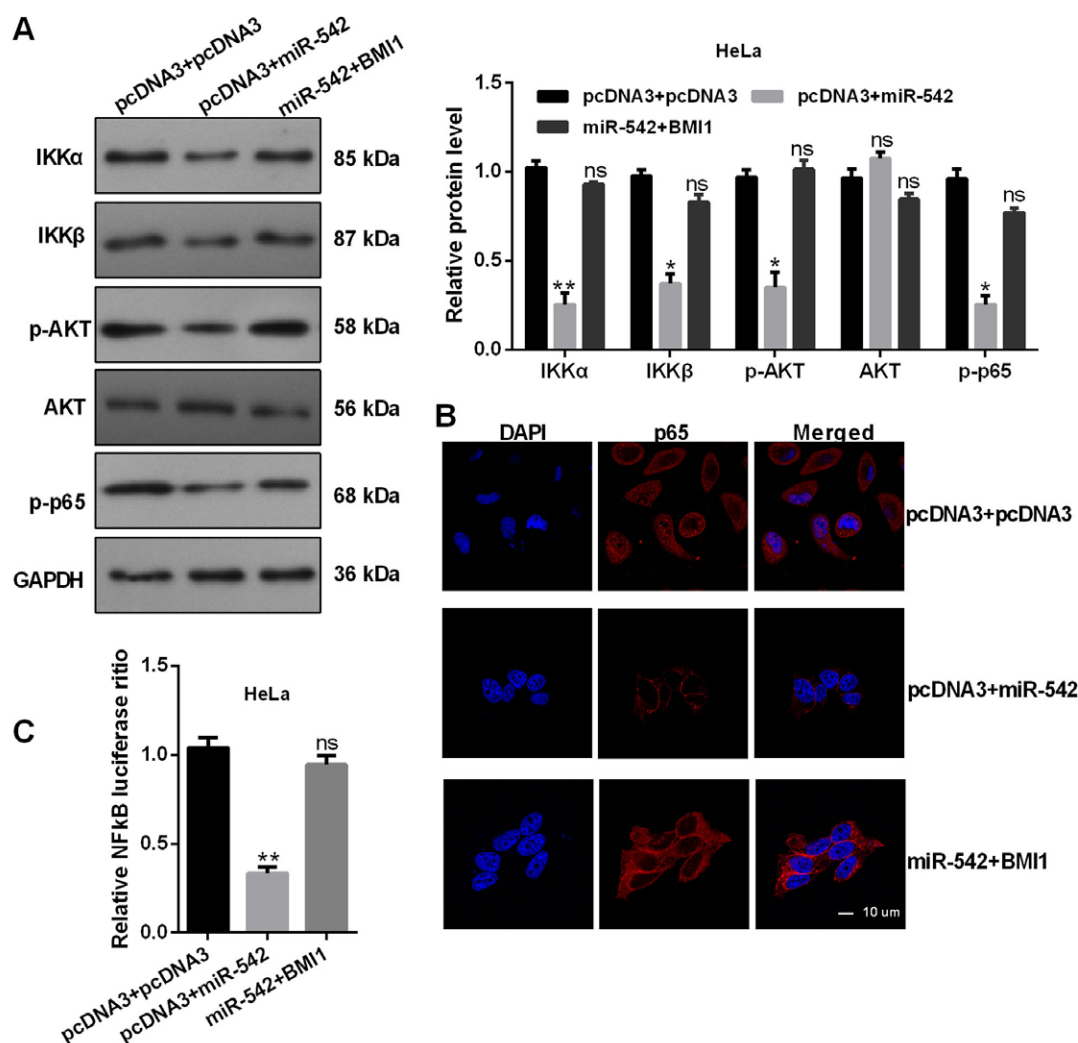


Fig. 5. miR-542 inhibited AKT/NFκB pathway through BMI1. **A)** Western blot showed the protein levels, including IKKα, IKKβ, pAKT, AKT, p-p65 and GAPDH, transfected with the indicated plasmids in HeLa cells. **B)** IF assay showed the distribution of p65 in HeLa cells transfected with the indicated plasmids. **C)** Luciferase reporter assays showed the NFκB pathway activity. Data are presented as means ± SD. * $P < 0.05$; ** $P < 0.01$; ns: not significant.

pri-miR-542 compared to cells transduced with pcDNA3 in a dose-dependent manner among 0 to 15 μM (Fig. 4B). To verify whether miR-542 regulates the tumor growth *in vivo*, stably transfected (Lenti-Con or Lenti-miR-542) HeLa cells were implanted subcutaneously into the right flank of mice. After 4 weeks, lenti-miR-542 treatment in HeLa cells markedly inhibited tumor growth and tumor volume (Fig. 4 C, D), following the down-regulation of KI67 expression by IHC in tumor tissues (Fig. 4E).

miR-542 regulates the AKT/NFκB pathway through BMI1

As shown in Fig. 5A, miR-542 overexpression markedly inhibited the phosphorylation of AKT and p65 and the expression of IKKα and IKKβ was also inhibited by transfected miR-542. BMI1 overexpression in HeLa cells transfecting pri-miR-542 blocked the miR-542-induced suppression of protein levels including p-AKT, IKKα, IKKβ, p-p50 and p-p65 (Fig. 5A). Additionally, IF assay

demonstrated the nuclear distribution of p65 transfected with miR-542 was reduced, compared with the pcDNA3 group. Furthermore, co-transfection with miR-542 and BMI1 rescued the suppressive role of miR-542 induced in HeLa cells (Fig. 5B). Finally, NF κ B luciferase reporter assays also identified the inactivation of NF κ B pathway by miR-542 overexpression in HeLa cells (Fig. 5C).

DISCUSSION

Emerging evidence has shown that miR-542 plays a crucial role in various biological processes (such as differentiation, cell proliferation, metastasis, transcriptional regulation) in many diseases, and differentially expressed miRNAs are closely related to many cancers (6, 7). In this study, we found down-regulated miR-542 may function as a suppressive gene in CC. As is known, miRNAs carry out their biological function via the regulation of their target genes (8). Our results revealed that BMI1 is the target gene of miR-542 by EGFP reporter assay. We also found that BMI1 was increased in CC tissues and cell lines, which was significantly positively associated with the serious TNM stage, large tumor size, distant metastasis and the poor prognosis in CC patients. Some papers have reported that BMI1 fulfilled important roles in many cancers through the regulation of various pathways. For example, You et al. reported that in gastric cancer, BMI1 targeted by miR-498 regulated the AKT pathway (9). In gastric cancer, BMI1 regulated stem cell-like properties through activated NF κ B and AKT pathway (10). Herein, we report that miR-542 inactivated the AKT/NF κ B pathway through the regulation of BMI1 in CC. In short, this study demonstrates that miR-542 is down-regulated in CC tissues and cell lines. The decreased miR-542 level is associated with advanced clinicopathological features of CC patients. In addition, miR-542 inhibited the proliferation, chemotherapy resistance and metastasis *in vitro* and tumor growth *in vivo* by directly targeting the BMI1-mediated AKT/NF κ B pathway. Thus, miR-542 function as a vital role in tumor growth and migration and invasion and may be a novel biomarker of prognosis and promising target of therapy for CC.

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LETTER TO THE EDITOR

Performance of prognostic nomogram in predicting long-term survival outcomes for osteosarcomaCY. Huang^{1*}, MY. Li^{2*}, W. Liu^{3*}, XX. Li¹, Y. Xu¹, JY. Li¹, QQ. Yao¹ and LM. Wang¹¹Department of Orthopaedic Surgery, Nanjing First Hospital, Nanjing Medical University, China;²Department of Cancer Recovery, Taikang Hospital, China; ³Department of Orthopaedic Surgery, Affiliated Jiangsu Province Hospital of Nanjing Medical University, China*Received February 21, 2020 – Accepted September 14, 2020*

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To the Editor,

With an incidence of 0.2-0.5%, osteosarcoma accounts for 35% of all the primary malignant bone cancers (1), and the 5-year survival rate of non-metastatic patients is reported to be above 60% (2). However, according to some studies, metastatic osteosarcoma still results in a much poorer prognosis (3). To improve osteosarcoma prognosis, it is of great importance to find significant prognostic factors. A previous case series reported that factors such as age, tumor size, gender and location affected overall survival (OS) and cancer-specific survival (CSS) (4-6). However, the criteria used for evaluation of surgical treatment vary among hospitals and even between surgeons, which may increase the heterogeneity of samples. Thus, it would be reasonable to establish a statistical prediction model that can integrate different individual prognostic factors to precisely predict osteosarcoma patient survival.

Recent research has confirmed that nomograms are a reliable tool to help clinicians make easy individual predictions of patient survival (7-9). Compared to the current limited American Joint Committee on Cancer (AJCC) staging system, we can estimate the individual survival rate more accurately by incorporating clinically essential

variables (10). The present study aimed to establish comprehensive prognostic nomograms based on the population data obtained from the SEER cancer registry program to better predict osteosarcoma patients' individualized survival. The ethics committee of Nanjing First Hospital (Nanjing, China) approved all related procedures.

MATERIALS AND METHODS*Data source and exclusion criteria*

The SEER program collects and publishes cancer survival data from cancer registries in the United States. Our study development cohort includes osteosarcoma patients diagnosed between 1999 and 2018 in the SEER program.

The exclusion criteria were patients with incomplete clinical characteristics, or other primary cancers, or patients with indefinite follow-up information. We collected the patients' clinical characteristics from the SEER database, including age, race, gender, surgery, tumor size, and primary site. SEER data were extracted using SEER*STAT (National Cancer Institute, Bethesda, MD, USA) software. The eligible patients were randomly divided into a training (n=3,623) cohort and a validation (n=3,227) cohort. CSS was calculated from the date of diagnosis to the date of death attributable to osteosarcoma. OS was defined as the

*Key words: cancer-specific survival; nomogram; osteosarcoma; overall survival; prognostic outcome**Corresponding Author:*

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interval between the date of diagnosis and death from any cause or the last date of follow-up.

Statistical analysis and nomogram construction

Categorical variables were compared by *Chi*-squared test, and continuous variables were evaluated by Student's *t*-test or Mann–Whitney U test. We used Kolmogorov–Smirnov to test continuous variable distributions and constructed Kaplan–Meier curves to estimate the CSS. We also used log-rank tests to assess statistical differences, and multivariate COX proportional hazards analyses to determine the CSS independent prognostic predictors. HR and the associated 95% CI were estimated. Subsequently, the significant clinical predictors were used to develop a nomogram for 3- and 5-year CSS in patients with osteosarcoma using R software packages.

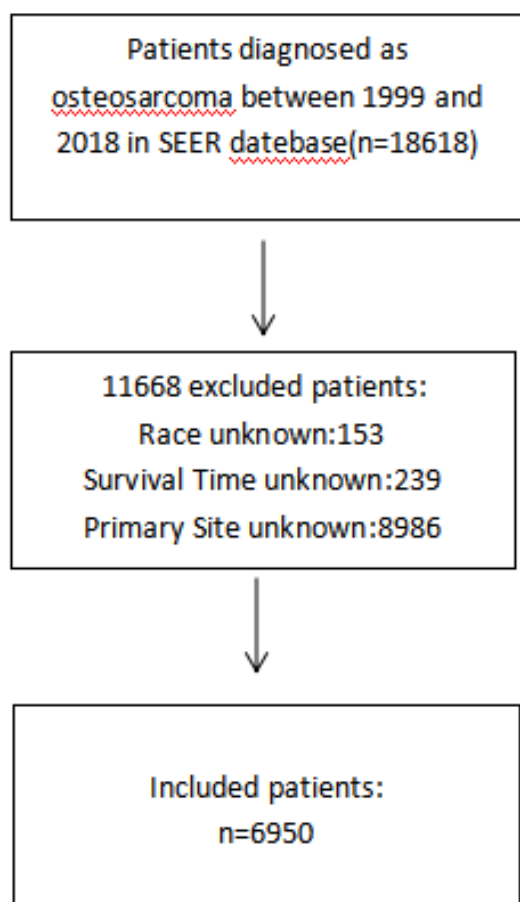


Fig. 1. Flowchart of the study cohort

Table I. Patients' clinical characteristics

Variables	SEER Cohort(n=3623)	Validation Cohort (n=3327)
Gender		
Male	1998	1934
Female	1499	1519
Age		
<19	1012	977
19--49	1137	1199
>49	1387	1238
Race		
Black	364	350
White	2857	2843
Other	288	248
Surgery		
Yes	2931	2789
No	597	633
Tumor Size		
<10	2475	2526
10--15	670	602
>15	367	310
First Indicator		
Yes	3066	3105
No	337	342
Primary Site		
Femur	1618	1782
Tibia	1297	1280
Other	496	477

Nomogram validation and performance evaluating

The validations were conducted both internally (training cohort) and externally (validation cohort) by discrimination and calibration and using bootstrap resampling (1,000 resamples). We used the C-index to evaluate the discrimination between survival probability and actual observations. The C-index value fluctuated between 0.5 and 1.0, with 0.5 representing random chance and 1.0 representing corrected discrimination. A calibration plot was constructed to determine whether the predicted survival and actual survival were in concordance.

Statistical analyses to identify risk factors were performed using SPSS 22.0 for Windows (SPSS, Inc., Chicago, IL, USA). A nomogram was formulated based on multivariate analysis results and by using the package of rms, cmprsk, survival and rmda packages in R version 3.6.2 (<http://www.r-project.org>).

RESULTS

Patient information

The present study identified 6,950 eligible osteosarcoma patients between 1999 and 2018, including 3,623 patients in the training cohort and 3,327 patients in the validation cohort. The flow

diagram of the study selection process is shown in Fig. 1. Table I lists the pathological characteristics and demographics of patients.

Factors associated with CSS

Data regarding age, race, gender, tumor size, primary site, surgery, and first indicator were collected in the training cohort. Other than gender and first indicator, these variables were included in the KM analysis as significant risk factors for poor survival (Fig.2). After receiving the data, the KM survival test and multivariate analysis with Cox regression were performed, and seven variables were identified as independent prognostic factors, including age, race, gender, primary site, surgery,

tumor size, and first indicator were then incorporated into the nomogram.

Nomogram construction

A nomogram that combined all the essential independent factors was constructed for predicting 3-, and 5-year CSS based on the reduced multivariate models of the training cohort (Fig. 3). According to the results, we found that race contributed most to prognosis, followed by the primary site, surgery, age, tumor size, gender, and first indicator. Each of the factors was given a score on the points scale. By summing and locating the scores, 3-, and 5-year survival of the patients with osteosarcoma could be predicted.

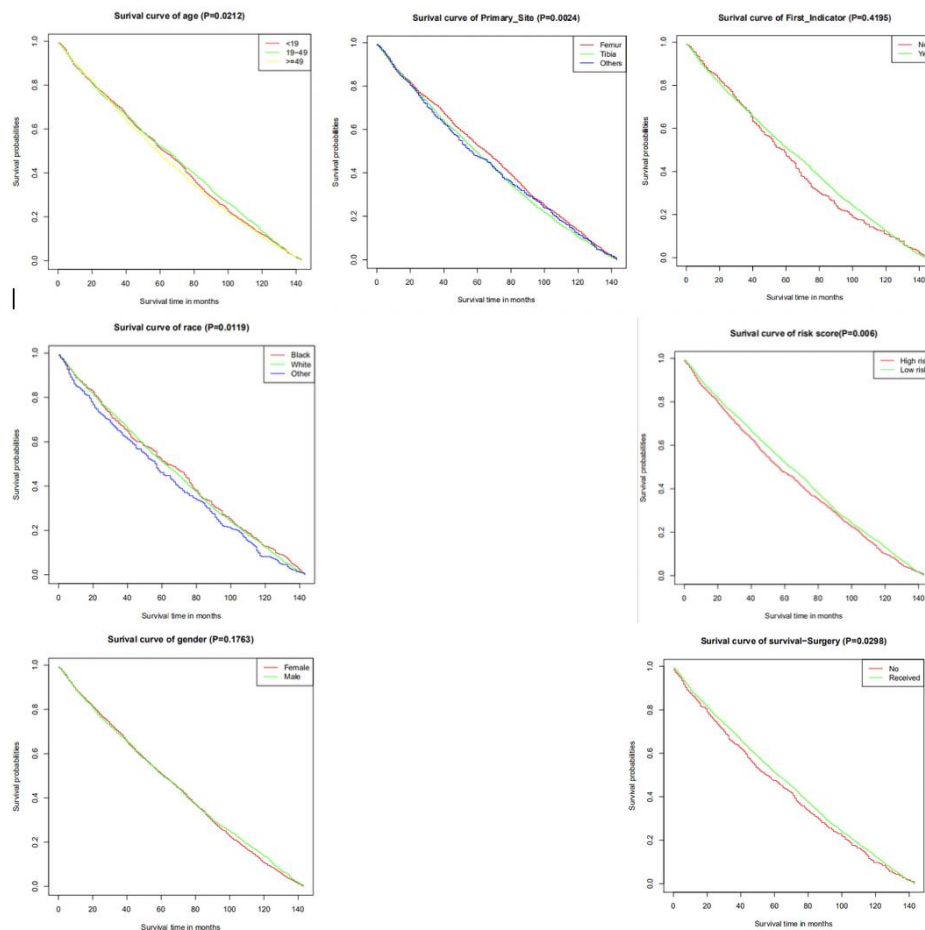


Fig. 2. Kaplan-Meier survival curves of the patients

Validation of the nomogram

All the independent prognostic predictors were integrated into the nomogram for predicting 3- and 5-year CSS. With C-indices of 0.751 (0.680–0.822) for CSS, the nomograms showed excellent accuracy when subjected to the internal validation. Both internal and external calibration plots for 3-year and 5-year CSS (Figs. 4 and 5) demonstrated fair agreement between actual probabilities and nomogram-predicted probabilities.

DISCUSSION

A nomogram is a statistical tool that can provide the most accurate predictions by a simple graphical presentation (11). It is easy to understand the nomogram, and it can be applied in clinical practice with high efficiency. Furthermore, the nomogram can help to find the individualized predictions,

so clinicians can use the model to assess patient participation in clinical trials.

Osteosarcoma is a malignant bone tumor, the incidence and mortality of which are steadily on the rise worldwide. Accurate and efficient information on prognosis is important for clinicians and patients to make individualized treatment decisions. Nomograms include simultaneous consideration of multiple factors, which provides an outcome for an individual patient (12).

To improve the nomogram accuracy, we used quite a large population-based cohort compared to previous osteosarcoma nomograms. Moreover, discrimination and calibration emphasized satisfaction in the presentation and validity of the model. We used seven easily-obtained variables, which reflect the normal status of the patients and their disease activities, thus providing clinically relevant information in osteosarcoma. However,

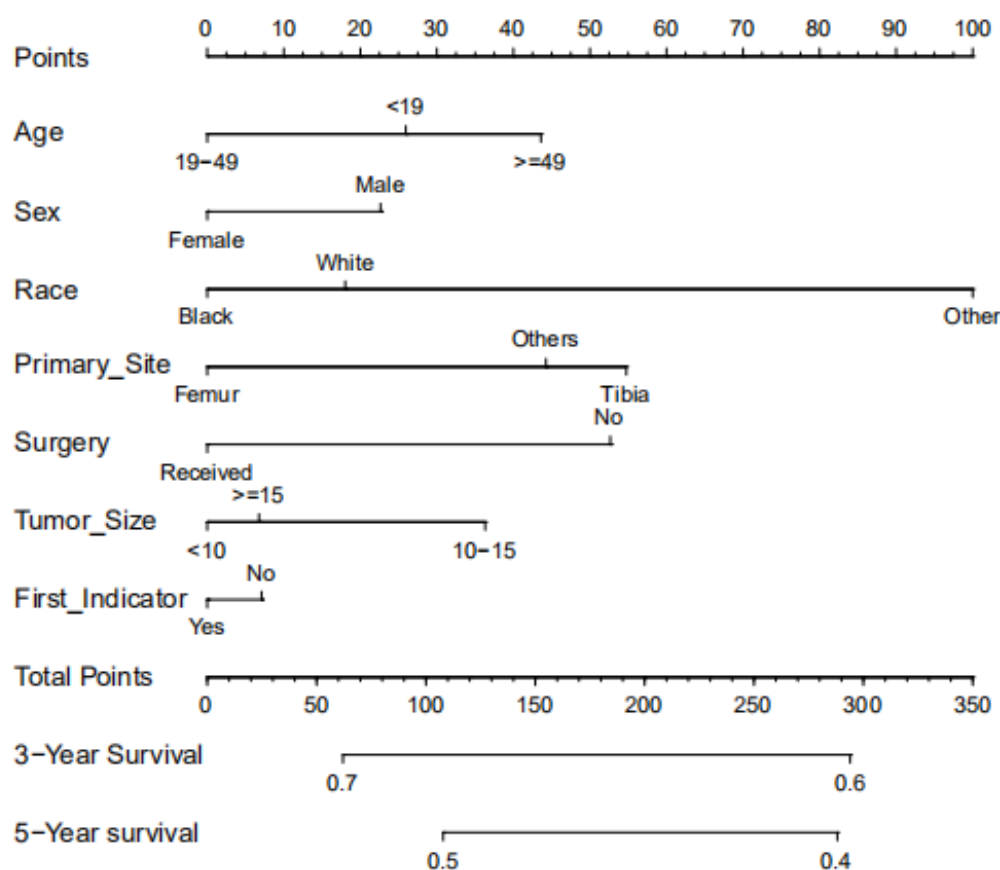


Fig. 3. Nomograms predicting 3-year and 5-year CSS in patients with osteosarcoma

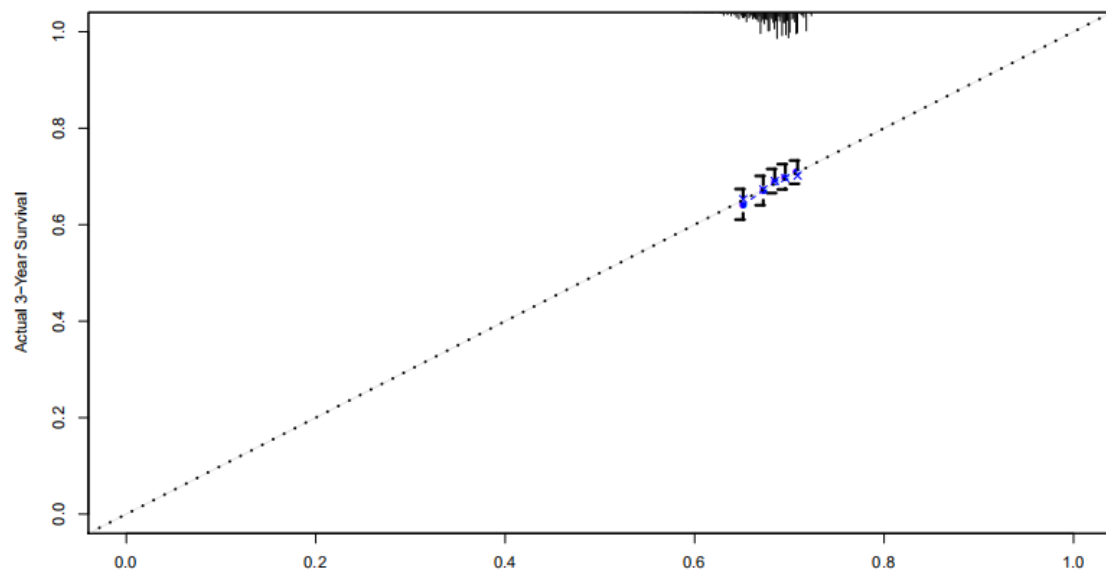


Fig. 4. Calibration curves of the nomogram predicting 3-year CSS.

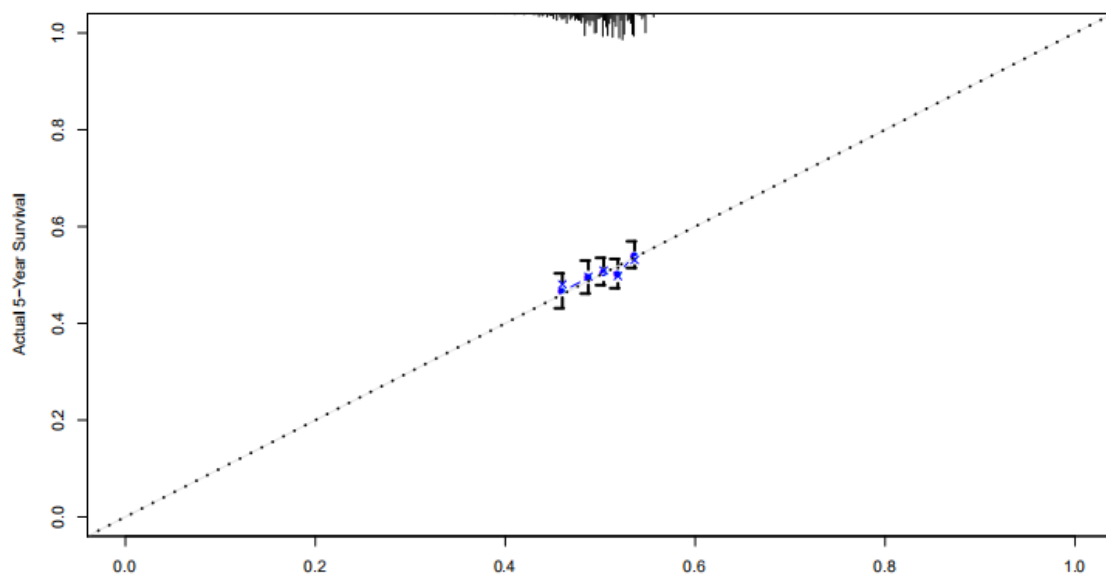


Fig. 5. Calibration curves of the nomogram predicting 5-year CSS.

the present study has some disadvantages. Firstly, some variables relevant to osteosarcoma prognosis were not studied because they were unavailable in the SEER database, such as morphologic pattern, vascular invasion, and serum tumor marker. In the future, we will include these variables in our research. Secondly, as with the other retrospective studies, both development and validation cohorts were subjected to selection bias. Thirdly, the RFS is not included in the SEER database. We need to do more additional studies with multi-institutional cohorts to validate the accuracy of the nomograms. However, despite the limitations, the present study pioneers the use of nomograms incorporating clinically feasible variables for predicting CSS in osteosarcoma patients, which were prominent during the patients' prognosis.

In medical decision-making progress, it is crucial to use the nomogram. Based on the SEER database, we developed and validated the nomogram of osteosarcoma prognosis with high accuracy. All of the established nomogram parameters show excellent performance, including the calibration plots, C-indexes, IDI, NRI, and DCA.

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LETTER TO THE EDITOR

Loteprednol combined with sodium hyaluronate in the treatment of dry eye disease and its effect on TNF- α and CXCL10 in tearsW. Chen^{1*}, X-L. Shi^{1*}, X-H He¹, Y-H. Mao¹, C. Li¹ and N. Dong^{1,2}¹Eye Institute & Affiliated Xiamen Eye Center, Xiamen University Medical College, Xiamen, Fujian, China; ²Department of Ophthalmology, Affiliated People's Hospital & Zhenjiang Kangfu Eye Hospital, Jiangsu University, Zhenjiang, Jiangsu, China*Received July 6, 2020 – Accepted September 15, 2020*

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To the Editor,

Dry eye syndrome, also known as keratoconjunctivitis sicca, refers to abnormal tear quality or kinetics due to various reasons. The inflammatory reaction occurs on the epithelial surface of the eye. The cornea, conjunctival epithelial cells, and goblet cells are stimulated by inflammatory factors and are damaged, eventually causing eye discomfort (1). There are many reasons for dry eyes: as age increases, the impact of life pressure and emotions will reduce the secretion of tears, and the deterioration of tear quality can be one cause (2); others are dry climate, increased environmental pollution and certain eye surface operations.

Increasing evidence shows that T cell-mediated inflammation, especially Th1 cells and Th17 cell-mediated immune inflammation, is the main pathway for the occurrence dry eye (3). When the ocular surface microenvironment is affected by external factors, ocular surface lymphocytes are infiltrated and activated, and the expression of inflammatory factors IL-1, TNF- α , IFN- γ increases (4). Chemokines and their receptors can refer to lymphocytes as specific sites of inflammation, and they play important roles in the inflammation process. In addition, increased

levels of CXCL9, 10, 11 have also been detected in the tears of patients with dry eye (5). Loteprednol is a glucocorticoid drug that not only reduces the toxicity of cortisol drugs, but also enhances the anti-inflammatory effect (6). Therefore, the efficacy of loteprednol combined with sodium hyaluronate in the treatment of dry eye was observed in this study.

MATERIALS AND METHODS*Study subjects*

From July 2018 to June 2019, 166 dry eye patients and people who received simple optometry with glasses in the ophthalmic outpatient department were involved in this study. For patients included in the study, the eye with severe dry eye symptoms was selected as the test eye. If the severity of dry eye was the same in both eyes, the right eye was selected as the test eye. For the control group, the right eye was selected as the test eye. The experimental protocol was reviewed and approved by the Ethics Committee of Xiamen University Medical College. All participants were informed of the purpose of this study and signed informed consents.

The 166 dry eye patients were randomly divided into sodium hyaluronate simple medication group (A group,

*Key words: loteprednol; sodium hyaluronate; dry eye; TNF- α ; CXCL10**Corresponding Author:*

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83 cases) and loteprednol plus sodium hyaluronate medication group (B group, 83 cases). Eighty-three people who received simple optometry with glasses in ophthalmic outpatient department were the normal control group (NC). Group A used 0.1% sodium hyaluronate eye drops, and group B used 0.1% loteprednol combined with 0.1% sodium hyaluronate eye drops for 1 month.

Dry eye symptom assessment (SPEED) questionnaire survey

The patients had symptoms of dryness, foreign body sensation, burning sensation and eye irritation. According to the frequency of symptoms, dry eye was divided into 4 grades (I: 0 score; II: 1 score; III: 2 scores; IV: 3 scores). The total score is calculated by adding the scores of the four symptoms. The maximum is 12 scores. A total score <10 points is the mild symptom group, and >10 points is the severe symptom group.

Tear breakup time

A fluorescein test paper was soaked in saline to make the fluorescein drop into the conjunctival sac. The patients blinked several times, and the time was calculated when the first dark spot appeared after the last blink, <10 s is abnormal.

Tear secretion test (Schirmer I test)

The tear test strip was placed in the conjunctival sac of the middle and outer $\frac{1}{3}$ of the patient's lower eyelid. Next, the patient looked down or closed his/her eyes gently. After about 5 min, the filter paper was taken out. The wet length was read and recorded.

Corneal fluorescein staining (FLS) score

The corneal fluorescein staining score is 12 points. The cornea is divided into 4 quadrants, each of which is 0 to 3 points. No staining is 0 points, 1 to 30 colored dots are 1 point, >30 colored dots are 2 points, and 3 points are corneal punctate fusion, and ulcers.

Tear collection

The tears were collected using the capillary tube method that was operated by the same person. Fifty μ l of sterile normal saline was put into the patient's conjunctival sac. The patient looked upward and 60 μ l of the tear saline mixture was collected in the conjunctival sac with a 25 ml capillary tube, without touching the ocular surface as

much as possible. The collected tears were put into EP tubes and stored at -80°C .

Expression of TNF- α and CXCL10 in tears

300 μ l washing solution was added to the microplate and left for 30 seconds. After discarding the washing solution, 50 μ l of detection buffer was added to each well. Next, 50 μ l of standard, sample and detection antibody was added. After blocking the membranes, the mixture was incubated at room temperature for 2.5 h. Then, 300 μ l of washing solution was added to each well to wash the plate 6 times. 100 μ l of horseradish peroxidase-labeled streptavidin was added to each well. Next, the mixture was incubated at room temperature for 45 min, then 100 μ l of the chromogenic substrate TMB was added to the wells and incubated in the dark at room temperature for 25 min. Finally, 100 μ l of stop solution was added to each well. OD value was measured at the 450 nm wavelength of the microplate reader. All experiments were repeated three times.

Statistical analysis

Statistical analysis was performed using Graphpad prism 6 and SPSS19.0 statistical software. The data of each test index in this study conforms to the normal distribution and is expressed as the mean $\pm$ standard deviation. The difference of each test index between groups was analyzed by independent sample *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Eighty-three normal controls (NC) and 166 dry eye patients participated in the study. Among them the dry eye patients, 83 received sodium hyaluronate alone (group A), and 83 received loteprednol combined with sodium hyaluronate (group B). As shown in Table I, the differences of age and gender between the three groups were not significant ($p > 0.05$, Table I).

This study observed the clinical data of tear breakup time (BUT), tear secretion function (Schirmer I test) and corneal fluorescence staining (FLS) before and after treatment in normal subjects and patients with dry eye. The dry eye symptom assessment (SPEED) questionnaire survey was carried out. The results showed that the SPEED score and FLS of dry eye patients (groups A and B) were significantly higher than those of

Table I. *Characteristics of the study population*

Group	Cases	Age (Years)	Gender (male/female)
Group A	83	51.07±11.83	15/68
Group B	83	47.37±10.98	11/72
NC Group	83	46.77±13.81	16/67
F		1.63	0.78
P		0.26	0.82

Table II. *Comparison of clinical data before and after treatment in normal persons and patients with dry eye syndrome.*

Group	Cases	SPEED score	BUT	Schirmer I	FLS
NC Group	83	4.51±1.83	8.36±2.13	12.03±3.66	0.18±
Group A before treatment	83	14.07±2.83 ^a	3.51±1.61 ^a	6.52±1.95 ^a	3.51±
Group A after treatment	83	11.07±2.13 ^b	4.33±1.83 ^b	7.24±2.11	2.88±
Group B before treatment	83	14.67±2.03 ^a	3.21±1.44 ^a	6.62±2.05 ^a	3.63±
Group B after treatment	83	10.27±1.73 ^{bc}	5.36±1.84 ^{bc}	8.72±1.65	2.36±

^a*P* < 0.01: compared with the normal group; ^b*P* < 0.05: compared with the same group before treatment; ^c*P* < 0.05: compared with group A after treatment

the control group (NC, *p* < 0.01, Table II). However, the BUT and Schirmer I test results of dry eye patients were significantly lower than those of the controls (*p* < 0.01, Table II). In addition, the SPEED scores, BUT and FLS scores of patients in groups A and B were improved compared with before treatment (*p* < 0.05, Table II). Although the Schirmer I test results after treatment in the two groups were improved compared with before, the difference was not statistically significant (*p* > 0.05, Table II). One month after the medication, the BUT, FLS and SPEED scores of the two groups (A and B) were compared. The results showed that the BUT, FLS and SPEED scores of group B improved more than group A (*p* < 0.05, Table II). Although the Schirmer I test results of the two groups were different, they were not statistically significant (*p* > 0.05, Table II). These results imply that loteprednol combined with sodium hyaluronate is an effective treatment for patients with dry eye.

Next, the expression of TNF- α and CXCL10 in the tears of normal controls and dry eye patients before and after treatment was detected. The results showed that the expression of TNF- α in groups A and B before treatment was higher than that in the control group (*p* < 0.05, Fig. 1 A, B). The expression of CXCL10 in group A and group B before treatment was higher than that in the control group, but the difference was not statistically significant (*p* > 0.05, Fig. 1 A, B). After treatment, the expression levels of TNF- α and CXCL10 in group A were lower than before treatment, but there was no significant difference (*p* > 0.05, Fig. 1 A, B). In addition, the expression of TNF- α in group B was lower than before treatment (*p* < 0.01, Fig. 1A). Although the expression of CXCL10 was lower than that of group B before treatment, there was no significant difference (*p* > 0.05, Fig. 1B). These results indicate that loteprednol combined with sodium hyaluronate can reduce the expression of TNF- α and CXCL10 in dry eye patients.

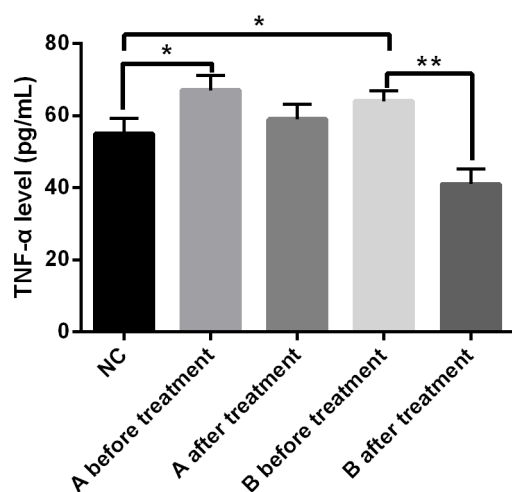
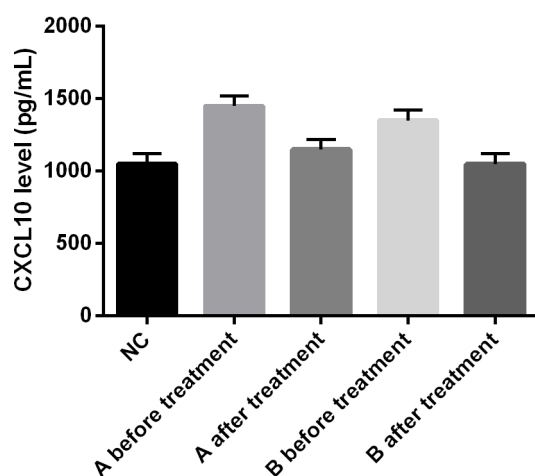
A**B**

Fig. 1. *A)* Expression of TNF- α was detected in dry eye patients' tears. *B)* Expression of CXCL10 was detected in dry eye patients' tears * $p < 0.05$, ** $p < 0.01$

DISCUSSION

Increased expression levels of inflammatory factors and chemokines have been detected in the ocular surface of patients with dry eye (7). Compared with normal controls, dry eye patients found high concentrations of the inflammatory cytokines IFN- γ and TNF- α (8). In addition, the chemokines CXCL-9, -10 and -11 levels have been found to be higher in tears (9). The study also found that the inflammatory

factor TNF- α level on the ocular surface of dry eye patients was higher than that of normal controls, and the difference was statistically significant. Compared with normal subjects, the expression of CXCL-10 in dry eye patients was also increased. It is further confirmed that inflammation and chemokines play important roles in the pathogenesis of dry eye.

In the present study, after treatment with sodium hyaluronate, the patient's tear film rupture time and corneal fluorescence staining were improved. As a new type of glucocorticoid drug, loteprednol can be rapidly metabolized into inactive products after being used in the eye, thereby reducing systemic toxicity (10). It has been reported that the metabolic site of loteprednol is in the human cornea, which has little effect on intraocular pressure and is suitable for the treatment of dry eye patients (11). This study found that the combined treatment of loteprednol and sodium hyaluronate can quickly and effectively improve the clinical symptoms of patients with dry eye, stabilize the tear film, and accelerate the recovery of corneal damage. The functional mechanism of loteprednol may be related to the inflammatory factor TNF- α and the chemokine CXCL10.

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LETTER TO THE EDITOR

Therapeutic effect and mechanism of icariin combined with calcium sensitive receptor on mouse gastric cancer cellsZG. Sun¹, ZF. Lang², YD. Mu³, J. Li⁴, CX. Xing⁵, L. Yan³ and S. Li²

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To the Editor,

Gastric cancer is one of the most common gastrointestinal tumors; there are more than 1 million newly diagnosed cases in the world each year, and the trend is increasing yearly. In the early stages of gastric cancer, surgical removal of cancerous tissue can effectively control its development, however, the disease has an insidious onset, rapid development, and a high mortality rate. For patients with advanced gastric cancer, there is no definite result of prolonging the survival time of patients through traditional radiotherapy and chemotherapy or emerging molecular targeted therapy or hypnotherapy.

An increasing number of experiments have proved that Chinese medicine has great advantages in inhibiting tumor growth, recurrence and metastasis, prolonging the survival of gastric cancer patients, regulating immunity, improving quality of life, and reducing the side effects and drug resistance of radiotherapy and chemotherapy. Icariin (ICA) is the main effective component of Epimedium. It has the effect of enhancing immunity and has few side effects. It can inhibit the proliferation of a variety of tumor cells, such as lung cancer, liver cancer,

leukemia, lymphoma, prostate cancer, cervical cancer, testicular stromal tumor, etc. (1-5).

In this study, we observed the growth of mouse forestomach carcinoma (MFC) cells under the intervention of ICA and explored the mechanism of ICA on gastric cancer cells. Icariin has a positive role in inhibiting the development of tumor cells of gastric cancer.

MATERIALS AND METHODS

Cell line and cell culture

MFC cells of gastric cancer were purchased from Beijing Vital River Laboratory Animal Technologies Co. Ltd. China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and incubated in a carbon dioxide incubator with 5% CO₂ at 37°C.

MTT assay

The cells in logarithmic growth phase were collected, the culture medium was eliminated, PBS (phosphate buffer saline) was added to clean the cells twice, trypsin was added to digest the cells for

Key words: icariin (ICA); calcium sensitive receptor (CaSR); murine gastric cancer; mouse forestomach carcinoma

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5 min, and then the cell suspension was centrifuged at 4000 r/min for 10 min to obtain cell precipitation. The cells were resuspended in serum-free medium, and then added to the counting plate. The dilution ratio was calculated according to the number of cells. The cell suspension was diluted to 3×10^4 cells/mL, 100 μ L well in a 96-well plate, and incubated in 5% CO₂ at 37°C for 24 h. 100 μ L RPMI-1640 culture medium containing different concentrations of ICA alone (25 mg/L, 50 mg/L, 100 mg/L, 200 mg/L, 400 mg/L), Calindol (10 μ mol/L), Calhex231 (10 μ mol/L), ICA (200 mg/L) combined with Calindol (10 μ mol/L), ICA (200 mg/L) combined with Calhex231 (10 μ mol/L) were added, respectively. Three parallel samples and blank control were set up in each group. After continuous culture for 24 h, 48 h and 72 h, respectively, the medium was absorbed and discarded. 100 μ L RPMI-1640 culture medium containing 5 mg/mL Methyl Thiazolyl Tetrazolium (MTT) was added into each well. After continuous culture for 4 h, the liquid was discarded. Then 100 μ L dimethyl sulfoxide (DMSO) was added into each pore, and shaken gently until completely dissolved. The absorbance at 570 nm was measured by enzyme-linked immunosorbent assay.

$$\text{Tumor cell inhibition rate (\%)} = \frac{1 - \text{experimental group}}{\text{control group}} \times 100\%$$

Hoechst staining

The MFC cells in logarithmic growth period were collected, and the cell concentration was adjusted to 1×10^5 cells/mL. The cells were inoculated with 2 mL/well in a 6-well plate with a cover glass, and cultured in a 37°C, 5% CO₂ incubator for 24 h. The medium in the plate was discarded, and ICA (200 mg/L) alone or ICA (200 mg/L) combined with Calindol (10 μ mol/L) was added, and the control group was set up. The cells of each group were cultured at 37°C and 5% CO₂ for 48 h. The culture medium was removed, and 0.5 mL fixed solution (4% paraformaldehyde) was added. The reaction lasted for 20 min, followed by PBS washing for 3 times. 1 mL Hoechst 3342 was added to each well for 15–30 min. After the reaction, the dye was discarded and washed with PBS for 3 times. The cells were observed and photographed under an inverted fluorescence microscope.

Tumorigenesis in mice

A mouse gastric cancer model was established. The collected MFC cells were adjusted to a cell suspension with a cell concentration of 5×10^7 cells/mL, and 200 μ L cell suspension was inoculated into the subcutaneous tissue of the right hind limb of the mice. One week later, the tumor grew to 0.5 cm³, which was a successful model of gastric cancer transplanted mice. After the successful molding, the four groups of mice were injected with 200 μ L normal saline, ICA (200 mg/L), Calindol (10 μ mol/L), ICA (200 mg/L) combined with Calindol (10 μ mol/L), respectively, along different sites around the tumor. The transplanted tumor was dissected and the cells and tissues were isolated for subsequent Western blot and RT-qPCR.

Western blot

The cells or tissues were collected, rinsed with PBS, added with corresponding volume of lysate, and left on ice for 5 min. The supernatant was obtained by centrifugation at 12000 rpm for 10 min. The protein concentration was determined by BCA assay. The protein samples were diluted with 5 $\times$ loading buffer and PBS, boiled in a water bath for 5 min and denatured. The protein liquid after denaturation was added to 12% SDS-PAGE gel and 80V constant pressure electrophoresis for 1 h. The SDS-PAGE glue was placed on the nitrocellulose membrane to carry out constant current transfer. 3% BSA (bovine serum albumin) was used for 4-degree closure. After closure, the nitrocellulose membrane was washed with PBS and CaSR monoclonal antibody, P16 monoclonal antibody and Caspase3 monoclonal antibody was added at 1:500 concentration, respectively. The membrane was incubated at room temperature for 45 min. Then the nitrocellulose membrane was washed with PBS buffer, and the incubation of the second antibody was started. The second antibody labeled with horseradish peroxidase was added at 1:1000 and incubated at room temperature for 30 min. Then, the nitrocellulose membrane was washed with PBS buffer and imaged by electrochemiluminescence analyzer Kodak X was used to expose the nitrocellulose membrane for about 3 min. The X-ray was scanned, and the

gray analysis of the strip was processed by Gel-Pro Analyzer software. The gray value of each band was measured by software in three independent repeated experiments, and the ratio of gray value of the target gene product to that of β -actin product was used as the relative expression of protein.

RT-qPCR

Total RNA was extracted from the samples using the total RNA extraction kit. The RNA was reverse-transcribed into cDNA using a reverse transcription kit. The reaction system was as follows: 1 μ L cDNA, 10 μ L mastermix, 0.5 μ L upstream and 0.5 μ L downstream primer (Table I) and 8 μ L ddH₂O were mixed. Each sample was provided with 3 multiple holes, and U6 was set as the internal parameter of miRNA. The reaction conditions were as follows: 95°C, pre-denaturation for 10 min; 95°C 10 s, 60°C 20 s, 72°C 30 s, the above operations were amplified by 40 cycles. The data obtained by quantitative PCR were compared with CT ($\Delta\Delta$ CT), and the average value of three repeated experiments was taken as the result.

Statistical analysis

SPSS version 17.0 (SPSS statistics, Chicago, Illinois, USA) was used for data analysis. The average value and standard deviation were used for

measurement data, while frequency and percentage were used for classification data. *T*-test was used to compare the two groups. Single factor analysis of variance was used to compare multiple groups. *Q*-test was used to compare the two groups. $P < 0.05$ was considered as statistically significant.

RESULTS

MTT result for cell proliferation

After the MFC cells were treated with different concentrations of ICA, the growth inhibition rate increased gradually with the increase of time and drug concentrations. When the concentration of ICA was 400 mg / L, the inhibition rate was 74.15% for 72 h (Fig. 1A). After ICA (200 mg/L) combined with Calindol (10 μ mol/L) acted on MFC cells, the cell growth inhibition rate of the combined drug group was significantly higher than that of ICA and Calindol alone, and with the extension of time, the inhibition rate was higher, in a time-dependent manner (Fig. 1B).

Hoechst staining result for apoptosis

Under the fluorescence microscope, compared with the negative control group, the nucleus of the apoptotic cells was significantly smaller after the treatment of ICA (200 mg/L) combined with Calindol (10 μ mol/L) on MFC cells (Fig. 2).

Table I. *Primer sequence information*

Name	Primer sequence (5'-3')	
CaSR	Upstream primers	TGACGAGCCTCAGAAGAATGC
	Downstream primers	CCTCCACCACTAATGACGAAGC
P16	Upstream primers	ACCAGAGGCAGTAACCATGC
	Downstream primers	CCTGTAGGACCTTCGGTGAC
Caspase3	Upstream primers	ATGGAGAACAATAAAACCT
	Downstream primers	CTAGTGATAAAAGTAGAGTTC
β -actin	Upstream primers	CTTAGTTGCGTTACACCCTTTCTTG
	Downstream primers	CTGTCACCTTCACCGTTCCAGTTT

Detection of CaSR, caspase 3, p16 protein and mRNA expression

In cell and tissues of tumorigenesis, the expression of CaSR and P16 protein was up regulated by ICA. For the ICA combined with Calindol group, the content of CaSR protein in cells was significantly

higher than that of ICA ($P < 0.01$), the content of Caspase3 protein in the ICA combined with Calindol group was significantly lower than that of normal saline (Negative Control) and ICA group ($P < 0.01$). The results of qPCR and Western blot indicated that ICA and Calindol could promote the up-regulation

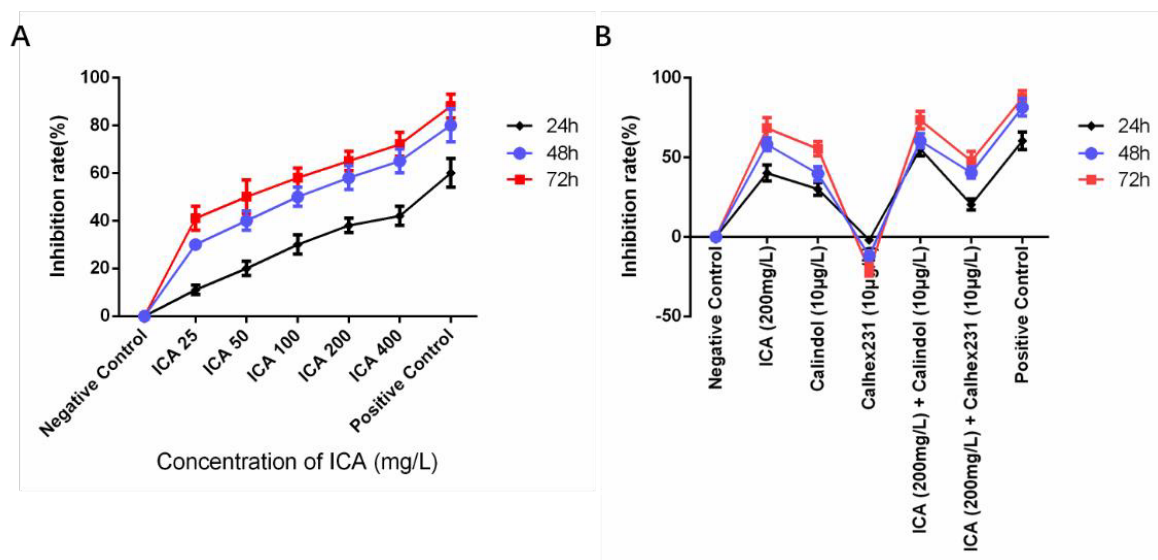


Fig. 1. MTT result of the proliferation of MFC cells. **A)** Effect of ICA on the proliferation of MFC cells; **B)** Effect of ICA combined with Calindol or Calhex231 on the proliferation of MFC cells.

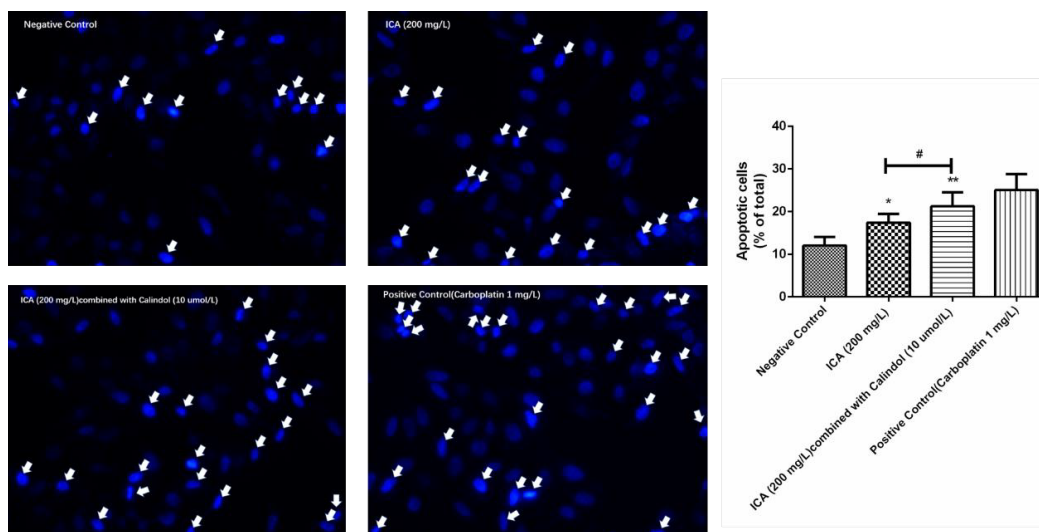


Fig. 2. Effect of ICA combined with Calindol on apoptosis of MFC cells. **A)** Negative control; **B)** ICA (200 mg/L); **C)** ICA (200 mg/L) combined with Calindol (10 µmol/L); **D)** Positive control (Carboplatin 1 mg/L). *Comparison between Negative Control and ICA ($P < 0.05$), ** Comparison between Negative Control and ICA + Calindol ($P < 0.01$), # Comparison between ICA and ICA + Calindol ($P < 0.05$).

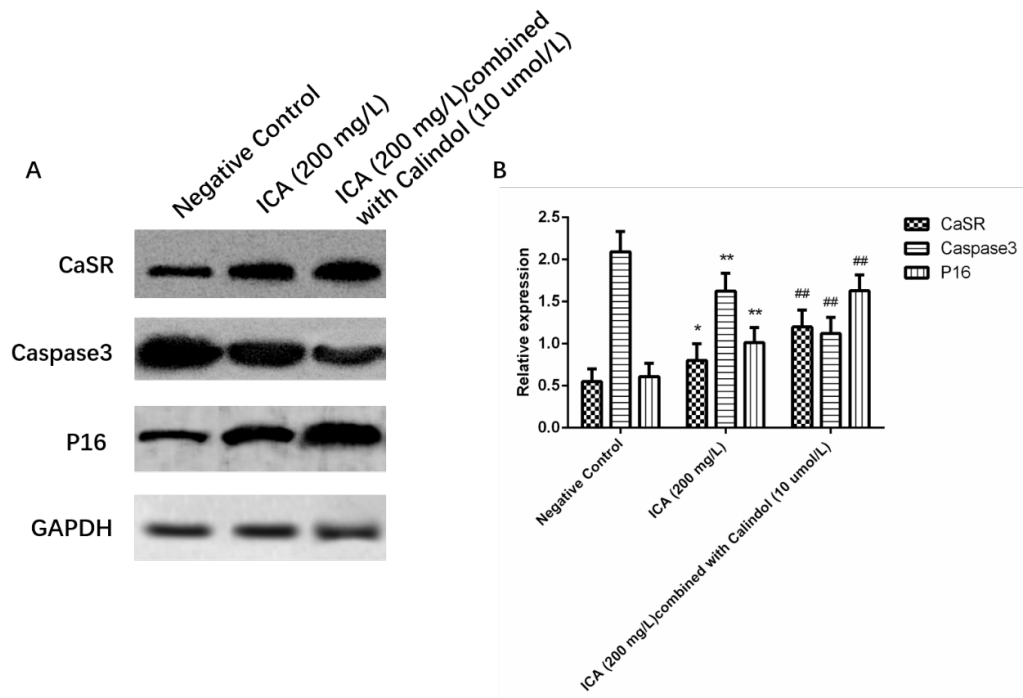


Fig. 3. Detection of CaSR, Caspase 3 and P16 protein and mRNA expression. **A)** Protein expression of CaSR, Caspase3 and P16 in Negative Control, ICA and ICA + Calindol group; **B)** mRNA expression of CaSR, Caspase3 and P16 in Negative Control, ICA and ICA + Calindol group. * Comparison between Negative Control and ICA ($P < 0.05$), ** Comparison between Negative Control and ICA ($P < 0.01$), # Comparison between ICA and ICA + Calindol ($P < 0.05$), ## Comparison between ICA and ICA + Calindol ($P < 0.05$).

of CaSR, P16 protein and mRNA expression in MFC cells, and the downregulation of Caspase3 protein and mRNA expression (Fig. 3).

DISCUSSION

Gastric cancer is one of the most aggressive malignant tumors of the digestive tract with high incidence rate and mortality (7). Because of its non-specific symptoms and highly invasive characteristics (8), it is urgent to develop more effective treatment for gastric cancer. It has been proved that ICA can inhibit proliferation and induce apoptosis of NB4 leukemia cell line. In the present study, MTT was used to confirm the inhibitory effect of ICA on MFC cell line proliferation. We showed that ICA can effectively inhibit the proliferation of

MFC cells in a time- and concentration-dependent manner, and this inhibition can be enhanced by the combination of ICA and Calindol.

Apoptosis is a physiological process of cell death, which is beneficial to the growth and maintenance of normal cells and tissues. The imbalance of apoptosis pathway will lead to the occurrence of malignant tumors and other diseases (9, 10). Therefore, Caspase3, which directly regulates apoptosis, plays an increasingly important role in the occurrence and development of tumors (11). P16 plays a key role in apoptosis, angiogenesis, epithelial mesenchymal transition, cell migration and invasion (12). However, the effects of ICA on the proliferation inhibition, apoptosis induction and caspase 3, p16 gene expression of gastric cancer cells have not been reported.

Substantial research has proved that ICA can also improve the cardiovascular and cerebrovascular system, anti-virus, anti-inflammatory, hypolipidemic, anti-tumor and other broad pharmacological effects. However, the development and research on the medicinal and health value of ICA is still in its infancy.

In conclusion, this study provides an experimental basis for the clinical application of ICA in the treatment of gastric cancer, as well as for the development of effective therapies, and has the advantage of effective components of traditional Chinese medicine in anti-tumor treatment.

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LETTER TO THE EDITOR

Acne scars treatment: A new topical successful proposal

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To the Editor,

The acne pathogenesis includes different aspects including propionibacterium acne, increasing sebum secretion, abnormal keratinization, inflammation and release of cytokines around the pilosebaceous glands. Acne scars (AS) usually occur in more than 85% of patients with previous acne history (1). Several treatments for AS have been proposed with different results (1-3). Peeling, fillers, cryotherapy, autologous fat implants, CO2 Laser, Erbium laser, microdermoabrasion and dermoabrasion, among others, have been used for treating atrophic AS (4, 5). Compressive medication and steroidal infiltration are mainly used for hypertrophic and keloidal AS. All the above-mentioned treatments give suboptimal results (1-3, 6, 7). In this study, the efficacy of topical treatment with a new cream based on Butyl 4 N Resorcinol + Metilsulfonilmetan was evaluated. This cross-sectional study was conducted on 100 patients aged 18-32 years. Improvement of scars was evaluated according to Goodman and Baron (8, 9). Written consent was obtained from all the patients and the study was conducted in accordance with the declaration of Helsinki.

Each and every previous treatment was stopped

60 days prior to the study. Fifty patients were randomly treated 2 times per day for 60 days with cream with active ingredients and 50 patients with cream containing only the carriers. Cream 1 included Butyl 4 N Resorcinol + Metilsulfonilmetan and cream 2 (placebo cream) consisted of vehicle of cream 1 without active ingredients. One hundred subjects with acne scars type 3 and type 4 were evaluated before and after 60 days of twice daily treatment with cream 1 and twice daily treatment with placebo cream 2. Evaluation of scars was made at time zero and after 60 days of treatment. SPSS version 24 was used as statistical analysis program. $P < 0.05$ was regarded as significant. Fifty-two females and 48 males were studied; 32% of the cases had boxcar and rolling scars. In 9% of the patients, ice-pick scar was observed. The overall severity scar scale included Goodman 3 in 39.7% of the patients and Goodman 4 in 60.3% of the study population. At the 2-month follow-up, grade 3 (Goodman 3) scars decreased from 81.1% at time zero to 50.7% after 60 days. Grade 4 scars decreased from 60.7% to 41.3%. The colour of the scars greatly improved (81%) and acne-related erythema improved by 31% compared with pre-treatment appearance (Fig. 1).

Key words: acne; acne scars; topical treatment

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Fig. 1. *a) After treatment with cream Butyl 4 N Resorcinol + Metilsulfonilmetan. b) Before treatment with cream Butyl 4 N Resorcinol + Metilsulfonilmetan*

The control group treated with cream 2 (placebo cream) did not show any improvement in any of the above-mentioned parameters. No side effects were reported in any cases, either in group 1 or group 2. In our study we have shown that the average Goodman and Baron's global scores was significantly lower with the treatment with active cream 1 (Butyl 4 N Resorcinol + Metilsulfonilmetan) compared to the treatment with placebo cream. Inflammatory phenomena associated with acne and acne scars showed satisfactory results with high patient satisfaction. Nor were any side effects noted in the study. Our study showed that treatment of acne scars of the face with Butyl 4 N Resorcinol + Metilsulfonilmetan cream was effective. Butyl 4 N Resorcinol + Metilsulfonilmetan cream can be considered as a safe, well-tolerated and effective approach for the treatment of acne scars.

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LETTER TO THE EDITOR

Transplantation of bone marrow stromal cells for ischemic stroke in ratsHM. Wang¹ and ML. Yan²

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To the Editor,

Ischemic stroke (IS) is a disorder with increased incidence nowadays, and is one of the main causes of major disability, especially in the elderly (1). Currently, the clinical methods for treating IS include early thrombolysis, protection of brain cells, and anti-platelet aggregation (2). Bone marrow stromal cells (BMSCs) are cells that can be differentiated into neural cells (3), making them good candidates for the treatment of IS. The relationship between inflammatory immune response and IS has noticeably attracted researchers' attention. After IS, abundant white blood cells flood into ischemic brain tissues, thereby activating the body inflammatory response. Stem cell transplantation can promote nerve regeneration and functional reconstruction after brain injury (4). Stem cells can also secrete related neurotrophic factors to promote the regeneration of the axonal nerve and myelin sheath at the injured site (5). Their ability to neutralize toxic metabolites and promoting the survival of damaged brain cells may determine the neuroprotective role of transplanted stem cells (6). Toxic metabolites comprise free radicals, neuroexcitatory toxins, inflammatory factors, and lipid peroxidases produced after ischemia. Among the inflammatory factors, interleukin (IL)-10 and transforming growth factor-beta 1 (TGF- β 1) influence

various immune cells (7). However, to date, these two factors have scarcely been studied in the field of stem cell transplantation. In this study, we started from the hypothesis that BMSCs can repair damaged neural function by influencing the secretion of anti-inflammatory factors and investigated these findings on a rat model of middle cerebral artery occlusion (MCAO) and reperfusion.

MATERIALS AND METHODS*Animals*

Forty-five male Sprague-Dawley (SD) rats (body weight, 260-300 g; reproductive age, 8 weeks) were used. The rats were randomly divided into 3 groups, 15 animals per group. Another 5 young SD rats (reproductive age, 4-5-weeks; and body weight, 90-100 g) were used for cultivation of BMSCs. The animals were kept in a ventilated and direct sunlight-free room with control environmental conditions. Food and water were provided once a day. The animal experiment was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University.

BMSCs were cultured from bone marrow cells of the five young male SD rats. The 45 8-week-old male SD rats were randomly divided into three groups: group A, group B, and group C; n=15 in each group).

Key words: bone marrow stromal cells; cell transplantation; ischemic stroke; brain injury; neural function

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Group A was the normal control group that received no treatment during the study period. Group B was the MCAO model control group that received normal saline after successful modeling in the same quantity and same conditions as BMSCs. Group C was the treatment group that received BMSC transplantation 24 h after successful MCAO modeling. The neurobehavioral scores and the spatial learning ability of the animals in each group were evaluated 1 week after BMSC transplantation. Then, the rats were sacrificed by decapitation after anaesthesia with 10% chloral hydrate (0.35 mL/100g) (Xi'an Tianzheng Kangyuan Biotechnology Co., Ltd., China), and blood and brain were collected to evaluate the volume of cerebral infarction in each group, and the levels of inflammatory factors (IL-10 and TGF- β 1) in the serum and brain tissues.

Isolation, cultivation, passage, and identification of BMSCs

The 5 young male SD rats were sacrificed by decapitation after anaesthesia with 10% chloral hydrate (0.35 mL/100 g). The bone marrow of femur and tibia were collected and prepared into a single-cell suspension. The samples were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 12% fetal bovine serum (FBS) (Hangzhou Lianke MeiXun Biomedical Technology Co., Ltd., China). The cells were passed when 80% of the flask was full [digested with trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25%)] (Youkang Hengye Biotechnology Co., Ltd., China). Alizarin Red staining and Oil Red O staining (Harbin zhongkesiens Biotechnology Co., Ltd., China) was used for osteogenic differentiation and adipogenic differentiation and to purify third-generation BMSCs. Expression levels of BMSC surface-specific markers (CD73 and CD90) and hematopoietic cell surface marker CD34 were detected by flow cytometry (BD, USA) to characterize the BMSC cells.

Count of transplanted cells

The third generation BMSCs were selected as the transplanted cells. The cells were digested with trypsin and centrifuged, and then suspended in physiological saline solution. Finally, the concentration of the cell solution was adjusted to 5×10^6 BMSCs in 1 mL culture medium.

Establishing a rat model of MCAO

According to the Zea Longa's suture-occluded method (8), a rat model of MCAO was established. During the

operation, an electric blanket was used to keep the anal temperature of the rats at the range of 37-37.5°C. Postoperative care was supplemented with soft materials and artificial feeding as appropriate.

Transplantation of BMSCs in the brain

The rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate (3.5 mL/kg), and were fixed on the animal stereotaxic frame in a prone position 24 h after right MCAO. The hair was removed from the top of the head. After routine skin disinfection, the skin was cut from the middle sagittal position of the top, with an incision length of about 1 cm. The periosteum was removed and the dura mater was exposed by drilling the right side of the skull with a dental drill with a diameter of 1 mm (2 mm apart from the right side of the midline after the coronal suture). The 25-gauge Hamilton microinjector with a capacity of 10 μ L was fixed on the stereotaxic frame, and BMSCs were slowly injected into the right striatum and cerebral cortex with a flow below 1 μ L/min with the bregma as the reference point. This location was roughly the location of the ischemic marginal zone. Each rat received 10 μ L of transplanted cells (5×10^3 cells/ μ L), including 7 μ L in the striatum and 3 μ L in the cerebral cortex. After injection, the microinjector remained *in situ* for 5 min and then withdrawn. The pinhole and bone hole were sealed with medical bio-protein glue to prevent overflow of transplanted cells. After injection, the incision was sutured layer-by-layer.

Modified neurological severity scores (mNSS)

The mNSS is an internationally recognized criterion for the evaluation of neurological dysfunction after ischemic stroke in rats. The highest score is 18 points, 1-6 points represent mild damage, 7-12 points indicate moderate damage, and 13-18 points denote severe damage. The neurological function of the rats was evaluated 24 h after MCAO, and the rat model with mNSS ranging from 7 to 12 was included in the experiment. mNSS were also assessed one week after treatment to evaluate the recovery of nerve function in each group.

Morris water maze test

Three weeks after the treatment, Morris water maze test (MWM) was used to evaluate the spatial learning

and memory ability of rats in each group. The program required animals to locate underwater platforms according to spatial cues in the environment. Moreover, during the experiment, the environment was kept quiet and the light was consistent, and the operation was gently carried out to avoid stimulation of unnecessary stress. The operation procedure is explained below.

The water depth was about 20 cm, the platform was hidden underwater, and the water temperature was 22–24°C. First, the positioning and sailing experiments were undertaken on days 1–5 to assess the learning ability of rats. The water maze was divided into four quadrants. Rats were randomly placed into the water from any of these quadrants with their faces facing the wall of the pool. The time required for rats to explore the hidden platform was recorded as the incubation period. The total duration of exploration was 80 s. If the rats could not explore the platform within the specified time, they were guided to swim onto the platform and stayed there for 10 s, and the recorded incubation period was 80 s. After that, the space exploration experiment on day 6 aimed to investigate the memory ability of rats: on the 6th day of the experiment, the underwater platform was removed. The diagonal quadrant of the platform was selected as the water entry point of the rats, and the number of times that the rats crossed the platform area and the duration that they stayed in the platform quadrant were recorded.

Specimen collection

After weighing the rats, 10% chloral hydrate (0.35 mL/100 g) (Xi'an Tianzheng Kangyuan Biotechnology Co., Ltd., China) was injected intraperitoneally for anaesthesia. The soft tissue of the skin was cut along the xiphoid process, and the diaphragm was punctured after entering the abdominal cavity. The thoracic cage was cut perpendicular to the ribs to fully expose the heart. The blood was collected with a disposable needle in the blood collection vessel (without heparin sodium), which was allowed to stand for 30 min and then centrifuged at 3000 rpm for 25 min to collect the upper serum. After the blood collection was completed, the rats were sacrificed by decapitation, and the whole brain was immediately removed. Brain tissues were cut using a stainless-steel blade into pieces of approximately 0.5 g weight and stored at -80°C for further analysis by Western blotting.

Detection of cerebral infarct volume

Brain tissues were frozen at -20°C for 20 min. The samples were coronally sectioned, from front to back, and a total of 5 sections were placed in 2% 2,3,5-triphenyl tetrazolium chloride (TTC) solution (Shanghai Kanglang Biotechnology Co., Ltd., China) and incubated at 37°C in the dark for 30 min. After coloration, the infarct area presented without colour, and the normal brain tissue was dyed red. Slides were then fixed with 40 g/L formaldehyde, and the infarct area was calculated based on the results of the pale area.

Serum levels of IL-10 and TGF- β 1

IL-10 and TGF- β 1 ELISA Kit were purchased from Shanghai Meilian Biotechnology Co., Ltd., China. The prepared samples and standards, according to the manufacturer instructions, were incubated at 37°C for 30 min. Then, the plate was washed 5 times before the sample was incubated with enzyme-labelled reagent at 37°C for 30 min. Then the plate was washed again for 5 times, and the colouring solutions A and B were added, and left to react at 37°C for 10 min. Finally, the termination liquid was added, and optical density (OD) of each hole was measured at a wavelength of 450 nm within 15 min. The OD values were used to set the standard curve.

Western blot analysis

Brain tissues were placed into an Eppendorf (EP) tube, cut into pieces, and the proteins were extracted by phenylmethylsulfonyl fluoride (PMSF) (Tianjin Weike Biotechnology Co., Ltd., China). The total proteins from each lane were mixed with an equal volume of 2×Loading buffer (135 mmol/L 20% Tris-Cl glycerol (pH 6.8), 0.02% bromophenol blue, 6% sodium dodecyl sulfate (SDS), and 10% β -mercaptoethanol) and denatured in boiling water for 5 min. A discontinuous 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins. The concentrated gel was electrophoresed at 60 V for 30 min, and the separated gel was electrophoresed at 120 V for 2 h. The proteins were electrotransferred onto the nitrocellulose membrane (0.45 mm pore size; Bio-Rad Laboratories Inc., Hercules, CA, USA) at a constant flow of 300 MA. The membrane was washed with TBST (TBS+Tween, TBS: Tris-Buffered Saline; Beijing Baiaolaibo Technology Co., Ltd., China) for 3 times, 15 min each time, and the

sample was sealed at room temperature for 2 h with 5% skim milk dissolved in TBST. Then the membrane was washed with TBST for 3 times, 15 min each time, and the filter membrane was incubated overnight at 4°C with primary antibody - TGF- β 1 Antibody (1:250), IL-10 Antibody (1:1000) and β -actin Antibody (1:200) (Santa Cruz, USA). After incubation, the membrane was washed with TBST for 3 times, 15 min each time, and incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:1000) (Beijing Boerxike Technology Co., Ltd., China) at room temperature for 1 h. After washing the membrane with TBST for 3 times, 15 min each time, enhanced chemiluminescence (ECL), X-ray exposure, development, and fixation were performed and the results were analyzed.

Statistical analysis

Measured data were expressed as mean $\pm$ standard deviation, and were analyzed using SPSS 19.0 software (IBM, Armonk, NY, USA). Analysis of variance (ANOVA) was used for comparison between the groups, and Student–Newman–Keuls test was used for comparison between the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Morphological characteristics and identification of BMSCs

The morphology of the early BMSCs under an inverted microscope was irregular fusiform (Fig. 1A). The osteogenic and lipogenic differentiation ability of the third-generation BMSCs was identified with the help of various induction factors, and the results revealed that BMSCs could be differentiated into osteoblasts and adipocytes (Fig. 1B) on the surface. CD34, CD73, and CD90 were used as surface markers, and their expression levels were detected by flow cytometry. Positive expressions of CD73 and CD90 were found, while the CD34 hematopoietic cell surface marker was negative (Fig. 1 D-F). In addition, CD34, CD73 and CD90 cell surface markers, as well as osteogenic and adipogenic differentiation, were used to show that the extracted cells were BMSCs.

Performance of the rat model of MCAO

The majority of rats showed typical neurological

deficits in the awake state 24 h after right MCAO. The performance of the rat model of MCAO was assessed as follows: firstly, the general state involved mental malaise and slow reaction; secondly, the same side of the Horner sign was investigated: two eyes were drooping compared with the right eyelid, crack of the eye was smaller, the pupil was narrowed, and the eyeball was sunken; thirdly, posture was evaluated: the left muscle tension decreased, while the right muscle tension was relatively increased. When the tail was suspended, the left limb was flexed, adducted, and stretched, and the forelimb was heavier, and the hind limb could simultaneously cause this phenomenon in severe cases. The body tilted to the left or made a circle during autonomous movement.

mNSS

Before treatment, there was no significant difference in mNSS between group B and group C, while this was significantly increased compared with group A ($P < 0.05$). One week after transplantation, mNSS in group C decreased significantly compared with group B ($P < 0.05$). Compared with the scores before transplantation, mNSS in group B increased at 1-week post-transplantation, without reaching the statistical significance while in group C the decreased was significantly (Table I).

MWM results

The evasive incubation period of MWM showed (Table I) that with the progress of the training, the evasive incubation period in each experimental group gradually decreased. In detail, the evasive incubation period in group C and group A decreased faster than that in group B, and there was no significant difference in the evasive incubation period between group C and group A on the first day ($P > 0.05$). On days 3-5 of the test, the evasive incubation period in group C and group A was markedly decreased compared with the value in group B ($P < 0.05$).

Cerebral infarct volume

There was no infarct in group A. The cerebral infarct volume was 52.95 ± 9.82 and 30.18 ± 7.36 mm³ in group B and group C, respectively ($P < 0.05$, Fig. 1 B, C).

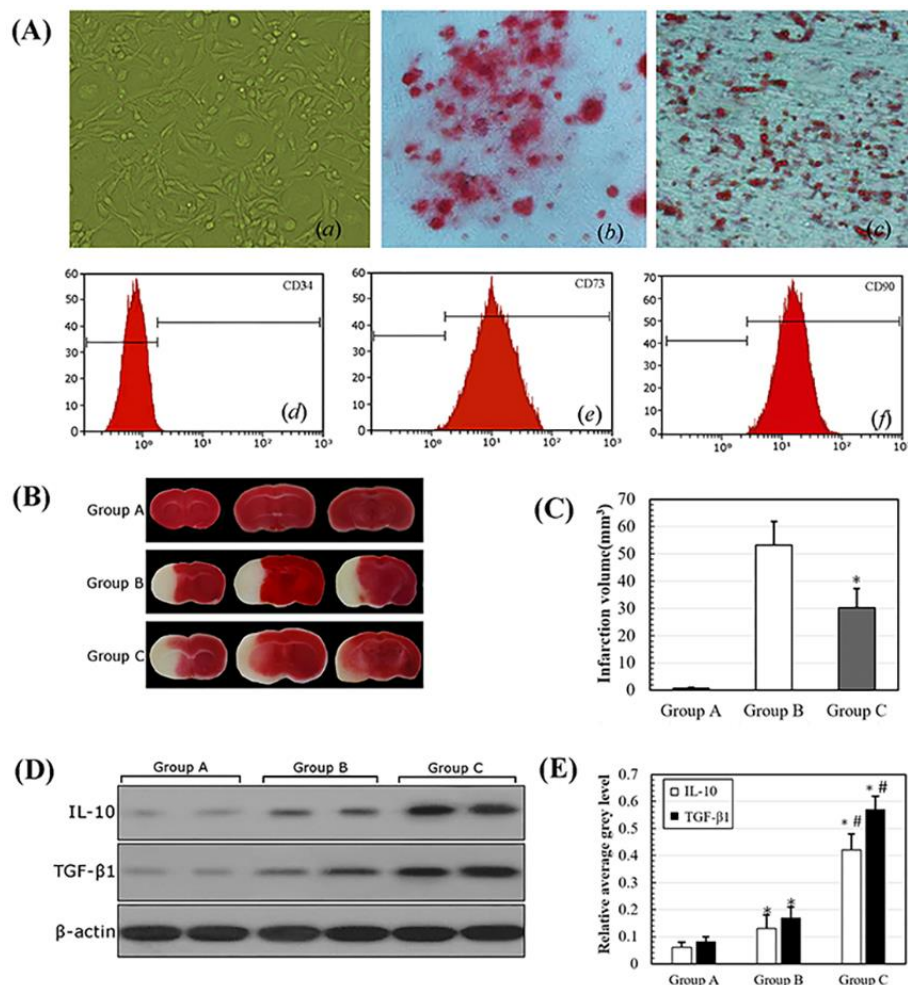


Fig. 1. *A)* Differentiation and identification of BMSCs (*a*: Morphology of BMSCs, 100x; *b*: Results of Alizarin red staining, in which Orange-Red staining within the cells indicated mineralized nodule formation, 100 x; *c*: Results of Oil red O staining of fat cells, 100 x; *d-f*: Results of flow cytometry for detecting the expression levels of CD34, CD73, and CD90)). *B)* Cerebral infarct volume; *C)* Comparison of cerebral infarct volume among the three groups, * $P < 0.05$ compared with group B); *D)* Expression levels of IL-10 and TGF- β 1 in brain tissues detected by Western blotting; *E)* Relative grey value statistical chart, * $P < 0.05$, compared with group A; # $P < 0.05$, compared with group B.

Serum levels of IL-10 and TGF- β 1

The serum levels of IL-10 and TGF- β 1 in group B were significantly increased compared with group A ($P < 0.05$), while those in group C were significantly increased compared with those in group B ($P < 0.05$) (Table I).

Expression levels of IL-10 and TGF- β 1 in brain tissue

Western blotting was used to detect the expression levels of IL-10 and TGF- β 1 in brain tissues. The expression of IL-10 and TGF- β 1 in the brain tissues

in group B were significantly increased compared with group A ($P < 0.05$). In contrast, in group C the expression of IL-10 and TGF- β 1 significantly increased compared with groups A and B ($P < 0.05$, Fig. 1 D, E).

DISCUSSION

Studies in the literature showed that embryonic stem cells, neural stem cells, BMSCs, and umbilical cord blood cells have the potential to differentiate

Table I. Values of mNSS before and after transplantation, the evasive incubation period(s), times of crossing platform area, serum levels of IL-10 and TGF- β 1 (ng/mL) in the three groups.

Parameter	Time-point	Group A	Group B	Group C
Values of mNSS	Before transplantation	1.4 $\pm$ 0.6	10.4 $\pm$ 1.4*	10.2 $\pm$ 1.0* [#]
	One week post-transplantation	0.4 $\pm$ 0.2	11.6 $\pm$ 1.8*	4.6 $\pm$ 0.6* ^{&}
	Day 1	39.64 $\pm$ 6.46	68.97 $\pm$ 21.41*	48.60 $\pm$ 11.33
The evasive incubation period(s)	Day 2	24.06 $\pm$ 3.77	45.05 $\pm$ 13.08*	33.08 $\pm$ 10.16
	Day 3	19.00 $\pm$ 5.65	42.20 $\pm$ 10.12*	23.60 $\pm$ 8.18 [#]
	Day 4	12.84 $\pm$ 4.25	39.38 $\pm$ 11.08*	22.71 $\pm$ 9.58 [#]
	Day 5	11.57 $\pm$ 3.16	33.16 $\pm$ 7.53*	15.97 $\pm$ 5.63 [#]
Times of crossing platform area	Day 5	6.6 $\pm$ 1.4	2.4 $\pm$ 0.5*	5.2 $\pm$ 1.2 [#]
IL-10 (ng/ml) (serum)	4 weeks	11.02 $\pm$ 0.17	16.38 $\pm$ 0.73*	28.17 $\pm$ 0.92* [#]
TGF- β 1 (ng/ml) (serum)	4 weeks	15.28 $\pm$ 0.39	31.28 $\pm$ 1.76*	41.29 $\pm$ 2.04* [#]

Note: * $P < 0.05$, compared with group A; [#] $P < 0.05$, compared with group B; [&] $P < 0.05$, compared with before transplantation.

into nerve cells and improve and repair nerve function after injury (9, 10). In the current study, BMSCs were successfully transplanted into infarction sites, and the results showed that the defects of neural function in group C were significantly improved, along with the reduction of infarcted brain area, which may be related to differentiation of BMSCs into nerve cells and glial cells. Simultaneously, no brain tissue damage was noted after BMSC transplantation. A previous study (11) showed that ischemic brain injury is closely associated with expression levels of TGF- β 1, which is only slightly expressed in normal brain tissues, while rapidly increases once ischemia and hypoxia occur in brain tissues. IL-10 is an important anti-inflammatory factor, which can inhibit the antigen delivery ability of macrophages, reduce the invasion of neutrophils in ischemic areas, and increase cerebral blood flow. Grilli et al. (12) found that the brain infarction area of rats with IL-10 knockout was 30% higher than that of rats without IL-10 knockout. This study showed that BMSCs transplantation for MCAO

could up-regulate the expression levels of the two anti-inflammatory factors (IL-10 and TGF- β 1) and exert immunomodulatory effects.

In conclusion, we showed that BMSCs transplantation could repair ischemic brain injury and improve learning and memory in rats. The mechanism of repairing nerve injury may be related to promoting the secretion of the anti-inflammatory factors (TGF- β 1 and IL-10) and reducing the inflammatory response. However, the specific mechanism in which BMSC transplantation promotes the generation of IL-10 and TGF- β 1 remains to be further explored.

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LETTER TO THE EDITOR

Long non-coding RNA FBXL19-AS1 serves as a competing endogenous RNA to regulate ZEB1 expression by sponging miR-431 in gastric cancer

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To the Editor,

Gastric cancer (GC) is a common malignant tumor worldwide, with the geographically highest incidence in East Asian countries (1). GC also ranks as the top reason for cancer-related deaths. Surgical resection and chemoradiotherapy are the main therapeutic approaches for GC (2), but unfortunately, most GC patients are diagnosed at advanced stages, and the long-term prognosis of these patients remains unfavorable (3). Accordingly, identification of new therapeutic targets for this fatal malignancy is still of great significance.

Long non-coding RNAs (lncRNAs) are a significant subtype of non-coding RNAs with more than 200 nucleotides in length (4). LncRNAs were previously regarded as “transcriptional noise”, but in recent years, accumulating studies have shown their involvement in various human diseases, particularly cancers (5). For example, FBXL19 antisense RNA 1 (FBXL19-AS1) is a recently identified lncRNA, and it was suggested to be expressed at a high level in many cancers, including colorectal (6) and osteosarcoma (7). In this study, we focused on the functions and mechanisms of FBXL19-AS1 in GC.

MATERIALS AND METHODS

Patients and tissue samples

A total of 107 matched pairs of GC and adjacent normal mucosa tissues were collected from patients who had undergone surgery at The Affiliated Hongqi Hospital of Mudanjiang Medical University. Before surgery, none of the patients had received chemotherapy, radiotherapy, or targeted therapy. The tissue samples were assessed by two experienced pathologists, freshly frozen in liquid nitrogen and stored at -80°C until use. The experiments were approved by the Ethics Committee of The Affiliated Hongqi Hospital of Mudanjiang Medical University. Written informed consent was obtained from all patients or their relatives.

Cell culture and transfection

The human GC cell lines (BGC-823, SGC-7901, MKN-45, and AGS) and normal human gastric epithelial cells GES-1 were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), while HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan City, UT,

Key words: gastric cancer; long non-coding RNA FBXL19-AS1; miR-431/ZEB1 axis; ZEB1 expression

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USA), supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

The FBXL19-AS1 small interfering (si)RNA (si-FBXL19-AS1), target-free siRNA (si-con), miR-431 mimic and control miRNA mimic (miR-con) were designed and chemically synthesized by Guangzhou RiboBio Co., Ltd., China. To construct FBXL19-AS1-overexpressing plasmids, FBXL19-AS1 cDNA was synthesized and cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen, USA). An empty vector was used as a negative control. Cells were seeded in 6-well plates and cultured to 70-80% confluence. Cell transfection was then performed using Lipofectamine 2000 Transfection Reagent (Invitrogen, USA).

Subcellular fractionation and RT-qPCR analysis

The cytoplasmic and nuclear fractions of cells were separated using a PARIS kit (Invitrogen, USA). Total RNA was extracted with TRIzol reagent (Invitrogen, USA). cDNA was generated with the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). qPCR reactions were then carried out using an SYBR Green PCR Kit (TaKaRa, Dalian, China) on an ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). Relative expression of genes was calculated using the 2^{-ΔΔCt} method. GAPDH and U6 acted as internal standards. The sequences of PCR primers are listed in Table I.

Western blot analysis

Total protein was extracted using radioimmunoprecipitation-assay buffer (Beyotime, Shanghai, China). Identical quantities of proteins were separated on SDS gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with specific primary antibodies against E-cadherin (catalog no. 14472; 1:2,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), N-cadherin (catalog no. 13116; 1:1,000 dilution; Cell Signaling Technology, Inc.), ZEB1 (catalog no. 3396; 1:2,000 dilution; Cell Signaling Technology, Inc.) or GAPDH (catalog no. sc-59540; 1:1,000

dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, followed by incubation with HRP-conjugated secondary antibody (catalog no. A0192; 1:5,000 dilution; Beyotime) at room temperature for 1 h. The bands were then visualized by using an enhanced chemilluminescence kit (GE Healthcare, Little Chalfont, UK). GAPDH was used as a protein-loading control.

MTT assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay. Cells were grown in 96-well plates at a density of 3,000 cells/well. At each time point, 20 µl of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the plates were incubated at 37°C for additional 4 h. Then, 150 µl of DMSO (Sigma-Aldrich) was added to terminate the reaction. The absorbance of each well was read at a wavelength of 570 nm using an ELx808 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Transwell assay

Cells were suspended in 200 µl of serum-free medium and plated into the uncoated or matrigel-coated upper chamber of Transwell plates (8 µm pore size; Corning Inc., Corning, NY, USA), and medium containing 10% FBS was added to the lower chambers. After 48 h, the cells on the lower membrane surfaces were fixed with 70% ethanol for 30 min, stained with 0.1% crystal violet for 10 min, and observed under a microscope.

Dual-luciferase reporter assay

The sequence fragments of FBXL19-AS1 and ZEB1 mRNA containing the predicted potential miR-431 binding sites were amplified by PCR and inserted into the psiCHECK-2 vector (Promega, Madison, WI, USA). The binding sites were mutated using a Mut Express II Fast Mutagenesis kit (Vazyme, Piscataway, NJ, USA). After transfection with the constructed luciferase plasmids and miR-431 mimic or mimic control using Lipofectamine 2000 Transfection Reagent for 48 h, the cells were lysed, and the luciferase activities were measured

using the Dual-Luciferase Reporter Assay System (Promega, USA).

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). All the experiments were performed in triplicates and the data was expressed as mean $\pm$ standard deviation (SD). The

differences between groups were analyzed using Student's *t*-test or one-way ANOVA. Survival curves were generated using Kaplan-Meier method and compared using log-rank test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

FBXL19-AS1 is overexpressed in GC

We first examined the expression levels of

Table I. The sequences of PCR primers.

Name	Sequence
FBXL19-AS1 Forward primer	GGTACAACCTACGGATATGA
FBXL19-AS1 Reverse primer	TACGTCTCGACCATTACGCA
GAPDH Forward primer	CTCTGCTCCTCCTGTTCGAC
GAPDH Reverse primer	CGACCAAATCCGTTGACTCC
miR-431 Forward primer	CAGGCCGTCATGCAAA
miR-431 Reverse primer	CGCTTCAGAATTTGCGTGTCAT
U6 Forward primer	CTCGCTTCGGCAGCACA
U6 Reverse primer	AACGCTTCACGAATTTGCGT

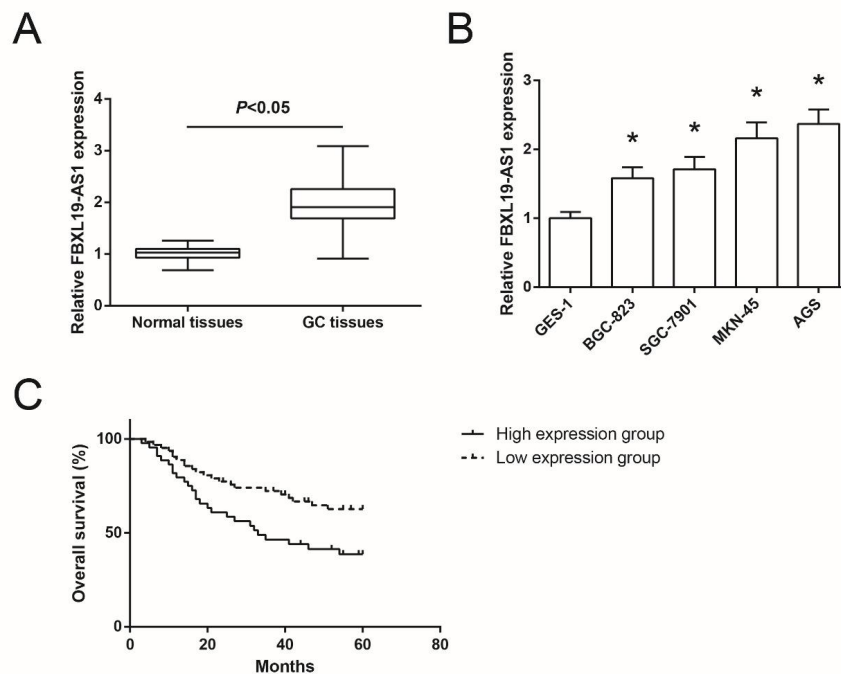
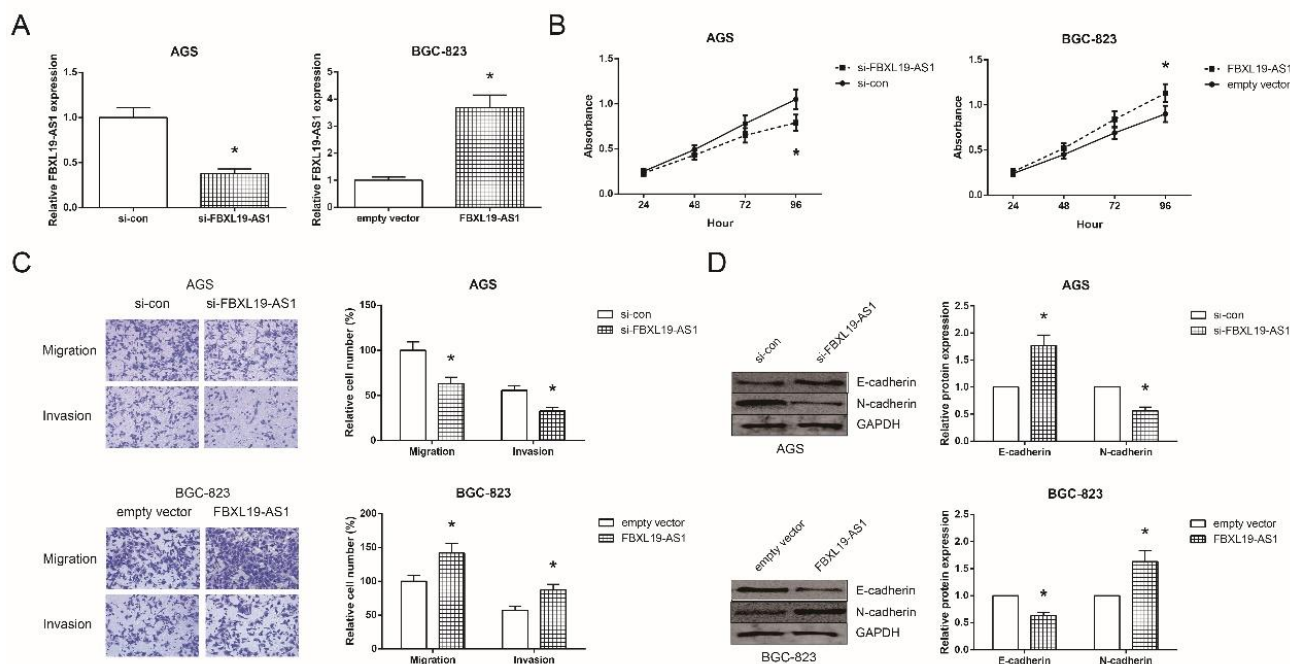


Fig. 1. *FBXL19-AS1 is overexpressed in GC. A)* *FBXL19-AS1* expression levels in GC tissues and adjacent normal tissues, detected by RT-qPCR analysis. **B)** *FBXL19-AS1* expression levels in GC cell lines and normal GES-1 cells. **C)** Kaplan–Meier survival curve of GC patients with low and high expression of *FBXL19-AS1*. **P* < 0.05 vs GES-1 cells.

Table II. Association of *FBXL19-AS1* expression with clinicopathologic characteristics of GC patients.

Characteristics	Total number (N=107)	FBXL19-AS1 expression		P value
		Low (N=63)	High (N=44)	
Age (years)				0.571
≥ 55	50	28	22	
< 55	57	35	22	
Gender				0.320
Male	67	37	30	
Female	40	26	14	
Tumor size (cm)				0.018
≥ 5	44	20	24	
< 5	63	43	20	
Location				0.823
Cardia+body	67	40	27	
Pylorus	40	23	17	
Distant metastasis				0.004
Yes	39	16	23	
No	68	47	21	
Lymph node invasion				0.163
Yes	62	33	29	
No	45	30	15	
TNM stage				0.016
I-II	61	42	19	
III-IV	46	21	25	

**Fig. 2.** *FBXL19-AS1* exerts an oncogenic role in GC cells. **A)** *FBXL19-AS1* expression levels in GC cells after transfection. **B)** The effects of *FBXL19-AS1* knockdown/overexpression on GC cell proliferation, detected by MTT assay. **C)** The effects of *FBXL19-AS1* knockdown/overexpression on GC cell migration and invasion, detected by Transwell assay. **D)** The effects of *FBXL19-AS1* knockdown/overexpression on EMT-related protein expression in GC cells, detected by western bolt analysis. * $P < 0.05$ vs si-con-transfected cells or empty vector-transfected cells.

FBXL19-AS1 in GC tissues and cell lines by RT-qPCR analysis. Compared to the adjacent normal tissues, FBXL19-AS1 expression was significantly increased in the GC tissues (Fig. 1A). In addition, we observed that GC cell lines (BGC-823, SGC-7901, MKN-45, and AGS) exhibited increased expression pattern of FBXL19-AS1 compared with normal GES-1 cells (Fig. 1B).

To estimate the clinical value of FBXL19-AS1 in GC, by using the median level of FBXL19-AS1 as a cutoff, 107 participants were allocated into a high expression group (N=44) and a low expression group (N=63). As shown in Table II, high FBXL19-AS1 expression was positively correlated with larger tumor size ($P=0.018$), distant metastasis ($P=0.004$) and advanced TNM stage ($P=0.016$) of GC patients.

Moreover, Kaplan-Meier analysis showed that GC patients with high FBXL19-AS1 expression exhibited poorer overall survival compared with those with low FBXL19-AS1 expression ($P=0.013$; Fig. 1C).

FBXL19-AS1 exerts an oncogenic role in GC cells

Loss-of-function and gain-of-function assays were then performed to evaluate the functional effects of FBXL19-AS1 on GC cells. RT-qPCR analysis confirmed the efficient knockdown of FBXL19-AS1 in AGS cells after transfection with si-FBXL19-AS1, while FBXL19-AS1 was effectively overexpressed in BGC-823 cells (Fig. 2A). MTT assay revealed that the proliferation of AGS cells was markedly suppressed by FBXL19-AS1 knockdown (Fig. 2B). Moreover, FBXL19-AS1 knockdown also notably

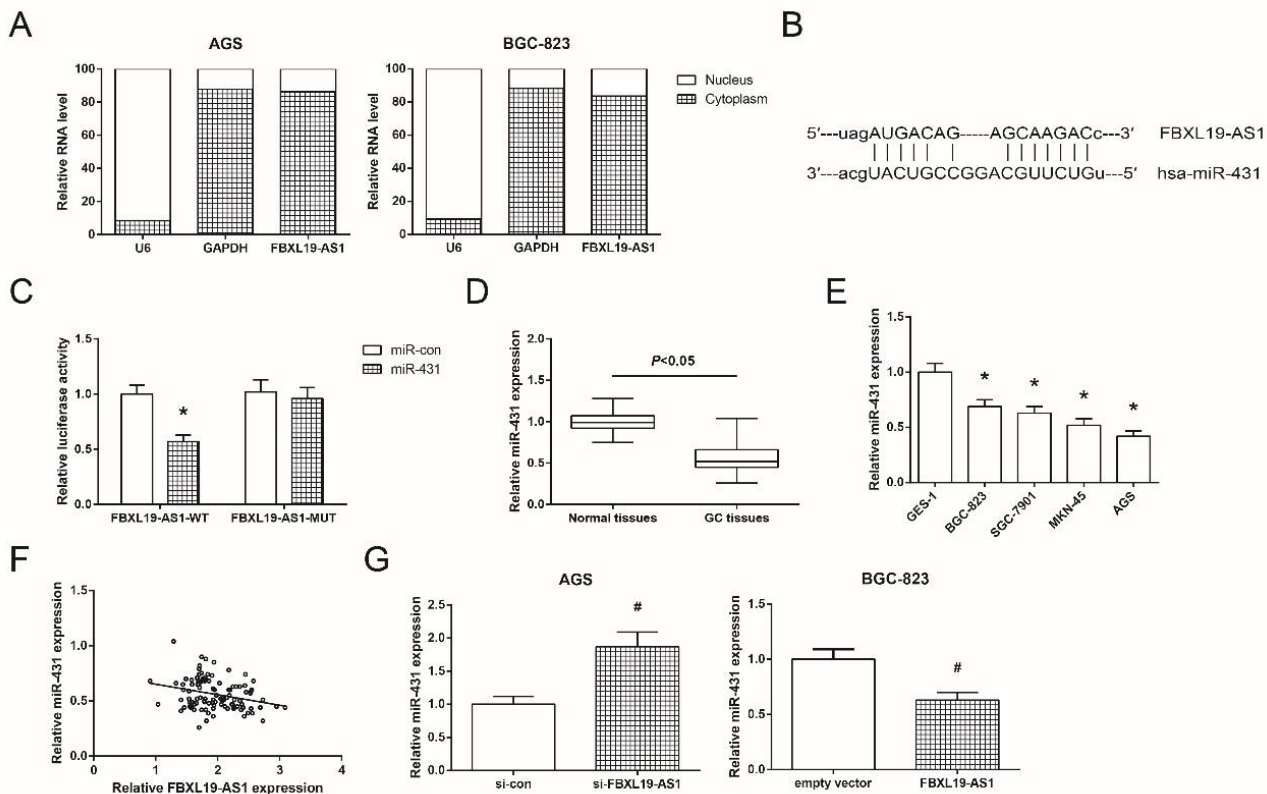


Fig. 3. *FBXL19-AS1 directly interacts with miR-431 in GC. A) The subcellular distribution of FBXL19-AS1 in GC cells. B) The predicted binding site of miR-431 within FBXL19-AS1 fragment. C) The direct binding relation between FBXL19-AS1 and miR-431, validated by dual-luciferase reporter assay. D) miR-431 expression levels in GC tissues and adjacent normal tissues. E) miR-431 expression levels in GC cell lines and normal cells. F) The correlation between the expression levels of FBXL19-AS1 and miR-431 in GC tissues. G) The effects of FBXL19-AS1 knockdown/overexpression on miR-431 expression levels in GC cells. * $P<0.05$ vs miR-con-transfected cells or GES-1 cells; # $P<0.05$ vs si-con-transfected cells or empty vector-transfected cells.*

weakened the migratory and invasive abilities of AGS cells (Fig. 2C). In contrast, FBXL19-AS1 overexpression promoted the proliferation, migration and invasion of BGC-823 cells. EMT

plays a critical role in cancer cell migration and invasion, and Western blot analysis further showed that FBXL19-AS1 knockdown increased the expression of epithelial marker (E-cadherin) and

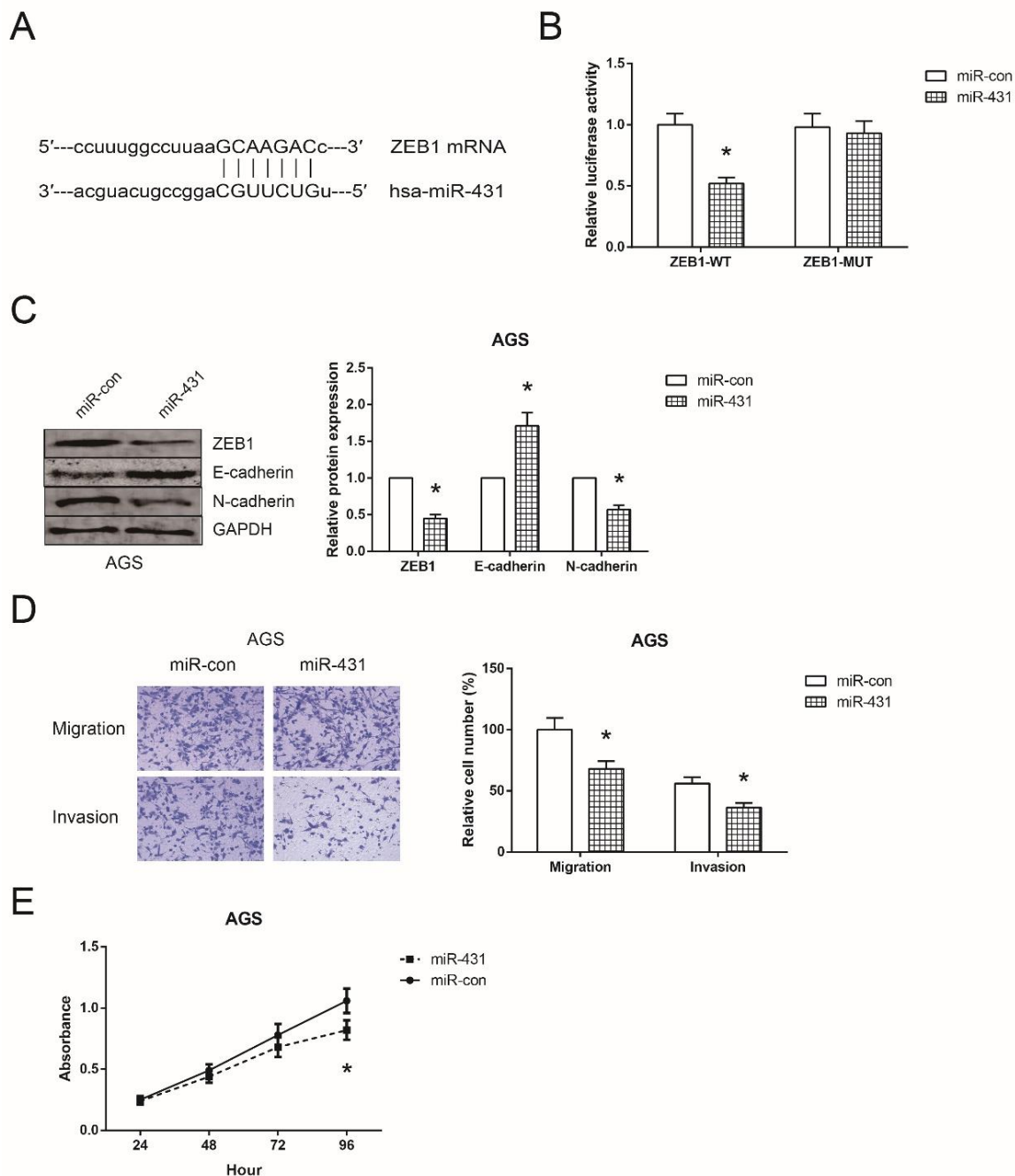


Fig. 4. ZEB1 is a direct target gene of miR-431 in GC. **A)** Predicted binding site of miR-431 within ZEB1 mRNA fragment. **B)** Direct binding relation between miR-431 and ZEB1 mRNA, validated by dual-luciferase reporter assay. **C)** Effects of miR-431 overexpression on EMT-related protein expression in GC cells. **D)** Effects of miR-431 overexpression on GC cell migration and invasion. **E)** Effects of miR-431 overexpression on GC cell proliferation. * $P < 0.05$ vs miR-con-transfected cells.

decreased the expression of mesenchymal marker (N-cadherin) in AGS cells (Fig. 2D). In contrast, FBXL19-AS1 overexpression promoted the EMT of BGC-823 cells.

FBXL19-AS1 directly interacts with miR-431 in GC

We observed that FBXL19-AS1 was preferentially localized to the cytoplasm of AGS and BGC-823 cells (Fig. 3A). By the Starbase database (<http://starbase.sysu.edu.cn/index.php>), we found that FBXL19-AS1

contains the potential complementary binding sites to miR-431 (Fig. 3B). To validate the hypothesis, dual-luciferase reporter assay was then performed, and the results revealed that miR-431 mimic obviously reduced the luciferase activity of FBXL19-AS1-WT in HEK293T cells, however, mutating the binding sites abolished this reduction (Fig. 3C). In contrast to FBXL19-AS1, miR-431 expression was notably decreased in the GC tissues and cell lines, compared with the normal counterparts (Fig. 3 D,

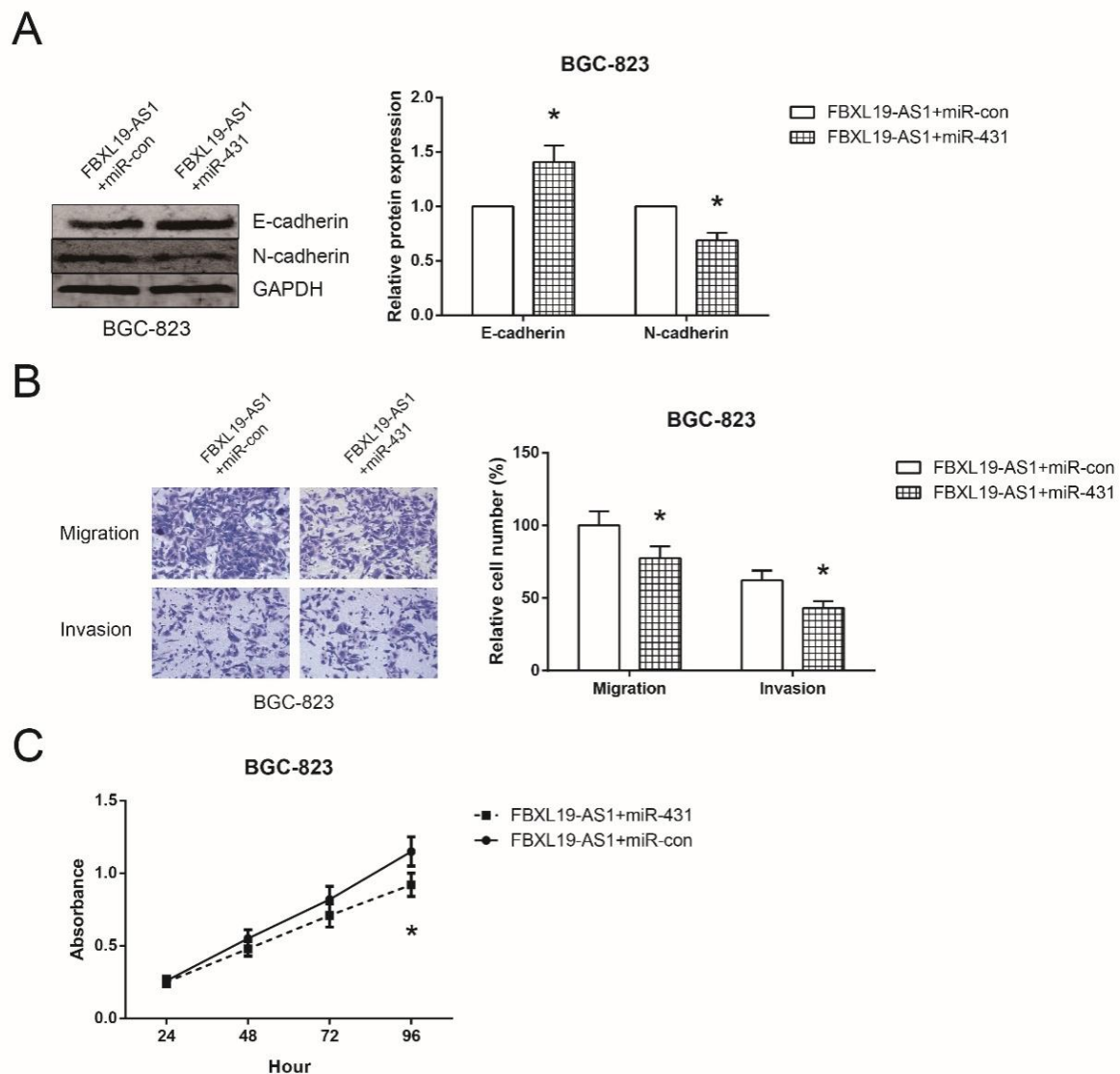


Fig. 5. miR-431 restoration mitigates the oncogenic role of FBXL19-AS1 in GC cells. **A)** Effects of miR-431 restoration on EMT-related protein expression in FBXL19-AS1-overexpressing GC cells. **B)** Effects of miR-431 restoration on the migration and invasion of FBXL19-AS1-overexpressing GC cells. **C)** Effects of miR-431 restoration on the proliferation of FBXL19-AS1-overexpressing GC cells. * $P < 0.05$ vs pcDNA3.1-FBXL19-AS1+miR-con-transfected cells.

E). An inverse correlation between FBXL19-AS1 and miR-431 expression was observed in GC tissues ($r = -0.275$; $P = 0.004$; Fig. 3F). We further found that FBXL19-AS1 knockdown remarkably increased miR-431 expression in AGS cells, while miR-431 expression was significantly decreased in FBXL19-AS1-overexpressing BGC-823 cells (Fig. 3G).

ZEB1 is a direct target gene of miR-431 in GC

We then predicted the target genes of miR-431 by using the TargetScan database (http://www.targetscan.org/vert_71/), and the potential binding sites of miR-431 were identified within the 3'-UTR of ZEB1 mRNA (Fig. 4A). In addition, the luciferase activity of ZEB1-WT in HEK293T cells was notably decreased by miR-431 mimic (Fig. 4B). We also observed that miR-431 overexpression decreased ZEB1 protein expression, thereby inhibiting the EMT of AGS cells (Fig. 4C). Furthermore, the proliferation, migration and invasion of AGS cells were significantly suppressed by miR-431 overexpression (Fig. 4 D, E).

miR-431 restoration mitigates the oncogenic role of FBXL19-AS1 in GC cells

Rescue experiments were further conducted by co-transfecting miR-431 mimic and pcDNA3.1-FBXL19-AS1 in BGC-823 cells, and the results indicated that miR-431 restoration markedly diminished the enhanced EMT (Fig. 5A), thereby inhibiting the migration and invasion of FBXL19-AS1 overexpressing BGC-823 cells (Fig. 5B). In addition, MTT assay showed that the effect of FBXL19-AS1 overexpression on the proliferation of BGC-823 cells was also notably blocked by miR-431 restoration (Fig. 5C).

DISCUSSION

GC progression is a complex process involving the accumulation of multiple genetic alterations. At present, many GC-related lncRNAs have been reported, some of which may function as diagnostic biomarkers or therapeutic targets for GC (8). The role of FBXL19-AS1 in diverse cancer types has been well studied, and herein, we identified a significant

upregulation of FBXL19-AS1 in GC samples. We then found that FBXL19-AS1 had a strong oncogenic effect in GC, with FBXL19-AS1 knockdown inhibiting GC cell proliferation, migration and invasion. As a key step in cancer metastasis, EMT is closely associated with GC initiation and progression (9), and this study also showed that FBXL19-AS1 could facilitate EMT in GC.

Increasing numbers of reports have found that lncRNAs can exert their functions by acting as competing endogenous RNAs (ceRNAs) to sponge miRNAs (10). This study confirmed that FBXL19-AS1 directly interacts with miR-431 in GC. Consistent with a previous study (11), we also found that miR-431 is downregulated in GC, and its expression could be negatively regulated by FBXL19-AS1. As a transcriptional factor, ZEB1 expedites EMT, thereby accelerating the metastasis and invasion in GC (12), and herein, ZEB1 was proved to be a target of miR-431 in GC. We further showed that miR-431 restoration mitigated the oncogenic role of FBXL19-AS1 in GC cells, partly by inhibiting ZEB1-mediated EMT.

In short, this study indicated that FBXL19-AS1 is highly expressed and exerts oncogenic effects in GC by regulating miR-431/ZEB1/EMT axis.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

Down-regulation of miR-365 suppresses cerebral ischemia injury by targeting IGF1RY. Chen^{1#}, L. Liu^{2#}, YY. Xing², Q. Li², GH. Zhao², YY. Lu² and JJ. Yang³

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To the Editor,

Ischemic stroke has become the second most common cause of death and the most common disability among adults in China (1). Currently, rapid reperfusion is still the standard treatment for cerebral ischemia. However, reperfusion can exacerbate tissue damage (2). Although rapid progress has been made in understanding the mechanisms of cerebral ischemia over the past few decades, there was no effective therapy for cerebral ischemia at the right time window (3). Therefore, looking for a target molecule to explore new therapeutic treatment for cerebral ischemia reperfusion is urgent.

Substantial evidence has indicated that miRNAs are involved in the development of cerebral ischemia and serve as its potential biomarkers (4). It was reported that miR-365 was up-regulated in the ischemic brain and it suppressed astrocyte-to-neuron conversion (5). MiR-365 was hypothesised to be involved in the development of cerebral ischemia/reperfusion injury.

IGF1R, a trans-membrane receptor tyrosine kinase, takes part in regulating IGF biological activity. IGF activation has been demonstrated to inhibit cell apoptosis induced by hypoxia and involved in regulating the development of ischemia stroke (6). In this study, whether IGF1R was a target

of miR-365 in regulating the development of cerebral ischemia/reperfusion injury was investigated.

The present study was aimed to explore the effect of miR-365 and IGF1R on ischemia/reperfusion injury and investigate the potential mechanism of miR-365 in regulating of OGDR-induced cell viability and apoptosis. Results showed that miR-365 was increased in SH-SY5Y cells while IGF1R was decreased. Moreover, down-regulation of miR-365 enhanced cell viability and inhibited cell apoptosis by targeting IGF1R.

MATERIALS AND METHODS

Human SH-SY5Y cells were purchased from the cell bank of the Chinese Academy of Sciences (Beijing, China). The cells were cultured in DMEM Medium (Thermo, USA) with 10% FBS (Gibco, Rockville, MD, USA) at 37°C in a humidified 5% CO₂ incubator. The cultured SH-SY5Y cells were transfected with miR-365 mimic or inhibitor to increase or decrease miR-365 expression following the manufacture's instructions. After infection for 48 h, SH-SY5Y cells were harvested for later studies. MiR-365 mimic, inhibitor or their corresponding negative controls were purchased from GenePharma Co. (Shanghai, China).

The OGD/R model was constructed as described previously with small modifications (7, 8). In brief,

Key words: miR-365, Ischemia/reperfusion injury, proliferation, apoptosis, IGF1R

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glucose-free DMEM was used to incubate SH-SY5Y cells and then transferred to a hypoxic atmosphere (1% O₂, 94% N₂, and 5% CO₂) quickly for 1 h at 37°C. Then, normal medium was applied for reoxygenation for 24 h instead of the glucose-free DMEM to culture the cells under normal conditions. The cells cultured under normoxic conditions and not deprived of oxygen and glucose served as control cell cultures.

With the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), siRNA IGF1R or control siRNA and IGF1R vector or control vector provided by Shanghai Genechem (Shanghai, China) were transfected into SH-SY5Y cells to decrease IGF1R expression.

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from SH-SY5Y cells. The complementary deoxyribose nucleic acid (cDNA) was performed using the Reverse-Transcription System Kit (Invitrogen, Carlsbad, USA). QRT-PCR was performed using the TaqMan PCR kit following a standard protocol. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. The $2^{-\Delta\Delta Ct}$ method was used to determine the expression changes. The primer sequences were: miR-365-F: 5'-CCGCTCGAGTTTCTTTAGGGACCAGG-3', miR-365-R: 5'-CCGACGCGTGCAAGTTTAATGTTTAATGCC-3'; IGF1R-F: 5'-CCCCCTCGAGGATCCTGAATCTGTGCAAAC-3', IGF1R-R: 5'-AAAAGCGGCCGCTTCCCA GCGAAATCATC-3' U6-F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', U6-R: 5'-CGCTTCACGAATTTGCGTGTGCAT-3'; GAPDH-F: 5'-CGACCACTTTG TCAAGCTCA-3', GAPDH-R: 5'-AGGGGAGATTCAAGTGTGGTG-3'.

Radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) was used to extract the total protein from SH-SY5Y cells. The total proteins were collected after homogenate for 30 min at 12,000 g at 4°C. Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) was applied to regulate the proteins concentration. The proteins were electrophoresed with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto the nitrocellulose (NC) membrane. After blocked with 5% skim milk for 2 h, the membrane was incubated by primary antibodies, including anti-IGF1R (Abcam, ab131476, 1:500), anti-Caspase-8 (Abcam, ab32397, 1:1000), anti-Bax (Abcam, ab32503, 1:1000), anti-Bcl-2

(Abcam, ab182858, 1:2000), anti-GAPDH (Abcam, ab9485, 1:2500), subsequently, the second antibodies for 2 h. The amount of protein was normalized with GAPDH values on the same lane.

SH-SY5Y cells were added to 96-well plates at a density of 5×10^3 cells/well. The cells were incubated for 48 h, and then 20 μ L MTT medium (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubation for 4 h at 37°C, MTT medium was replaced by 100 μ L dimethyl sulfoxide (DMSO) and incubated for additional 10 min. Cell viability was measured by detecting the absorbance of each well at 24 h at a wavelength of 490 nm.

The IGF1R-3'UTR fragment was amplified and cloned into the pGL3-reporter vectors. For reporter assays, SH-SY5Y cells were co-transfected with miR-365 mimic and WT or Mut vectors. After transfection for 48 h, the luciferase activity was tested by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

All data are presented as mean $\pm$ SD (standard deviation) of at least three experiments. Analysis of variance (ANOVA) followed by Student's *t*-test (when there were two groups) or Tukey's post-hoc test (when there were more than two groups) was carried out with Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). A *P*-value of *P* < 0.05 was considered significant differences.

RESULTS

To assess the roles of miR-365 and IGF1R in cerebral ischemic injury, miR-365 and IGF1R expression were first detected in OGDR SH-SY5Y cells. The results showed that miR-365 expression was raised in OGDR model (Fig. 1A), while IGF1R expression was decreased (Fig. 1B). The results indicate that the expressions of miR-365 and IGF1R are opposite in OGDR cells.

Next, miR-365 expression was decreased by miR-365 inhibitor or increased by miR-365 mimic in OGDR SH-SY5Y cells. As shown in Fig. 1C, miR-365 expression was increased obviously in SH-SY5Y cells after treatment with miR-365 mimic and decreased after being treated with miR-365 inhibitor. Then, cell viability was measured

after OGDR SH-SY5Y cell transfection with miR-365 mimic or inhibitor by MTT. As Fig. 1D shows, the viability of SH-SY5Y cells subjected to OGDR model was decreased significantly. MiR-365 inhibitor facilitated cell viability, and miR-365 mimic decreased the viability of OGDR SH-SY5Y cells. Western blot was applied to evaluate cell apoptosis by detecting Caspase-8, Bax and BCL2 expression. As Fig. 1E shows, the expression level of Caspase-8 in SH-SY5Y cells that were subjected to OGDR model was increased significantly. MiR-365 inhibitor decreased Caspase-8 expression, while miR-365 mimic increased Caspase-8 expression in OGDR SH-SY5Y cells. Moreover, the expression of

Bax was also increased in cells subjected to OGDR model. Treatment with miR-365 inhibitor inhibited Bax expression while treatment with miR-365 mimic facilitated its expression. BCL-2 showed a decreased expression in OGDR model. Treatment with miR-365 inhibitor promoted BCL-2 expression while treatment with miR-365 mimic inhibited its expression (Fig. 1F).

IGF1R is a potential target of miR-365 identified by TargetScanHuman 7.2 (Fig. 2A). Dual luciferase reporter assay was then used to further confirm whether IGF1R was a direct target of miR-365 in SH-SY5Y cells. As shown in Fig. 2B, miR-365 mimic decreased the luciferase activity whereas

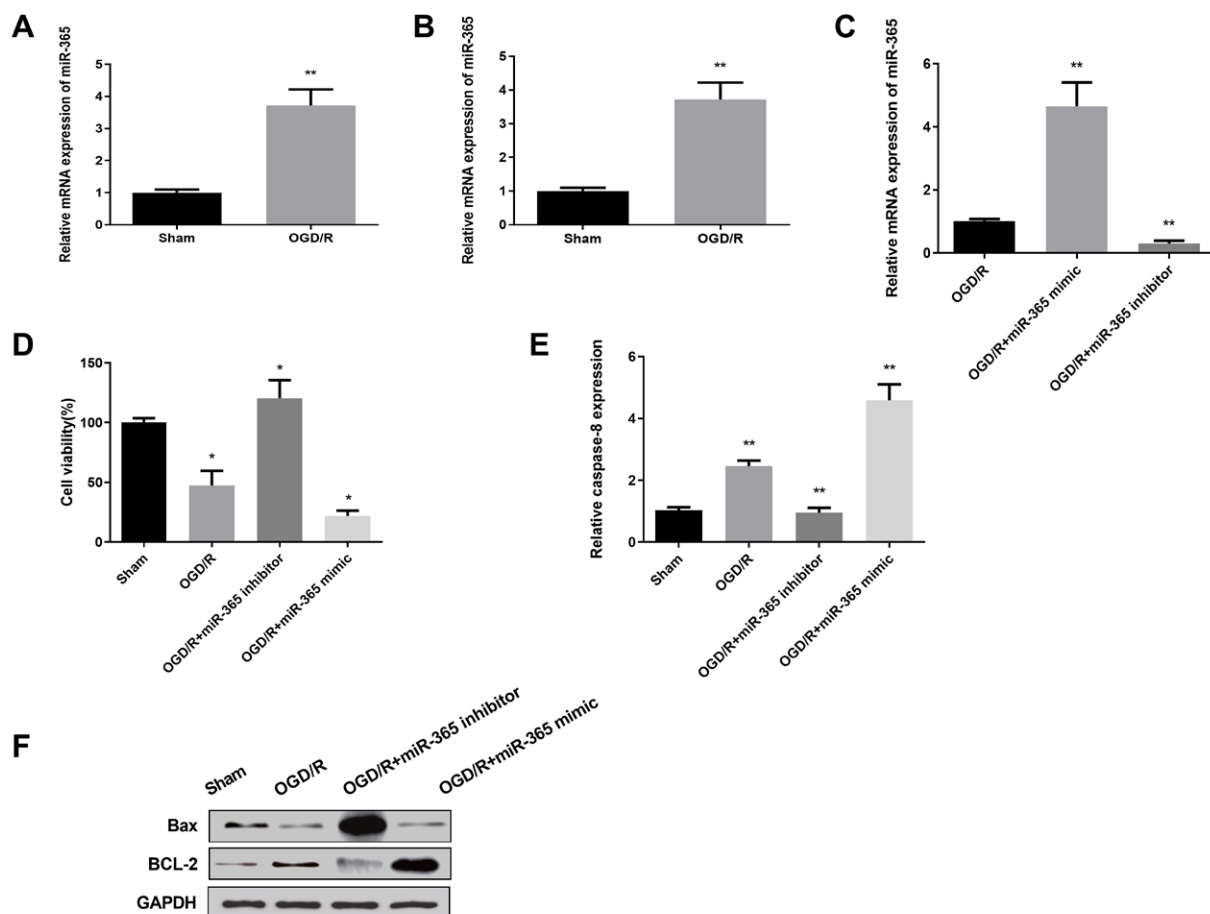


Fig. 1. Down-regulation of miR-365 promoted cell viability and inhibited cell apoptosis. **A)** miR-365 expression in OGDR cells. **B)** Relative IGF1R expression in OGDR cells. **C)** Relative miR-365 expression in OGDR cells treated with miR-365 inhibitor/mimic. **D)** The percentage of cell viability in OGDR cells. **E)** Relative expression of Caspase-8 in OGDR cells. **F)** Bax and BCL-2 detected in OGDR cells. * $P < 0.05$, ** $P < 0.01$

miR-365 inhibitor increased the luciferase activity. Furthermore, qRT-PCR and Western blot analysis showed that miR-365 mimic had an inhibitory effect on IGF1R expression, while miR-365 inhibitor showed an opposite effect (Fig. 2 C, D).

IGF1R expression was increased by IGF1R vector in OGDR SH-SY5Y cells. As Fig. 2E shows, IGF1R expression was increased obviously after cells were treated with IGF1R vector. Cell viability was then tested after OGDR SH-SY5Y cells were

transfected with IGF1R vector by MTT. As shown in Fig. 2F, the viability of SH-SY5Y cells that were subjected to OGDR model was decreased significantly. Overexpression of IGF1R facilitated cell viability of OGDR SH-SY5Y cells. Western blot was applied to evaluate cell apoptosis by detecting Caspase-8, Bax and BCL2 expression. As Fig. 2G shows, the expression of Caspase-8 in SH-SY5Y cells that subjected to OGDR model was increased significantly. IGF1R vector decreased Caspase-8

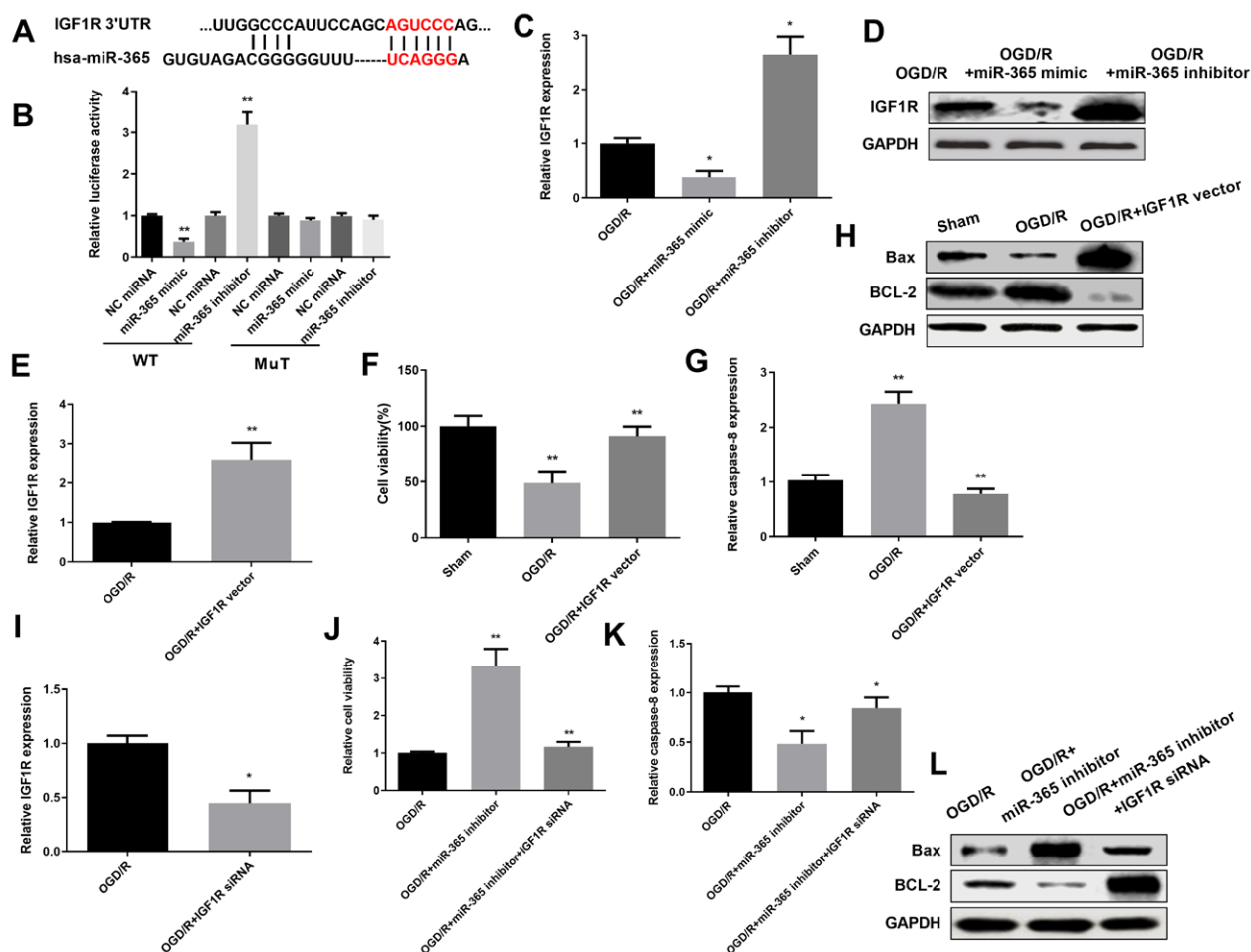


Fig. 2. MiR-365 modulated cell viability and inhibited apoptosis via targeting IGF1R. **A)** The binding sites of miR-365 and IGF1R showed by bioinformatic analysis. **B)** The relative luciferase activity of WT 3'-UTR IGF1R. **C)** Relative expression of IGF1R detected by qRT-PCR. **D)** IGF1R protein expression tested after treated with miR-365 inhibitor/mimic. **E)** Relative IGF1R expression in SH-SY5Y cells. **F)** The percentage of cell viability in OGDR cells treated with IGF1R vector. **G)** Relative expression of Caspase-8 in OGDR SH-SY5Y cells treated with IGF1R vector. **H)** Bax and BCL-2 detected in OGDR SH-SY5Y cells treated with IGF1R vector. **I)** Relative IGF1R expression in OGDR SH-SY5Y cells. **J)** Relative cell viability in OGDR SH-SY5Y cells treated with miR-365 inhibitor or both miR-365 inhibitor and IGF1R siRNA. **K)** Relative expression of Caspase-8 in OGDR SH-SY5Y cells treated with different transfection. **L)** Bax and BCL-2 detected in OGDR SH-SY5Y cells treated with different transfections. * $P < 0.05$, ** $P < 0.01$

expression in OGDR SH-SY5Y cells. Moreover, the expression of Bax was also increased in cells subjected to OGDR model. Treatment with IGF1R inhibited Bax expression. BCL-2 showed a decreased expression in OGDR model. Treatment with IGF1R vector promoted BCL-2 expression (Fig. 2H).

The effect of IGF1R on OGDR SH-SY5Y cell proliferation and apoptosis regulated by miR-365, knockdown of IGF1R and its expression is shown in Fig. 2I. Cell viability was detected in OGDR SH-SY5Y cells co-transfected with IGF1R siRNA and miR-365 inhibitor; the results showed that IGF1R siRNA markedly reversed miR-365 inhibitor-mediated promotion of cell proliferation in OGDR SH-SY5Y cells (Fig. 2J). The results showed that the expression of Caspase-8 was increased in OGDR SH-SY5Y cells co-transfected with IGF1R siRNA and miR-365 inhibitor compared with the cells transfected with the miR-365 inhibitor alone (Fig. 2K). Moreover, IGF1R siRNA reversed the inhibitory effect of miR-365 inhibitor on Bax level and the promoting effect on BCL-2 level (Fig. 2L).

DISCUSSION

The results of this study showed that miR-365 expression was elevated in OGDR SH-SY5Y cells, whereas IGF1R expression was declined. Moreover, miR-365 and IGF1R mRNA expression were negatively correlated, and IGF1R siRNA reversed the promotion effect of miR-365 inhibitor on OGDR SH-SY5Y cell proliferation and the inhibitory effect of miR-365 inhibitor on cell apoptosis. All these findings provide an insight into the treatment of cerebral ischemia reperfusion via miR-365/IGF1R axis.

According to many previous reports, miR-365 was demonstrated to be anomalous in brain related diseases (9, 10). In brain stroke, miR-365 was found to express higher and it regulated astrocyte conversion into neuron (5). In our study, the findings discovered that miR-365 was up-regulated in OGDR SH-SY5Y cells, and down-regulation of miR-365 facilitated cells viability and inhibited cells apoptosis.

IGF1R was reported by many researchers that it not only participated in the development of cancers, but also in brain diseases, including cerebral

ischemia injury (11, 12). In our study, results showed that miR-365 expression was decreased in OGDR SH-SY5Y cells, and IGF1R inhibited cell apoptosis and facilitated cell viability. In addition, IGF1R was first found to be the target of miR-365 in OGDR SH-SY5Y cells.

There are still some limitations to this study. Based on the present results on cell models, an experimental animal model was then applied to construct cerebral I/R injury models, which aims to investigate the role of miR-365 in cell apoptosis *in vivo* and explore the role of IGF1R. Moreover, the correlation between clinical factors and miR-365 expression in patients with stroke was not investigated.

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Effectiveness of neurolinguistic programming in reducing anxiety and phobia

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To the Editor,

Neurolinguistic programming (NLP) was developed in the 1970s by John Grinder (a professor of linguistics) and Richard Bandler (a mathematician) as a neurological and behavioral approach for effective communication, personality development, counseling and psychotherapy (1). It is defined as a “detailed operational model of the processes involved in human behavior and communication”. The principles of NLP may be employed to identify and alter the human being’s experience and actions. In other words, it is a therapeutic technique that is used to understand and reprogram the unconscious patterns of thoughts and behavior in order to alter the psychological response. The principles of NLP are based on the studies from cybernetics, psychophysiology, linguistics, and information services (2).

There are three important terms in NLP, which have specific meanings: ‘neurological’ specifies that neurological processes are responsible for all types of behaviors in individuals; ‘linguistic’ specifies that language is the key for organizing thoughts and communication; and ‘programming’ specifies that methods may be applied systematically to change the behavior of an individual for better results. In other words, there is a close relationship between thinking (neurological or mental), speech/language (linguistics), and systematic change in the behavior (programming) (3). Apart from the employment of

NLP principles in different fields, including business, education and law, there have been studies showing the key role of NLP in influencing health outcomes and medicine, weight balance, dentistry, smoking cessation and pain management. More importantly, the key role of NLP has been explored in altering the behavior of individuals. Accordingly, it has been successfully employed in altering the behavior of individuals in depression, anxiety and phobia (3-5). The present study discusses the research describing the effectiveness of NLP training in altering the behavior of individuals in anxiety and phobia of different types.

Claustrophobia

Due to the closed, cylindrical nature of whole-body MRI scanners, the development of claustrophobia is very common in patients undergoing MRI scans and about 37% of patients develop symptoms of mild-to-severe anxiety undergoing the scan. Accordingly, about fourteen percent of patients are given sedatives before MRI to reduce the incidence of phobia. In certain cases, it becomes severe enough to cause MRI scan failure. More specifically, the patients feel distressing symptoms in response to spatial (space) restriction, temperature, long duration and acoustic noise. One study was performed on 50 people (24 males and 26 females) who had failed MRI examinations due to the development of claustrophobia with an aim

Key words: phobia; anxiety; neurolinguistic programming; PTSD; behavior

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to explore the effectiveness of NLP in reducing the need for general anesthetic in performing MRI scans. The duration of the scan was between 20 to 30 minutes and around 1 hour was spent by the NLP radiographer with each patient. In this study, the NLP technique named, "Clare's fast phobia cure" was employed (2). In this method, patients were asked to recount the unpleasant feelings experienced in the MRI scan chamber; thereafter, they were asked to associate (anchor) those unpleasant feelings to something present in that room. It was followed by a period in which patients were asked to think about pleasant/good memories. During that thought process (of good feelings), the NLP radiographer touched the patients on their hands or shoulders to anchor the experience. This procedure led to the development of comfort in patients with feelings of phobia and anxious thoughts associated with MRI scans (2). The development of anxiety was assessed using Spielberger's State-Trait Anxiety Inventory-based questionnaire. The results depicted that 38 (76%) patients out of 50 were able to complete the MRI scan and examinations were of good quality for further clinical reporting. Moreover, the anxiety levels were significantly less in the patients who completed the MRI. Although the anxiety levels were also reduced in the remaining 12 patients who were unable to complete the MRI scan, the anxiety score could not become low enough to complete the scan primarily due to very high preexisting anxiety prior to NLP. More importantly, due no need of the use of general anesthetic, the overall cost of MRI scanning was also reduced significantly, adding to the value of employing NLP before MRI to reduce the anxiety and claustrophobic feeling in patients (2).

Acrophobia and other phobias

Acrophobia refers to fear of heights and, as with other phobias, it may hamper the quality of life in a significant manner. There have been many behavioral interventions that have been tested in patients including desensitization, *in vivo* exposure, virtual reality exposure, negative practice, occupational actions and neurolinguistic programming. A randomized clinical trial was conducted to reveal the effectiveness of a single session of NLP on rapid

phobia cure in 96 subjects suffering from acrophobia (4). Another study revealed the effectiveness of NLP procedures in treating snake phobia (6). A study by Einspruch and Forman also explored the role of NLP in patients suffering from a phobia. They enrolled 31 patients (and 17 completed) and employed Mark's Phobia Questionnaire and Fear Inventory and the Beck Depression Inventory to quantify and assess phobia. The results depicted that eight weeks of NLP training led to a significant reduction in score of phobia and marked improvement in their disease state, signifying the effectiveness of NLP in phobia management (7).

Public speaking anxiety

Public speaking anxiety is very common in different populations and may hamper the academic and professional progress of students/professionals. The study performed by Krugman et al. (5) aimed to explore the efficacy of a single session of NLP of one 1 hour duration in controlling/attenuating the symptoms of anxiety associated with public speaking. The test was carried out on 55 subjects (28 male and 27 females). NLP training for these subjects was imparted by students of clinical psychology and counseling psychology. In one hour of NLP training, subjects were asked to re-experience the previous situations in which they acted (behaved) in a proficient manner, and during the thought process, they were touched on the shoulder to anchor the feelings and reduce the anxiety. Thereafter, they were asked to imagine a movie of their childhood with fear of public speaking. It was followed by instruction in which they were told to mix the images of acting in a proficient manner and fear of public speaking. The results depicted a significant reduction in the different parameters of anxiety in subjects undergoing NLP training, thus showing its effectiveness in attenuating public speaking anxiety (5).

Trait anxiety

Trait anxiety is a personality trait and it reflects the proneness to develop anxiety in individuals. It is a stable, long-term predisposition to respond to stressful stimuli with the development of anxiety. It is in contrast to state anxiety, which is, in fact,

a state of anxiety, apprehension, tension and worry. Konefal et al. (8) performed a study on 61 individuals (31 male and 30 females) to examine the influence of NLP training on trait anxiety. Considering trait anxiety of individuals as stable and long-lasting, the NLP training was given for a long period. Indeed, the participants were subjected to NLP training for 21 days, with three sessions each day, comprising demonstration, practice, and discussion. More precisely, for the first 10 days, the training consisted of building a rapport with others, analyzing the pattern of languages, understanding internal and external manifestations of behavior and communication, analyzing the changes in the physiological parameters (respiratory rate, color of skin, size of pupil) with changes in the affective state and developing specific goals for personal change. For the next 11 days, techniques were focused on minimizing/eliminating phobic responses to previous unpleasant events, developing new behaviors so as to avoid the undesired behavior in response to given situations, organizing the life experiences of individuals in a linear timeline to facilitate the change in behavior. The parameters of trait anxiety were assessed using Spielberger State-Trait Anxiety Inventory before and after NLP training. The results showed that there was a significant difference in the pre-test and post-test trait-anxiety scores, suggesting the beneficial effects of long-term NLP training in reducing the parameters of trait anxiety (8).

Occupational stress

Occupational stress i.e. stress at the workplace (job stress) is becoming very common these days and results due to unfavorable interactions taking place between the individual and the work environment. Along with other jobs, there is considerable stress in the nursing profession and nurses tend to experience work-related stress. There has been a study aiming to explore the effect of NLP training in reducing occupational stress in critical care nurses. In the study, sixty critical care nurses who participated were divided into a control and an experimental group. The experimental group received 18 sessions of NLP training over a period of 6 months, each session lasting for 180 minutes. The NLP training sessions included tasks such as

goal setting, time management, assertiveness skills, and representational system. The stress levels among nurses were assessed using Expanding Nursing Stress Scale (ENSS). The results showed a significant decline in stress score levels in the experimental group (64.53) following NLP training in comparison to the control group (120.96), receiving no training. Moreover, the stress score level was also significantly lower in the experimental group after treatment in comparison to basal levels (120.88) suggesting the efficacy of NLP training in attenuating occupational stress in critical care nurses (9).

Social anxiety

Social anxiety is also a common type of anxiety in which there is a marked and persistent fear in unfamiliar social situations. As in other types of anxiety, the role of NLP training has been evaluated in persons suffering from social anxiety (10). One study was performed on 28 participants (15 men and 13 women) with ages ranging from 20 to 60 years. NLP training was given for 21 days, which was further divided into 10 days and 11 days training. The basic procedure of NLP training was the same as explained in previously. The assessment of social anxiety and avoidance behavior was carried out using the Liebowitz Social Phobia Scale. The result depicted that there was a significant decline in fear (12.9) and avoidance (14.5) scores in patients following NLP training in comparison to basal, before treatment fear (20.3) and avoidance (20.1) scores suggesting the effectiveness of NLP in reducing the symptoms of social anxiety (10).

Conclusion

The employment of NLP as a therapeutic technique to understand and reprogram the unconscious patterns of thoughts and behavior has been very effective in bringing positive changes in the behavior of individuals suffering from claustrophobia, acrophobia, snake phobia, social anxiety, public speaking anxiety, trait anxiety, and occupational stress. However, more detailed studies at clinical levels are required to firmly establish it as a form of alternative therapy in managing subjects suffering from anxiety and phobia.

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LETTER TO THE EDITOR

Correlation analysis between polymorphisms of IL-2 gene and preeclampsiaQ. Yang^{1*}, X.-Y. Wei^{1*}, X.-H. Tang² and X.-Y. Chen¹¹*Department of Obstetrics, Maternal and Child Health Hospital of HuBei Province, Wuhan, China;*²*Department of Clinical Laboratory, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China**Received March 17, 2020 - Accepted June 11, 2020*

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To the Editor,

Eclampsia is one of the main causes of maternal and neonatal deaths. The pathogenesis of preeclampsia has not been fully elucidated. However, it is not only related to the eating and everyday habits of pregnant women, but is also associated with the difference in physique (1, 2). Some studies have confirmed that the main inflammatory factors in the blood of patients with preeclampsia change. For example, in some studies, the levels of interleukin (IL)-2, interferon (IFN)- γ and IFN- α were significantly elevated in comparison with those in normal control groups, while IL-10 and IL-4 were lowered compared with those in control groups (3, 4). The expression level of factors in serum is regulated by the upstream related gene, and if the structure or the promoter of regulatory gene is changed, it will inevitably affect the expression level of the factors (5). At present, the main way to influence gene structure is the single nucleotide polymorphism (SNP) caused by mutations in nucleotide bases. Many studies have demonstrated that SNP has a closer relationship with the occurrence of disease (6, 7). IL-2, as a main indicator for measuring autoimmunity and the degree of disease, is one of the frequently explored inflammatory factors (8, 9). Although IL-2 is notably increased in preeclampsia, the SNPs of IL-2

and the relationship between SNPs and preeclampsia are rarely reported. This study aims to investigate the relationship between IL-2 gene polymorphisms and preeclampsia by analyzing their distributions in a cohort of pregnant women.

MATERIALS AND METHODS

General data

A total of 150 patients with preeclampsia admitted to our hospital from August 2015 to August 2019 were randomly enrolled as the case group, while 200 healthy pregnant women during the same period were selected as the control group. No statistically significant differences were found in age, gestational week and living habits between the two groups ($P>0.05$), thus they were comparable (Table I). This study was approved by the Ethics Committee of Maternal and Child Health Hospital of HuBei Province. Signed written informed consents were obtained from all participants before the study.

Genomic deoxyribonucleic acid (DNA) extraction

A total of 5 mL of venous blood was collected using an EDTA (ethylenediaminetetraacetic acid) anticoagulant blood collection tube. Next, the

Key words: preeclampsia; IL-2; gene polymorphism; distribution frequency; onset risk

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genomic DNA extraction kits (Beijing CoWin Biosciences Co., Ltd., Beijing, China) were applied to extract the genomic DNA from cells in the blood. Subsequently, the concentration and purity of DNA were measured by Nanodrop (Roche Diagnostics, Basel, Switzerland). Finally, each subject's genomic DNA was equally divided into five 800 μ L Eppendorf (EP) tubes and stored at -20°C.

SNP typing via polymerase chain reaction (PCR)

The primer sequences for typing were generated through analysis by Oligo6.0, and the primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. Primer sequences were shown below rs2067006: Forward: 5'-GCAATTAGCTCATTGTGTGG-3' HEX: 5'-CCTGTTTTATCAACAAATC-3'; Reverse: 5'-TTGTGGTGGACAAGAGCAAG-3' F A M : 5'-CCTGTTTAATCAACAAATC -3'; rs2069776: Forward: 5'-CCTTCTGAGTG ATTCTGGT-3'; HEX: 5'-CTGAACTCTGACCAAAGCT-3'; Reverse: 5'-ACTGTATCTGAGTCCCAGCA -3' FAM: 5'-CTGAACTCTGACCAGAGCT -3'. The DNA solution, upstream and downstream primers as well as probe primers were added into the pre-configured TransStart Probe quantitative PCR (qPCR) SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China), and they were placed in the CFX96 fluorescence qPCR instrument (Bio-Rad,

Hercules, CA, USA) for reaction under the following conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. The experimental results were produced via the built-in software of the instrument. Three replicate wells were set for each sample, diethyl pyrocarbonate (DEPC)-treated water was adopted for the negative control, and a positive plasmid [synthesized by Sangon Biotech (Shanghai, China) Co., Ltd.] containing this sequence was selected for the positive control.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. The differences in the genotype distributions between the case group and the control group were statistically analyzed by *chi*-squared test. Logistic regression analysis was applied to test the correlation of each genotype with onset risk. $P < 0.05$ suggested that the difference was statistically significant.

RESULTS

Genotype distributions of rs2067006 and rs2069776 in the two groups

There were no statistical differences in the genotype frequencies of rs2067006 and rs2069776

Table I. Comparisons of general conditions between the two groups of patients

Index	Case group (n=150)	Control group (n=200)	t/χ^2	P
Age (years)	26.6±6.5	27.8±5.8	1.406	0.161
Gestational week	28.4±5.3	26.5±5.2	1.038	0.301
Smoking				
Yes	36 (24%)	34 (17%)	1.503	0.220
No	114 (76%)	166 (83%)		
Drinking alcohol				
Yes	58 (38.7%)	66 (33%)	0.706	0.401
No	92 (61.3%)	134 (67%)		
High glucose				
Yes	62 (41.3%)	76 (38%)	0.228	0.633
No	88 (58.7%)*	124 (62%)		
High salt				
Yes	49 (32.7)	58 (29)	0.321	0.571
No	101 (67.3)	142 (71)		

between the two groups ($P>0.05$), and all the gene distributions conformed to the Hardy-Weinberg equilibrium law (Rs2067006: Frequency=0.015; $P=0.241$ and rs2069776: Frequency=0.065; $P=0.327$) (Table II).

Onset risks of preeclampsia in different genetic models of rs2067006

The odds ratio (OR) value was 1.014 ($P=0.964$) in the dominant model (AT+AA/TT), 1.956 ($P=0.238$) in the recessive model (AA/TT+AT), and 1.877 ($p=0.242$) in the additive model (AA/TT) (Table III).

Onset risks of preeclampsia in different genetic models of rs2069776

The OR value was 1.671 ($P=0.071$) in the dominant model (AG+GG/AA), 1.976 ($p=0.103$) in the recessive model (GG/GG+AG), and 3.59 ($P=0.05$) in the additive model (GG/AA) (Table III).

Relationship between combination of rs2067006 with rs2069776 and preeclampsia

When pregnant women's venous blood carried both rs2067006 (A) and rs2069776 (G), the risk value of preeclampsia was 3.59 ($P=0.027$) (Table IV).

Table II. Distribution of genotype frequencies

Genotype	Case group (n=150)		Control group (n=200)		χ^2	P
	n	%	n	%		
rs2067006						
TT	94	62.7	126	63	0.002	0.964
AT	42	28	64	32	0.381	0.537
AA	14	9.3	10	5	1.393	0.238
T	230	76.7	316	79	0.153	0.696
A	70	23.3	84	21		
rs2069776						
AA	59	39.3	104	52	3.25	0.071
AG	64	42.7	76	38	0.459	0.498
GG	27	18	20	10	2.658	0.103
A	182	60.7	284	71	2.359	0.125
G	118	39.3	116	29		

Table III. Comparisons of onset risks of preeclampsia among different genetic models of rs2067006 and rs2069776

Model	Genotype	Control (n=200)	group	Case group (n=150)	OR (95% CI)	P
rs2067006						
Dominant model	TT	126 (63)		94 (62.7)	1	0.964
	AT+AA	74 (37)		56 (37.3)	1.014 (0.752-1.109)	
Recessive model	TT+AT	190 (95)		136 (90.7)	1	0.238
	AA	10 (5)		14 (9.3)	1.956 (1.213-2.324)	
Additive model	TT	126 (63)		94 (62.7)	1	0.242
	AA	10 (5)		14 (9.3)	1.877 (1.415-2.133)	
rs2069776						
Dominant model	AA	104 (52)		59 (39.3)	1	0.071
	AG+GG	96 (48)		91 (60.7)	1.671 (1.393-1.952)	
Recessive model	AA+AG	180 (90)		123 (82)	1	0.103
	GG	20 (10)		27 (18)	1.976 (1.471-2.331)	
Additive model	AA	104 (52)		59 (39.3)	1	0.05
	GG	20 (10)		27 (18)	2.38 (1.914-2.736)	

Table IV. Genotype frequency distributions and OR values of combination of rs2067006 with rs2069776.

Genotype		Control group (n=200)		Case group (n=150)		OR (95% CI)	P
		n	%	n	%		
rs2067006	rs2069776						
T	A	78	39	60	40	1	0.884
T	G	52	26	34	22.7	0.85 (0.651-1.232)	0.586
A	A	56	28	26	17.3	0.71 (0.632-0.909)	0.072
A	G	18	9	30	20	3.59 (2.814-5.026)	0.027

DISCUSSION

It has been reported that in addition to the common symptoms, the levels of immune factors in the blood of patients with preeclampsia will change significantly, such as ILs, IFNs, anti-tumor necrosis factors and other common inflammation-related factors. It is mentioned in a large number of studies that IL-2 has an increased tendency in the onset of preeclampsia (10). Rs2067006 and rs2069776 are two base sites related to the IL-2 gene. Rs2067006 mainly exists in the population as bases T and A, and rs2069776 in the form of A and G. Rs2067006 is located in the upstream promoter region, while rs2069776 is in the downstream region of the gene, and both are responsible for regulating the non-coding regions of the gene. It is reported in one study that rs2069776 polymorphism affects sleep in adults with acquired immunodeficiency syndrome (11). This study exhibited that the rs2067006 and rs2069776 were separately counted in eclampsia-affected populations and healthy populations. Meanwhile, there were no significant differences in the two polymorphisms in the dominant, recessive and additive models between the two groups. However, when considering the linkage equilibrium effect of two SNPs, it was found that if the pregnant women had both rs2067006 (A) and rs2069776 (G), the risk value of preeclampsia would be higher. Moreover, the proportion of people carrying these two types of mutant bases is nearly 20%, therefore attention should be paid to the prevention and occurrence of diseases in such pregnant women. There were also limitations to this study. First of all, there was no distinction between ethnic groups in the Chinese population. As different ethnic groups may

have genetic differences, comparisons between ethnic groups were not available. Secondly, the sample size of the case-control study was relatively small. Thirdly, the environmental information was insufficient, but as a matter of fact, the interaction between genes and the environment is also an important cause of preeclampsia. IL-2 gene polymorphisms are partly correlated with preeclampsia, especially displaying a higher onset risk in people who carry both rs2067006 (A) and rs2069776 (G).

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LETTER TO THE EDITOR

Exploring the anti-cancerous and anti-inflammatory potential of bovine meat by-product hydrolysates

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To the Editor,

The meat processing industry produces large quantities of waste by-products on a yearly basis which pose potential pollution threats to the environment. Meat industries usually generate 60% of animal by-products based on the live weight of the animal, out of which 40% is edible and 20% consists of inedible by-products. Waste generated by the meat industry serves as a valuable source of proteins and has gained more attention among food processing sectors for production of bioactive peptides through addition of animal by-products (1).

Dietary proteins have also been recognized for their bioactive properties in hydrolyzed form on the basis of consumption. Bioactive peptides in terms of structural confirmation comprise amino acids ranging from 2-30 units with specific sequences that exhibit functional and therapeutic potential (2). Inflammation affects a wide range of cells, tissues and organs as it is a complex and multisystem phenomenon. Oxidative stress in the body leads to the production of oxygen reactive species resulting in chronic inflammation and cancer, damage and alteration of DNA structure, mutation in gene expression and inhibition of normal apoptosis (3). Protein hydrolysates, as a novel chemotherapeutic

agent, have the ability to reduce oxygen reactive species and cancer cell growth and also enhance the induction of apoptosis in cancer cells (4). Keeping in mind the importance of meat by-product hydrolysates, the present study was designed to evaluate the anti-inflammatory and cytotoxic effect against RAW 264.7 and HCT-116, respectively.

MATERIALS AND METHODS

This research was conducted at the Meat Science and Technology Laboratory, NIFSAT, University of Agriculture, Faisalabad-Pakistan and the Department of Food Science, University of Massachusetts, Amherst, MA, USA. Meat by-products (liver, kidney and heart) were purchased from a local butcher's shop in Faisalabad-Pakistan while the reagents and chemicals were procured from local suppliers of Sigma-Aldrich (Germany), Merck (Germany) and Oxide (UK).

Bioactive peptides were generated through enzymatic hydrolysis by following the method of Jang et al. (5) with slight modifications. Blended meat samples were homogenized and diluted with 1:10 w/v with phosphate buffer (0.02M) at pH 7.4 by using the homogenizer (VELP, Scientifica, OV5). Extracted protein was centrifuged at 11,000 rpm for 30 min and collected supernatants

Key words: meat by-products, enzymatic hydrolysis, cytotoxicity, anti-inflammatory

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were introduced with constant substrate to enzyme ratio 100:1. Hydrolysis of meat by-products was performed in triplicate at predetermined time intervals (0, 2, 4, 6, 8 h) in a shaking water bath with the agitation speed of 150 rpm. The degree of hydrolysis of meat by-products was determined by following the method of Stadnik (6):

$$\text{Degree of Hydrolysis (\%)} = \frac{10\% \text{ Trichloroacetic Acid TCA} - \text{Soluble Nitrogen} \times 100}{\text{Total Protein}}$$

The method of Telez et al. (7) was used to determine the anti-inflammatory activity of hydrolyzed proteins from bovine meat by-products by adopting a slight modification.

Effect of bovine meat by-products against cancer line

The method of Jang and Lee (8) was used to determine the cytotoxic effect of hydrolyzed protein of meat by-products by using the MTT-assay with slight modification. For this purpose, human colon cancer HCT-116 cells (15000 cells /ml) were used as cancer cell model.

Statistical analysis

All the experiments were carried out in triplicate to explore the bioactivities of bioactive peptides. SPSS software (version 19) was used to check the level of significance at 5% confidence interval.

RESULTS

Degree of hydrolysis

Fig. 1 shows that enzymatic activity and hydrolysis time at 0, 2, 4, 6 and 8 h had a highly significant

($p < 0.01$) effect on generation of bovine meat by-product (liver, kidney and heart) hydrolysate. The highest enzymatic activity was observed in liver and varied from 7.31% to 30.55% which further increased significantly with increase in hydrolysis time, while minimum hydrolysis was noted in heart ranging from 3.51% to 20.23% which also showed a similar trend to that observed in liver hydrolysis.

Determination of anti-inflammatory activity

Meat by-product hydrolysates and hydrolysis time had non-significant ($p > 0.05$) effect on cell viability. The overall cell viability varied from 87.40% to 89.6% during different hydrolysis times, as shown in Table I.

The results presented in Table I show that protein hydrolysates had significant ($p < 0.01$) reduction of nitric oxide (NO) production during hydrolysis. It is also obvious from the results that maximum NO production inhibition was observed in liver after 6 h of hydrolysis whereas, minimum inhibition was noted in heart hydrolysates.

Effect of bovine meat by-products hydrolysates against cancer line HCT-116

The cytotoxic activity of protein hydrolysates, hydrolysis time and concentration of hydrolysates are depicted in Fig.2. The cytotoxic activity of liver hydrolysates varied significantly ($p < 0.01$) for different concentrations of hydrolysates as compared to kidney and heart hydrolysates. As the degree of

Table I. Mean values (%) of meat by-product hydrolysates concentrations (mg/mL) and hydrolysis time (hr) on cell viability and on NO production

Hydrolysis Time (hr)	Cell Viability				Nitric oxide Production			
	Liver	Kidney	Heart	SEM	Liver	Kidney	Heart	SEM
0	87.80 ^{aA}	87.70 ^{aA}	87.40 ^{aA}	1.5732	95.00 ^{aA}	97.00 ^{aA}	98.00 ^{Aa}	2.5103
2	88.50 ^{aA}	88.30 ^{aA}	87.60 ^{aA}	1.5412	54.60 ^{cB}	59.30 ^{cA}	62.50 ^{Ca}	1.3238
4	88.90 ^{aA}	88.70 ^{aA}	87.80 ^{aA}	2.248	50.30 ^{cdB}	54.10 ^{dB}	58.30 ^{cdA}	1.255
6	89.60 ^{aA}	89.20 ^{aA}	88.30 ^{aA}	1.8333	44.90 ^{dC}	49.70 ^{eB}	53.20 ^{Da}	0.9157
8	88.10 ^{aA}	87.90 ^a	87.70 ^a	2.4465	62.80 ^{bC}	69.20 ^{bB}	75.30 ^{bA}	1.384
SEM	2.0077	2.2447	1.575		1.9137	1.0329	1.6433	

For cell viability, ^{acd} column-wise comparison (within a column) and ^{ABC} show comparison of means (within a row).

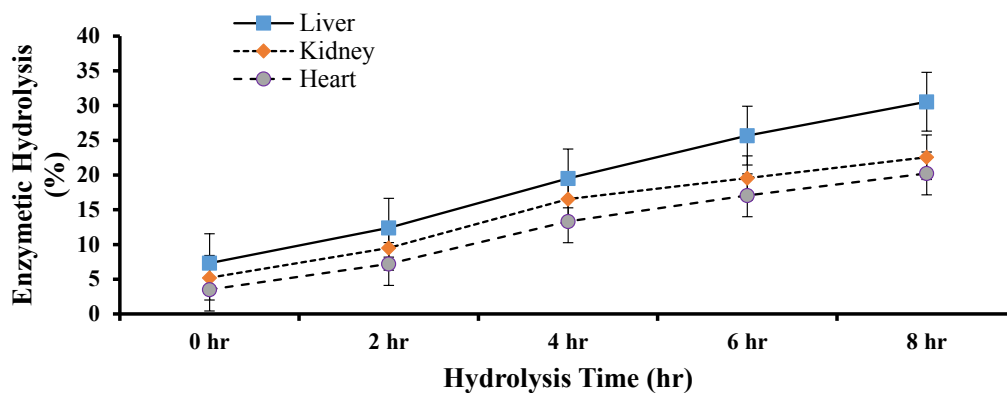


Fig. 1. Interactive effect of meat by-products and hydrolysis time (hours) on degree of hydrolysis

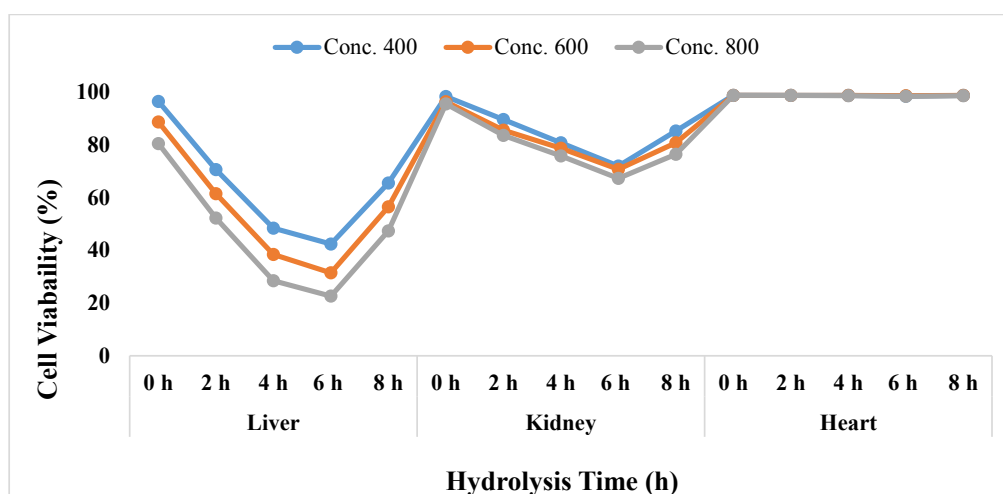


Fig. 2. Interactive effect of liver, kidney and heart hydrolysates concentrations (ug/mL) and hydrolysis time (hours) for cytotoxic effect (%) against colon cancer cells (HCT-116).

hydrolysis increased the number of peptides having low molecular weight increased, showing more cytotoxic activity than kidney and heart. The highest cytotoxic activity of liver hydrolysates (22.60%) was observed at a concentration of 800ug/ml after 6 h of hydrolysis. The results expressed in Fig. 2 show the cytotoxic activity of kidney hydrolysates (67.20%) at a concentration of 800ug/ml after 6 h of hydrolysis while heart hydrolysates showed minimum cytotoxic activity after different hours of hydrolysis.

DISCUSSION

Molecular weight of bioactive peptides as well as their amino acid sequence and bioactivities

are mainly affected by the substrate, proteolytic enzymes and hydrolysis conditions. In one study, the effect of plant enzymes on sarcoplasmic and myofibrillar proteins was probed and similar results were observed that high degree of hydrolysis results in maximum solubility of proteins (9).

Minimum cells were killed by bioactive peptides generated by the enzyme hydrolysis after different hour showing that type of peptide and amino acids sequence, friendly to cells and did not induce any DNA damage and toxicity. However, presence of NO leads to inflammation related tissue damage and cells therefore suppression of NO production is important for the prevention and treatment of inflammatory diseases. So, the decrease of NO production in Raw

264.7 macrophage cells without cell toxicity was due to low mol. wt. bioactive peptides generated by enzymatic hydrolysis that inhibits the tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and NO synthase (10). The results regarding decrease in NO production are in line with the earlier finding of (11), who also observed the decrease in NO production for macrophages cells (Raw 264.7) by using bovine lung hydrolysates.

Several studies have confirmed that fractions of meat proteins obtained after enzymatic hydrolysis have potent anti-inflammatory and cytotoxic activity against colorectal cancer (HCT-116) cells (12). In another study, similar results were noted that fish protein hydrolysates possess anti-proliferative activity against colon (HCT-116) and breast cancer (MCF-7) cells (11).

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LETTER TO THE EDITOR

Effect of curcumin on wound healing in a murine model of diabetic footX. Deng¹, F. Li², Y. Li³, B. Yang³, R. Han³, C. Tang³ and F. Fang³

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To the Editor,

Curcumin (CUR) is a yellow acidic phenolic substance extracted from the rhizomes of *Curcuma longa* with many applications in traditional Chinese medicine that have attracted the attention of scientists (1). A large number of studies have proved that CUR has many therapeutic properties such as anti-inflammatory, antioxidant, anti-cancer, promotion of wound healing and is effective in prevention and treatment of cardiovascular diseases and neurodegenerative diseases (1, 2). CUR has antioxidant and anti-inflammatory effects, but its specific mechanism is not fully elucidated. CUR can antagonize the effect of a variety of reactive oxygen species (ROS), increase the activity of antioxidant enzymes, and block the activity of ROS synthetase (3). Recent studies have shown that the phenolic group and methoxy group on the phenyl group from the structure of CUR are important for its antioxidant activity (4). Due to the ability to remove ROS, CUR can reduce oxidative damage of proteins, fats, and DNA (5). CUR can also improve the activity of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione, glutathione transferase, and glutathione peroxidase (5). Modulation of p38MAPK, JNK, and NF- κ B levels can play an important role

in the antioxidant effect of CUR (6). In diabetic patients, local skin tissues are in a state of high glucose for a long time, leading to the accumulation of oxidation products that determine the appearance of oxidative stress responsible for promoting the apoptosis of skin tissue cells and determining nerve and microvascular damage (7). Changes in skin histology may aggravate the progression of diabetic foot (DF), thus resulting in difficulty in wound healing. Therefore, reducing oxidative stress is an important strategy for promoting the healing of DF wounds. In this study, we aimed to evaluate the therapeutic effect of CUR on DF ulcer and the underlying mechanism in a murine model.

MATERIALS AND METHODS*Animal model of diabetic food ulcer*

Forty-six CD - SD male rats (200 $\pm$ 20g, aged 8-10 weeks) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., China. The animal experiment was approved by the Ethics Committee of Chongqing Traditional Chinese Medicine Hospital.

Ten rats were randomly selected from the 46 rats and used as a control group. The rat from the control group received normal rat feed during the experiment. The other 36

Key words: CUR; type 2 diabetes mellitus; healing mechanism; phosphorylation JNK; oxidative stress

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rats were used for obtaining the diabetic food ulcer model. The animals received high-sugar and high-fat diets for 8 weeks followed by 14 h of fasting and one intraperitoneal injection of Streptozotocin (Sigma, USA)(35 mg/kg bw). After 8 weeks of normal rat feed and 14 h of fasting, the control group of rats received one intraperitoneal injection with an equal volume of citric acid-sodium citrate buffer (Nanjing Senbeiga Biotechnology Co., Ltd., China). After 1 week, the Glucose (GLU) levels were measured by a glucose meter (ACCU-CHEK Performa Connect; Roche, Germany). The rats with fasting GLU above 11.1 mmol/L were selected for the DF model group. On each selected animal, a rectangular full-thickness skin (2mm×5mm) was cut on the back of the left foot to prepare the DF model. The same wound was also carried out on the rats from the control group.

The thirty successful DF rats (6 rats were eliminated) were randomly divided into 3 groups based on fasting GLU value and wound area: model group, alpha-lipoic group, and CUR group, 10 rats per group. Alpha-lipoic acid, a known antioxidant compound with anti-inflammatory effects, was used as a positive control to compare and analyze the effects of CUR. The treatment started one day after obtaining the wound. The rats from the alpha-lipoic group received alpha-lipoic acid (Hubei Dixin Chemical Manufacturing Co., Ltd., China) in doses of 100 mg/kg bw/day for 18 days. The rats in the CUR group received CUR (Shaanxi Jianhe Biotechnology Co., Ltd., China) in doses of 200 mg/kg bw/day for 18 days. The rats in the control group and the model group received the same amount of normal saline for 18 days. During the treatment, the rats from the control group received standard rat diet, while the rats from the model group, alpha-lipoic group and CUR group received high-sugar and high-fat diet.

Observation of wound healing

The wound healing was evaluated every day, and the complete epithelial coverage was considered as the basis for wound healing. The ulcer wounds of rats were photographed before the start of treatment (0 day) and after 4, 8, 13, and 18 days, respectively. Image-pro Plus, 6.0 Image analysis software, was used to measure the ulcer wound area and calculate the wound healing rate using the following equation: wound healing rate = [(original wound area - unhealed wound area)/original wound area] × 100%.

Detection of GLU, lipids, and insulin levels

After 18 days of treatment, the rats were fasted for 12 h, anaesthetized with 10% chloral hydrate (0.3g/kg) (Guangzhou Peiyu Biological Products Co., Ltd., China), and the blood was collected from the common carotid artery. After collection, the blood was kept at room temperature for 1 h, centrifuged at 4°C for 10 min at 3500 rpm, and then the supernatant was separated into EP tubes, and frozen at -20°C for later use. GLU was measured by micro GLU meter (ACCU-CHEK Performa Connect; Roche, Germany), blood lipid level (TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-Density Lipoprotein Cholesterol; HDL-C: High-Density Lipoprotein Cholesterol) of rats in each group was detected by an automatic biochemical analyzer (Chengdu Simate Technology Co., Ltd., China), and insulin level was detected by radioimmunoassay (RIAS) (Kit: Shanghai Xinfan Biotechnology Co., Ltd., China; radioimmunoassay: Zhongcheng Electromechanical Technology Development Co., Ltd., China). Insulin resistance index (IRI) ($\text{GLU} \times \text{blood insulin} / 22.5$) was calculated.

Detection of hemorheology index

Whole blood containing 3.8% sodium citrate was collected, and a semi-automatic hemorheological instrument measured the whole blood viscosity. After centrifugation at 1000 rpm for 10 min, platelet-rich plasma (250 µL) was collected and induced by 10 µL ADP (Adenosine diphosphate; Shanghai Boao Biotechnology Co., Ltd., China) (final concentration: 5.54 µg/mL). Platelet aggregation rate (PAgT) was determined by a platelet aggregation coagulation factor analyzer (Beijing Zhongqinshidi Scientific Instrument Co., Ltd., China). After centrifugation for 10 min at 3000 rpm, the platelet-poor plasma was collected and used to determine the prothrombin time (PT), thrombin time (TT), and activated partial thromboplastin time (APTT).

Detection of oxidative stress markers

On the 18th day of treatment, the rats were anaesthetized with 10% chloral hydrate (0.3g/kg) and sacrificed by spinal dislocation, and the full-thickness tissue of the wound was excised. Xanthine oxidase method and 5-dithiobis-2 -nitrobenzoic acid (DTNB) staining method were used to determine the levels of superoxide dismutase (SOD) and glutathione (GSH) in tissues.

Hematoxylin & eosin staining

The wound tissues were fixed in 10% paraformaldehyde fixative solution (Wuhan Kanuosi Technology Co., Ltd., China) for 24 h, then dehydrated in an automatic dehydrator for 12 h, followed by paraffin embedding. Sections with a thickness of 5 μ m were prepared for hematoxylin & eosin (H&E) staining and observed under the microscope (Shenzhen Boshida Optical Instrument Co., Ltd., China) and photographed.

Immunohistochemistry

The expressions of VEGF and p-JNK in wound tissues were detected by SP immunohistochemical staining. The wound tissues of rats were immobilized with 10% neutral formaldehyde for 48 h and then paraffin sections were prepared and stained according to the instructions of SP immunohistochemical kits (Wuhan Moshake Biotechnology Co., Ltd., China). The samples were observed and photographed under a light microscope.

Western blot

Approximately 100 mg of tissue was used to extract total tissue proteins. Western blot was used to detect the expression of VEGF and p-JNK in wound tissue (VEGF, p-JNK, β -actin primary antibody and horseradish peroxidase labelling the second antibody; Shanghai Biyuntian Biotechnology Co., Ltd., China). The antibody binding zone was detected by chemiluminescence. The film was scanned, and the strip was scanned for

absorbance integration under Labworks software (UVP, USA). β -actin was used as an internal reference and the ratio of the film gray value of the target gene/gray value of β -actin internal reference film was used to calculate the relative expression of the target protein.

Statistical analysis

The data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$) and statistical analysis was performed using SPSS 19.0 software (IBM, USA). Comparison between groups was made by one-way analysis of variance (ANOVA), and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Wound healing rate

On the 18th day of treatment, the wounds of the rats in the control group were basically healed, while the wounds in the model group healed slowly ($P < 0.01$). Compared with the model group, both the alpha-lipoic acid group and the CUR group had significantly improved wound healing rate at each time point ($P < 0.05 \sim 0.01$) (Table I).

GLU, lipids, and insulin levels

The glucose (GLU), blood insulin (INS) and insulin resistance index (IRI) in the model group were significantly increased compared to the control group ($P < 0.05$). After treatment with CUR, GLU, INS, and IRI decreased significantly compared

Table I. The wound healing rate

	Before treatment (Day 0)	Day 4	Day 8	Day 13	Day 18
Control group	6.86 $\pm$ 1.02	33.94 $\pm$ 2.36	63.97 $\pm$ 7.12	80.98 $\pm$ 5.23	93.92 $\pm$ 3.53
Model group	7.02 $\pm$ 0.97	19.74 $\pm$ 3.14**	27.05 $\pm$ 7.13**	39.89 $\pm$ 8.03**	56.88 $\pm$ 6.24**
Alpha lipoic group	6.97 $\pm$ 0.89	27.7 $\pm$ 3.91*#	50.10 $\pm$ 7.51***	69.65 $\pm$ 7.68***	85.22 $\pm$ 3.31***
CUR group	7.89 $\pm$ 0.91	30.43 $\pm$ 7.35***	50.22 $\pm$ 7.47***	73.93 $\pm$ 3.97***	87.1 $\pm$ 3.34***

Note: * $P < 0.05$; ** $P < 0.01$ compared with the control group, # $P < 0.05$, *** $P < 0.01$ compared with model group.

Table II. Effects of treatment on biochemistry and oxidative stress markers

	Control group	Model group	Alpha-lipoic group	CUR group
GLU(mmol/L/ml)	6.34±1.13	29.51±1.65**	13.25±2.16*	12.89±2.71*
INS(uIU/ml)	8.75±0.88	11.11±2.64*	5.98±0.47	5.79±0.35
IRI	2.47±0.58	14.57±3.77**	3.52±0.53	3.32±0.62
TG(mmol/L)	0.32±0.03	0.98±0.07**	0.44±0.04*##	0.41±0.03*##
TC(mmol/L)	2.53±0.09	6.19±0.12**	3.83±0.08*##	2.98±0.04##
LDL-C(mmol/L)	1.17±0.06	4.75±0.08**	1.32±0.05*#	1.27±0.06*#
HDL-C(mmol/L)	1.80±0.08	0.79±0.06**	1.36±0.07*##	1.41±0.09*##
PT/s	20.71±2.07	14.98±0.94*	18.25±1.61#	18.53±1.58#
TT/s	37.35±1.46	28.47±2.46*	34.31±1.01#	34.54±1.24#
APTT/s	19.88±1.01	14.31±1.89*	18.05±1.34#	18.37±0.87#
SOD/(U/mgprot)	58.3±3.42	34.41±4.02**	56.26±3.44##	56.54±3.22##
GSH(U/mgprot)	16.35±1.25	10.32±1.4**	15.54±1.23##	15.65±1.56##

Note: compared with the control group, * indicates $P < 0.05$, ** indicates $P < 0.01$; compared with the model group, # indicates $P < 0.05$, ## indicates $P < 0.01$. GLU: Glucose; INS: Insulin; IRI: Insulin resistance index; TG: Triglyceride; TC: Total cholesterol; LDL-C: Low-Density Lipoprotein Cholesterol; HDL-C: High-Density Lipoprotein Cholesterol; PT: prothrombin time; TT: thrombin time; APTT: activated partial thromboplastin time; SOD: superoxide dismutase; GSH: glutathione.

to the model group ($P < 0.05$). The levels of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) in the model group were all increased compared to the control group. In comparison, the level of high-density lipoprotein cholesterol (HDL-C) were lower compared to the control group ($P < 0.01$). The treatment with CUR and alpha-lipoic acid significantly decreased the levels of TC, TG, and LDL-C and significantly increased the level of HDL-C compared to the model group ($P < 0.01$) (Table II).

Hemagglutination indexes in rats

PT, TT, and APTT in the model group were significantly decreased compared to the control group ($P < 0.01$). The treatment with alpha-lipoic acid and CUR significantly increased the levels of PT, TT, and APTT compared with the model group, with no differences between the two (Table II).

Activity of SOD and GSH in wound tissue

SOD and GSH levels in the wound tissue of the model group were significantly decreased compared

to the control group ($P < 0.01$). The treatment with alpha-lipoic acid and CUR significantly increased the levels of SOD and GSH compared with the model group ($P < 0.01$) (Table II).

HE staining of local ulcer tissues

For the pathological observation of HE staining, the maximum diameter of the ulcer surface was selected as far as possible. The results showed that compared with the control group, the model group had less granulation tissue and more inflammatory cells. Compared with the model group, the alpha-lipoic group and CUR group had abundant granulation tissue, less inflammatory cell infiltration, and fibroblasts with increased regular arrangement (Fig. 1A).

Changes of VEGF and p-JNK expression in local ulcer tissue

Immunohistochemical results showed that the positive expression of VEGF protein was brownish yellow staining. Compared to the control group, the expression of VEGF in local ulcer tissues was

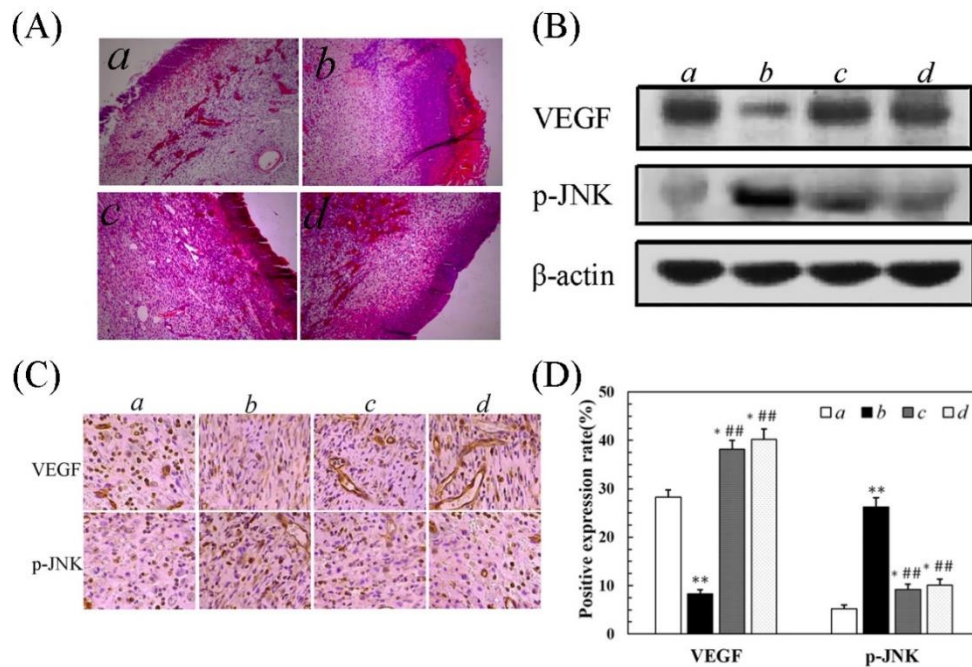


Fig. 1. *A)* H&E staining; *B)* Expression of VEGF and p-JNK in local ulcer tissues detected by Western Blot; *C)* Expression of VEGF and p-JNK in local ulcer tissues detected by immunohistochemistry ($\times 400$); *D)* the positive expression of VEGF and p-JNK; (a: control group; b: model group; c: alpha-lipoic group; d: CUR group;) (* $P < 0.05$, ** $P < 0.01$ compared to the control group; *** $P < 0.01$ compared to the model group)

decreased in the model group. Compared to the model group, the expression of VEGF in the alpha-lipoic group and CUR group was increased, suggesting that the treatment with alpha-lipoic acid and CUR could promote angiogenesis in the local tissues of DF ulcer. On the contrary, compared to the control group, the expression of p-JNK in the model group was significantly increased ($P < 0.05$). The expression of p-JNK in both the alpha-lipoic group and the CUR group was significantly decreased compared to the model group ($P < 0.05$) (Fig. 1 C, D).

Western Blot results showed that the expression of VEGF in the CUR group (0.389 ± 0.012) and the lipoic acid group (0.372 ± 0.016) increased compared to the model group (0.131 ± 0.013) ($P < 0.05$), and decreased compared to the control group (0.412 ± 0.015) ($P < 0.05$). The expression of p-JNK in the CUR group (0.208 ± 0.034) and the lipoic acid group (0.210 ± 0.025) decreased compared to the model group (0.503 ± 0.097) ($P < 0.05$), and significantly increased compared to the

control group (0.116 ± 0.091) ($P < 0.05$). There was no significant difference in various indexes between the CUR group and the alpha-lipoic group ($P > 0.05$) (Fig. 1B).

DISCUSSION

Recent studies on hemorheology have found that hemorheological disorders caused by hyperglycemia and hyperlipidemia are one of the risk factors for the formation of DF (8). The high adhesion and aggregation state of platelets caused by high blood lipid and GLU and the increase of blood viscosity can cause microcirculation disorder, lead to capillary occlusion, tissue ischemia, and hypoxia, and promote the occurrence of diabetes (8). This study showed that the PT, TT, and APTT time in rats from the model group was significantly decreased compared with the control group, indicating that the wound healing speed was slow. Treatment with alpha-lipoic acid and CUR significantly accelerated the healing of

foot ulcers. Other studies have found that oxidative stress is involved in the occurrence and development of diabetes and its complications (9). SOD and GSH are adequate free radical scavengers *in vivo*, and their catalytic activity is essential for maintaining the oxidation balance system (10). Alpha-lipoic acid is a powerful antioxidant, which has good antioxidant stress and anti-inflammatory effects (11). In this study, CUR had similar effects to alpha-lipoic acid in reducing the level of blood lipid, accelerating the wound healing of DF, inhibiting the formation of oxidative stress in the body, and enhancing the body antioxidant capacity by increasing the levels of SOD and GSH in the wound tissue. The mechanism through which CUR promotes microangiogenesis and wound healing in DF ulcer is associated with its ability to improve the VEGF level in the local tissues and down-regulate the expression of p-JNK.

VEGF is a multifunctional specific cytokine that promotes the proliferation and migration of endothelial cells, increases the multiplication times of vascular endothelial cells, and induces angiogenesis (12).

JNK signalling pathway plays an important role in the pathogenesis of T2DM. In the state of oxidative stress and acute and chronic inflammation, JNK has an inhibitory effect on insulin signal transduction (13). In conclusion, CUR can promote the healing of foot ulcers in T2DM rats by modulating oxidative stress through up-regulation of VEGF and down-regulation of the JNK signalling pathway.

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LETTER TO THE EDITOR

**Evaluation of SARS-CoV-2 in the female reproductive tract of COVID-19 patients:
A prospective study**

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To the Editor,

Infection of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as the cause of the 2019 novel coronavirus disease (COVID-19) (1). The newly identified virus is a highly infection disease, causing outbreak all over the world (2). By April 23, 2020, this virus had affected more than 2,500,000 people worldwide and caused more than 170,000 deaths. About half of those infected are females (3). COVID-19 can cause multiple system dysfunction. The latest study reported that the virus affects the male reproductive system (4), however, the data about the effects of SARS-CoV-2 on the female reproductive system is limited.

SARS-CoV-2 has approximately 85% sequences similar to SARS virus, which caused an outbreak of severe acute respiratory syndrome (SARS) in 2003 (5). The related research on the female reproductive system mainly focused on the mother-to-child transmission. A study of pregnant women diagnosed

with COVID-19 pneumonia also showed no virus found in amniotic fluid, cord blood, neonatal throat swab or breast milk (6). However, the potential route of SARS-COV-2 to the reproductive system remains unclear.

In this study, the risk factors associated with reproductive system infections were investigated in young female patients diagnosed with common COVID-19 pneumonia. The study and similar investigations will provide further insight into the transmission route of this new type of highly infectious virus.

MATERIALS AND METHODS

This prospective interventional case series study was reviewed and approved by the Medical Ethics Committee of The Fourth Affiliated Hospital, Zhe Jiang University School of Medicine (approval number K20200025 and K2020030). Written informed consent was signed by all enrolled patients.

Key words: COVID-19; vaginal fluids; reproductive system; SARS-CoV-2

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Study subjects

Seven confirmed COVID-19 female patients diagnosed at the Fourth Affiliated Hospital of Zhejiang University from January 26, 2020 to March 29, 2020 were included in the study. Diagnosis of COVID-19 pneumonia was based on the New Coronavirus Pneumonia Prevention and Control Program (5th edition) published by the National Health Commission of China. Respiratory samples of all seven women with COVID-19 pneumonia were tested for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection by RT-PCR.

All respiratory, vaginal, blood, and feces samples were taken from each patient at 3-4 day-intervals. All samples diagnosed with the novel coronavirus were collected for reverse-transcription polymerase chain reaction (RT-PCR) assay.

Study protocol

A disposable sampling swab was used to collect the patients' secretions from the posterior vaginal fornix. Respiratory secretions included sputum specimens and nasopharyngeal swabs. When each sample was taken, the

Table I. Summary of patient characteristics

	All patients(n=7)
Age Median (range)-yrs	46.57 (24-72)
Age groups-no./total no. (%)	
15-45yrs	4/7 (57.14)
≥45yrs	3/7 (42.86)
Female reproductive system complications	
Symptoms (e g, abnormal leucorrhea, itching)	0/7 (0)
B-Mode Ultrasound Image/CT (e g, Uterine fibroids, ovarian tumors)	0/7 (0)
Exposure history within 14 days - no./total no. (%)	
Recently been to Wuhan	1/7 (14.29)
Contact with infected person	6/7 (85.71)
Starting symptoms - no./total no. (%)	
Fever	4/7 (57.14)
Cough	3/7 (42.86)
Myalgia or fatigue	2/7 (28.57)
Dyspnoea	0/7 (0)
Sore throat	1/7 (14.29)
Diarrhea	1/7 (14.29)
Diagnosis[#] (Clinical type) - no./total no. (%)	
Mild	0/7 (0)
Common type	7/7 (100)
Severe	0/7 (0)
Critical type	0/7 (0)
Treatment	
Antiviral therapy	7/7 (100)
Use of corticosteroid	0/7 (0)
By the time of first collection of vaginal discharge, the mean onset time (days)	9.3
Patient1	8
Patient2	10
Patient3	7
Patient4	10
Patient5	6
Patient6	14
Patient7	10
The average body temperature of the patients (°C)	36.63
Patient1	36.63
Patient2	36.74
Patient3	36.87
Patient4	36.91
Patient5	36.23
Patient6	36.42
Patient7	36.67
[#] Diagnosis of COVID-19 pneumonia was based on the New Coronavirus Pneumonia Prevention and Control Program (5th edition) published by the National Health Commission of China.	

Baseline information, laboratory diagnosis, and treatment process of the included cases were obtained from the electronic medical record system. All information was obtained and cured with a standardised data collection form. Two researchers independently reviewed the data collection forms.

Statistical analysis was carried out using the SPSS software, version 22.0. Continuous data were expressed as mean $\pm$ standard deviation (SD) if they were normally distributed, or as median and interquartile range (IQR) if they did not follow a normal distribution. Categorical variables were expressed as counts (%). For laboratory results, whether the measurements were outside the normal range was also assessed.

The detailed characteristics of the study participants are provided in Table I. All seven female patients included in our study were diagnosed with the common type of COVID-19 pneumonia and had a history of epidemiological exposure to SARS-CoV-2. Four of the patients had fever. Other symptoms at admission included cough (3/7), myalgia (2/7), sore throat (1/7), and diarrhea (1/7). None of the patients had female reproductive system conditions, such as abnormal leucorrhea, itching, uterine fibroids, or ovarian tumors. All patients received antiviral treatment. The patients were aged 24-72 years [average (46.6±19.6) years], of whom

Patient	Confirmatory Test	Sampling Numbers	Specimen				
			Sputum	NS	Feces	Blood	VF
1	NS (+)	2	+	+	-	-	-
2	NS (+)	4	+	+	-	-	-
3	NS (+)	5	+	+	-	-	-
4	NS (+)	6	+	-	+	-	-
5	NS (+)	4	+	+	-	-	-
6	NS (+)	5	+	+	-	-	-
7	NS (+)	5	+	+	+	-	-
Total-no./total no. (%)		31	7/7 (100)	6/7 (85.7)	2/7 (28.6)	0/7 (0)	0/7 (0)

“+”: Positive; “-”: Negative; NS: Nasopharyngeal swab; VF: Vaginal Fluids

57.14% were aged between 20-45 years. When the vaginal discharge was collected for the first time, the mean onset time of the symptoms was (9.3±2) days.

SARS-CoV-2 nucleic acid detection

The results of SARS-CoV-2 nucleic acid detection in different specimens by RT-PCR are summarized in Table II. All patients were positive for SARS-CoV-2 nucleic acid in nasopharyngeal swab samples before admission. Each patient was tested an average of 4-5 times during hospitalization. All 31 blood and vaginal fluids samples obtained from the seven patients produced negative RT-PCR results. The positive detection rates in sputum, nasopharyngeal swab, and feces were 100%, 85.7%, and 28.6%, respectively.

DISCUSSION

The clinical data characteristics of the patients included in our study were consistent with those in previous studies. None of the patients showed symptoms or signs of diseases related to the female reproductive system during the short-term observation period.

SARS-CoV-2 belongs to the same subgenus as the severe acute respiratory syndrome virus (SARS-CoV), the Middle East respiratory syndrome virus (MERS-CoV) (7), and the mumps virus (8), which may cause long-term complication in the testes, such as orchitis and infertility. However, no evidence showed the presence of the virus in the female reproductive system.

This study has certain limitations. Firstly, the number of cases included in our analysis was relatively small. Moreover, the follow-up time was short and the evaluation was limited.

Our study provides further insight into the effects of SARS-CoV-2 infection on the reproductive system of female COVID-19 patients. Currently no evidence shows that SARS-CoV-2 virus exists in the vaginal fluids and serum of the common type COVID-19 patients. However, the lack of evidence of SARS-CoV-2 infection in the vaginal fluids does not mean that other parts of the female reproductive system, such as the uterus, ovaries, and fallopian

tubes, are not affected by the virus. Large cohort comprehensive studies are needed to evaluate the effect of the virus more systematically on the female reproductive system in the future.

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LETTER TO THE EDITOR

Effects of different doses of methylprednisolone on TNF- α , IL-6, and IL-13 in serum and bronchoalveolar lavage fluid of children with severe mycoplasma pneumoniae pneumoniaXF. Qu^{1#}, Z. Xu^{2#} and X.F. Lin³

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To the Editor,

Mycoplasma pneumonia (MP) is a common virulence factor of community-acquired pneumonia (CAP) of school-age children and is also a dominant factor that leads to lower respiratory tract infection. In the pediatric population, MP accounts for 10-40% of lower respiratory tract infection, and is one of the main reasons of child deaths (1). Although MP infection is a self-limited disease, patients with severe mycoplasma pneumoniae pneumonia (MPP) are often accompanied with multiple organ damage (such as hepatitis, myocarditis, encephalitis) or severe pulmonary diseases, such as obliterative bronchiolitis, bronchiectasis, or atelectasis (2). Macrolide antibiotics are often used to treat MPP, however, in recent years, the resistance to these drugs is on the increase, thus the efficacy is badly affected (3). Methylprednisolone is a type of glucocorticoid that contributes to good anti-inflammatory effects and can improve the immune function as well as alleviate the clinical symptoms of patients (4).

At present, the pathogenesis of severe MPP is still unclear. Previous studies have shown that cytokines excessively released by the body and immune

responses may be involved in its development (5), and that the level of cytokines in serum and bronchial alveoli is associated with the severity of pulmonary inflammation (6). Tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine that can lead to inflammation and death of apoptotic cells and can stimulate and mediate the release of some cytokines, such as interleukin(IL)-6 and IL-1 β (7). The level of IL-6 in bronchoalveolar lavage fluid (BALF) or plasma of patients with pneumonia is significantly higher than that in healthy people (8). IL-13 is a Th2-type cytokine closely related to the clinical outcomes of patients with systemic inflammatory response syndrome or sepsis (9). In the present study, children with severe MPP were treated with methylprednisolone and the effects of TNF- α , IL-6, and IL-13 were investigated.

MATERIALS AND METHODS

Study subjects

One hundred and twenty-eight children with severe MPP, who were diagnosed in Qingdao Women and Children Hospital from April 2015 to August 2017, were

Key words: dose; IL-6; IL-13; methylprednisolone; pneumonia; severe mycoplasma pneumoniae TNF- α

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used as study subjects. Children who were treated with a small dose of methylprednisolone were included in group A (63 children), whilst those treated with a large dose of methylprednisolone were included in group B (65 children). There were 30 males and 33 females in group A aged 2-12 years, average (5.8 ± 1.5) years. Their disease course was between 7 days and 30 days, average (13.2 ± 4.4) days. Group B included 29 males and 36 females aged 2-13 years, average (5.6 ± 1.3) years. Their disease course was between 8 days and 32 days, average (13.6 ± 4.7) days. This study was approved by the medical ethics committee of the hospital. The guardians of the study subjects were informed and gave informed consent.

Inclusion criteria: patients were in accordance with the diagnostic criteria of MPP (10); patients still had hyperpyrexia for less than 10 days after being treated with macrolide antibiotics (body temperature $\geq 38^\circ\text{C}$) and were accompanied with frequent cough; age was between 1 and 14 years; radiology showing there were necrotizing pneumonia changes or pulmonary large lobe consolidation in the body, accompanied with pleural effusion; complication with systemic inflammatory response syndrome and obliterative bronchitis; positive MP-IgM. Exclusion criteria were: severe liver or kidney dysfunction, malignant tumors, epilepsy, tuberculosis, congenital heart diseases, endocrinal and metabolic diseases, nervous system diseases, connective tissue diseases, respiratory system tumors; patients with severe malnutrition; psychosis or a family history of mental illness.

Treatment

Routine treatment was carried out on children in the two groups, and included azithromycin and gamma globulin. Additionally, a symptomatic treatment was carried out for all children, including hypothermia, relieving cough, reducing phlegm, and relieving asthma. Apart from these treatments, children in group A were intravenously injected with a small dose of methylprednisolone for 5 days (Belgium Pharmacia & Upjohn Company, batch number: H20060054) [$2 \text{ mg}/(\text{kg} \cdot \text{d})$]. Then they were given methylprednisolone orally (Pfizer Italia s.r.l, Latina, Italy, batch number: H20110064) [$2 \text{ mg}/(\text{kg} \cdot \text{d})$]. Children in group B were intravenously injected with a large dose of methylprednisolone for 3 days [$30 \text{ mg}/(\text{kg} \cdot \text{d})$], then given methylprednisolone orally [$2 \text{ mg}/(\text{kg} \cdot \text{d})$]. Children in the two groups stopped using this medication after a

week. The incidence of adverse reactions of children in two groups was observed during the treatment.

Efficacy evaluation

The efficacy of treatment in the two groups was evaluated in a week after the children were treated. Marked effective: X-ray showed that pneumonia lesions were absorbed and clinical signs and symptoms completely disappeared; effective: X-ray films showed that pneumonia lesions were reduced and clinical signs and symptoms disappeared; ineffective: X-ray showed that pneumonia lesions did not reduce or increased, and clinical signs and symptoms did not improve or were aggravated. (marked effective + effective)/the number of cases in a group $\times 100\%$ = effective rate. The time taken for disappearance of fever, wheezing, cough, and rale of children in the two groups was observed.

Detection methods

BALF and peripheral blood of children in the two groups were collected on the day before treatment and after treatment. 5 mL of peripheral blood was collected and was placed in a vacuum tube without anticoagulant 30 min before the bronchoalveolar lavage fluid was collected. The serum was separated from the blood by a centrifuge at $1000 \times g$ for 10 min, then it was stored at -70°C . BALF collection method: The lesion areas were confirmed by radiology, and the largest infiltrated shadow area was selected. The catheter of BF-1T260 Olympus electronic bronchoscope was placed in the lesion areas to lavage alveoli (Wuhan Shengshida Medical Equipment Co., Ltd., China); 2 mL of lidocaine with a concentration of 2% was used to locally anesthetize the areas that were lavaged, then sterile syringes were used to rapidly inject sterile saline at 37°C into the suction hole of fiber bronchoscope, the total dosage was about 80 mL, 20 mL was injected each time. After this step, BALF was absorbed by negative pressure, the mucus and sputum in it were removed, then it was placed in sterile test tubes. The supernatant was separated by a centrifuge at $1000 \times g$ for 10 min, then it was stored at -70°C . Enzyme linked immunosorbent assay (ELISA) was used to detect the concentrations of TNF- α , IL-6, and IL-13 in BALF and serum. This step was carried out according to the instructions of TNF- α ELISA kits, IL-6 ELISA kits, and IL-13 ELISA kits (Shanghai Hengfei Biotechnology Co., Ltd., China, CSB-EQ023955HU-1,

CSB-E16524h-1, CSB-E04601h-1). Standard holes, test sample holes, and blank control holes were established (samples and enzyme-labeling reagent were not added into them). 50 μ l of standard samples was added into enzyme-labeling coating plates. 40 μ l of sample diluent was added into the test sample holes, then 10 μ l of test samples was added (the final dilutability of the samples was 5 times). Next, the holes were covered with film and were incubated at 37°C for 30 min. Then the liquid in each hole was removed, the holes were dried and were washed for 5 times. Next, 50 μ l of enzyme-labeling reagent was added into each hole, except for blank control holes. The holes were covered with films and were incubated at 37°C for 30 min. Firstly, 50 μ l of A color-developing agent was added into each hole, then 50 μ l of B color-developing agent was added into each. After the two agents were mixed, the color was developed at 37°C for 10 min in the dark. Next, 50 μ l of stop solution was added into each hole to terminate reactions. The optical density value (OD value) of each hole was measured sequentially at the wavelength of 450 nm by a BioTek automatic microplate reader, (Beijing IMAGE Trading Co., Ltd., China) and the concentrations of TNF- α , IL-6, and IL-13 were calculated.

Statistical analysis

Statistical analysis was performed by SPSS 21.0 (IBM Corp, Armonk, NY, USA). The count data were expressed as the number of cases/percentage [n(%)]. *Chi*-squared test was used to compare the count data between groups. When the theoretical frequency in *chi*-squared test was less than 5, continuous calibration *chi*-squared test was used. The measurement data were expressed as mean value $\pm$ standard deviation ($\bar{x} \pm sd$). Independent samples *t*-test was used to compare the measurement data between groups. Paired *t*-test was used to compare the data of children in groups before and after treatment. The ROC curve was used to evaluate the diagnostic value and efficacy of the concentrations of TNF- α , IL-6, and IL-13 in serum of children before treatment. When $P < 0.05$, the difference was statistically significant.

RESULTS

There was no significant difference in general data of children in the two groups. The general data included gender, age, body mass index (BMI), disease

course, residence, lesion area (lung lobes), the nature and degree of cough, history of allergic rhinitis, history of atopic dermatitis, body temperature, white blood cells (WBC) count, platelet (PLT) count, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) ($P > 0.05$, Table I).

In group A, after the children were treated, the efficacy of treatment in 28 children (44.44%) was marked effective, in 21 children (33.33%) was effective, and in 14 children (22.22%) was ineffective; the treatment effective rate was 77.78%. In group B, after the children were treated, the treatment in 40 children (61.54%) was marked effective, in 18 children (27.69%) was effective, and in 7 children (10.77%) was ineffective; the treatment effective rate was 89.23%. There was no significant difference between the treatment effective rate of children in group A and group B ($P > 0.05$, Table II). The disappearance time of fever, wheezing, cough, and rales of children in group B was significantly shorter than that in group A ($P < 0.05$, Table III).

In group A, 6 children (9.52%) vomited during the treatment. The incidence of complications was 9.52%. In group B, 7 children (10.77%) vomited, 1 child (1.54%) had allergy. The incidence of complications was 10.77%. There was no significant difference in the incidence of complications of children between group A and group B ($P > 0.05$, Fig. 1).

There was no significant difference between the concentrations of TNF- α , IL-6, and IL-13 in BALF

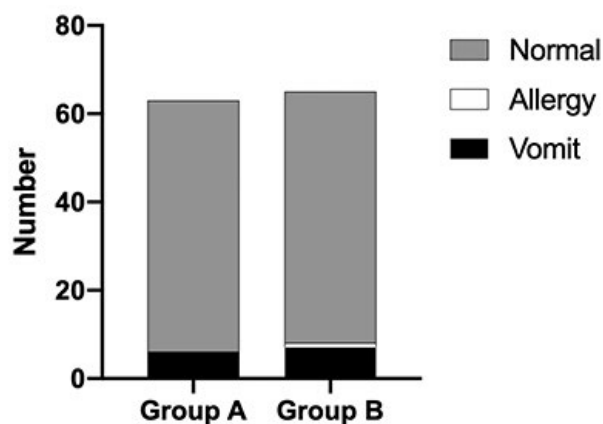


Fig. 1. The comparison of the incidence of adverse reactions of children in group A and group B.

Table I. General data, the concentrations of TNF- α , IL-6, and IL-13 in BALF and serum of children in group A and group B ($\bar{x}\pm sd$)

	Category	Group A(n=63)	Group B(n=65)	t/ χ^2 value	P value
General data	Gender			0.116	0.733
	Male	30(47.62)	29(44.62)		
	Female	33(52.38)	36(55.38)		
	Age(year)	5.8 $\pm$ 1.5	5.6 $\pm$ 1.3	0.807	0.421
	BMI(kg/m ²)	15.84 $\pm$ 1.19	15.92 $\pm$ 1.23	0.374	0.709
	Disease course(d)	13.2 $\pm$ 4.4	13.6 $\pm$ 4.7	0.497	0.62
	Residence			2.532	0.112
	City	36(57.14)	28(43.08)		
	Country	27(42.86)	37(56.92)		
	Lesion area (lung lobes)			0.778	0.378
	One lobe	22(34.92)	18(27.69)		
	Two lobes and more than two lobes	41(65.08)	47(72.31)		
	The nature of cough		/	1.227	0.268
	Dry	40(63.49)	35(53.85)		
	Wet	23(36.51)	30(46.15)		
	The degree of cough			0.124	0.94
	Slight	7(11.11)	6(9.23)		
	Serious	20(31.75)	21(32.31)		
	Fierce	36(57.14)	38(58.46)		
	The history of allergic rhinitis			0.289	0.591
	Yes	13(20.63)	16(24.62)		
	No	50(79.37)	49(75.38)		
	The history of atopic dermatitis			0.28	0.597
	Yes	14(22.22)	12(18.46)		
	No	49(77.78)	53(81.54)		
	Body temperature($^{\circ}$ C)			0.836	0.658
	Moderate heat(38.0-39.0)	26(41.27)	22(33.85)		
	Hyperpyrexia(39.1-41.0)	35(55.56)	40(61.54)		
	Ultrahyperpyrexia(39.1-41.0)	2(3.17)	3(4.61)		
	WBC($\times 10^9$ /L)	10.06 $\pm$ 3.28	10.25 $\pm$ 2.97	0.344	0.732

Table II. Comparison of the treatment effective rate and the disappearance time of clinical symptoms of children between group A and group B

Group	Group A	Group B	χ^2 value	P value
n	63	65	-	-
Marked effective	28 (44.44)	40 (61.54)	-	-
Effective	21 (33.33)	18 (27.69)	-	-
Ineffective	14 (22.22)	7 (10.77)	-	-
Effective rate (%)	77.78	89.23	3.06	0.08
Disappearance time of fever (d)	4.6 $\pm$ 0.8	2.9 $\pm$ 0.6	13.63	<0.001
Disappearance time of wheezing (d)	4.1 $\pm$ 1.1	2.8 $\pm$ 0.9	7.328	<0.001
Disappearance time of cough (d)	10.5 $\pm$ 3.9	7.1 $\pm$ 2.3	6.03	<0.001
Disappearance time of rales (d)	7.3 $\pm$ 1.3	5.8 $\pm$ 1.2	6.786	<0.001

Table III. *Diagnostic value of the concentrations of TNF- α , IL-6, and IL-13 in children before treatment*

Indicator	AUC	95%CI	Std. Error	Cut-off (pg/mL)	Sensitivity (%)	Specificity (%)
TNF- α	0.747	0.644-0.850	0.053	21.20	80.95	63.55
IL-6	0.817	0.733-0.901	0.043	45.47	85.71	68.22
IL-13	0.769	0.672-0.866	0.049	9.91	90.48	54.21

and serum of children in group A and those of children in group B before treatment ($P>0.05$). The concentrations of TNF- α , IL-6, and IL-13 in BALF and serum of children in group A and group B after treatment were significantly lower than those before treatment ($P<0.05$). The concentrations of TNF- α , IL-6, and IL-13 in BALF and serum of children in group B were significantly lower than those of children in group A after treatment ($P<0.05$, Table I).

Children whose efficacy was marked effective and effective in group A and group B were included in an improved group (107 children). Children whose efficacy was ineffective in group A and group B were included in an ineffective group (21 children). The concentrations of TNF- α , IL-6, and IL-13 in serum of children in the improved group were (19.25 ± 6.18) pg/mL, (42.63 ± 7.85) pg/mL, and (8.12 ± 2.69) pg/mL before treatment. The concentrations of TNF- α , IL-6, and IL-13 in serum of children in the ineffective group were (24.63 ± 7.48) pg/mL, (47.63 ± 8.56) pg/mL, and (11.18 ± 3.58) pg/mL before treatment. The concentrations of TNF- α , IL-6, and IL-13 in serum of children in the improved group were significantly lower than those of children in the ineffective group before treatment ($P<0.05$). The ROC curves of the concentrations of TNF- α , IL-6, and IL-13 were drawn in the diagnosis of treatment efficacy before treatment. The sensitivity of TNF- α was 80.95%, the specificity was 63.55%. The sensitivity of IL-6 was 85.71%, the specificity was 68.22%. The sensitivity of IL-13 was 90.48%, the specificity was 54.21% (Table III and Fig. 2).

DISCUSSION

MPP is a common pediatric disease. Clinically, macrocyclic ester antibiotics are used to treat MPP, but the pathogenesis of severe MPP is complicated. The pathogenesis of severe MPP may be related to genetic mutation, infection of drug resistant strains, pathogen damage, immune inflammatory reaction caused by infection, etc. Methylprednisolone is a glucocorticoid drug that can block the activation and chemotaxis of inflammatory cells, and inhibit the immune inflammatory response as well as reduce the time of inflammatory response (8). It has been demonstrated that the efficacy of methylprednisolone was effective in the treatment of children with severe CAP, and that methylprednisolone could shorten fever time and reduce levels of CRP and WBC (10). It was reported that IL-6 and IL-13 might play a role in the development of inflammation caused by fatal acute respiratory infections and alveolar fibrosis (11). Thus, controlling levels of TNF- α , IL-6, and IL-13 may be the key to treating children with severe MPP. In this study, levels of TNF- α , IL-6, and IL-13 decreased obviously in BALF and serum of children in group A and group B after they were treated. Moreover, levels of TNF- α , IL-6, and IL-13 decreased obviously in BALF and serum of children in group B compared to those of children in group A. This result shows that methylprednisone can obviously inhibit the inflammatory response of children with severe MPP, the reason may be that levels of TNF- α , IL-6, and IL-13 are inhibited. Previous study indicated that corticosteroids and macrolides

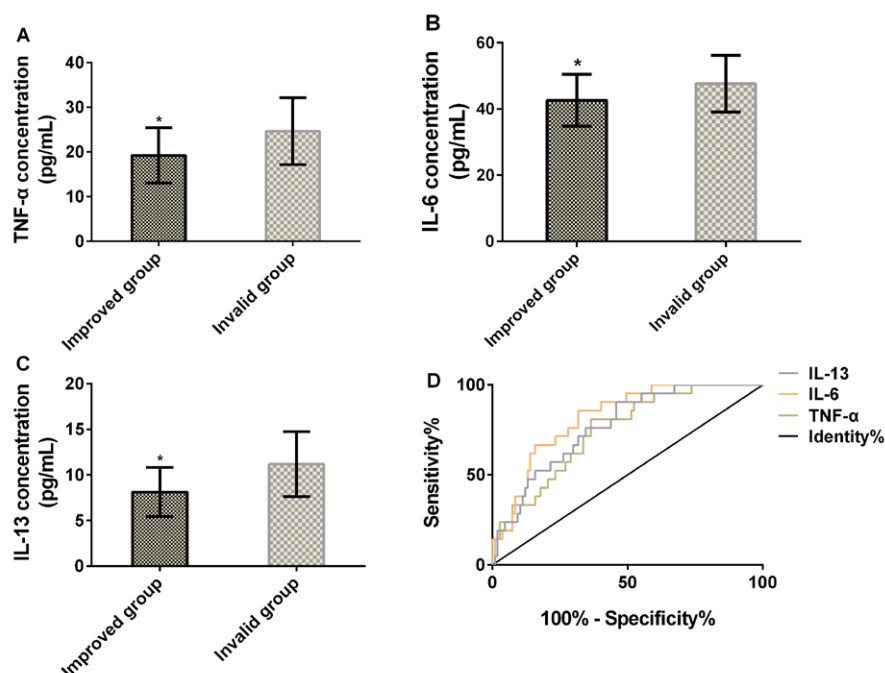


Fig. 2. The diagnostic value of the concentrations of TNF- α , IL-6, and IL-13 for the efficacy of treatment in children in the improved group and the ineffective group before they were treated. The comparison of the concentration of TNF- α in serum of children in the improved group and the ineffective group before they were treated (A). ELISA showed that the concentration of TNF- α in serum of children in the improved group was significantly lower than that of children in the ineffective group before they were treated ($P < 0.05$). The comparison of the concentration of IL-6 in serum of children in the improved group and the ineffective group before they were treated (B). ELISA showed that the concentration of IL-6 in serum of children in the improved group was significantly lower than that of children in the ineffective group before they were treated ($P < 0.05$). The comparison of the concentration of IL-13 in serum of children in the improved group and the ineffective group before they were treated (C). ELISA showed that the concentration of IL-13 in serum of children in the improved group was significantly lower than that of children in the ineffective group before they were treated ($P < 0.05$). The ROC curves (D) of the concentrations of TNF- α , IL-6, and IL-13 in the efficacy of the children before they were treated. Note: compared to the ineffective group, $*P < 0.05$.

could reduce levels of inflammatory biomarkers in blood and BALF of patients with non-responding pneumonia, such as IL-6, TNF- α , IL-10 (12). This result is similar to the result of our study. The difference is that both a large dose of methylprednisolone and a small dose of methylprednisolone were used to treat children in this study. It was found that a large dose of methylprednisolone could obviously inhibit inflammatory factors, the reason of which may be that a large dose can quickly inhibit the inflammatory response of the body in the treatment of children with MPP, thus the clinical symptoms and signs recover rapidly. However, there are still some drawbacks to this study. Firstly, the risk factors affecting the treatment efficacy in children with severe MPP were not observed.

Secondly, the specific mechanisms of TNF- α , IL-6, and IL-13 in MPP were not investigated deeply. These defects need to be corrected in later studies.

In summary, the clinical efficacy of methylprednisolone is good in the treatment of children with severe MPP. The mechanism is in inhibiting the levels of TNF- α , IL-6, and IL-13. Detecting levels of TNF- α , IL-6, and IL-13 in serum of children with severe MPP can predict the efficacy of treatment in children with severe MPP before they are treated.

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LETTER TO THE EDITOR

Correlation analysis of *Helicobacter pylori* infection, inflammatory activity and serum high sensitivity C-reactive protein level in elderly patients with chronic gastritisWW. Yang¹, L. Li², S. Tan¹, ZY. Liu¹, and Y. Jiang¹

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To the Editor,

Gastritis is an inflammation of the gastric mucosa caused by various reasons, and is one of the most common digestive diseases (1). Gastritis is divided into two categories, acute and chronic, according to the severity of the disease (2), chronic being the most common.

Clinical studies have shown that the continuous infection of *Helicobacter pylori* (*H. pylori*) affect the occurrence and development of gastric cancer (3). *H. pylori* stimulates epithelial cells and promotes the secretion of inflammatory factors by combining with gastric mucosal epithelial specific receptors. High-sensitivity C-reactive protein (hs-CRP) is a commonly used clinical inflammatory response marker, which can reflect the body's inflammation (4). Interleukin (IL)-2, IL-8, IL-10 and IL-27 are common pro-inflammatory cytokines in the human body and play important roles in the development and progression of diseases (5).

Previous studies have reported that *H. pylori* infection is extremely harmful to health, therefore, it is necessary to investigate its relationship with various inflammatory cytokines and hs-CRP levels in patients with various stages of chronic gastritis. In the present study, the relationship was analyzed

by ELISA between *H. pylori* infection, various inflammation cytokines and hs-CRP levels in elderly patients with chronic gastritis to provide data support and relevant reference basis for clinical diagnosis and treatment.

MATERIALS AND METHODS

Clinical data

A total of 86 patients who were treated at Guizhou Second Provincial People's Hospital from August 2016 to August 2019 due to gastrointestinal symptoms were selected as research subjects. All patients were diagnosed by gastroscopy and biopsy. Twenty-seven patients (average age 55.21±4.74 years) had superficial gastritis; 35 cases (average age 57.91±4.55 years) were accompanied by atrophic gastritis; and 24 patients (average age 62.39±6.61 years) were accompanied by precancerous lesions. A total of 70 healthy elderly subjects (average age 59.78±5.39 years) who underwent physical examination in the above hospital during the same period were selected as the control group. There was no statistically significant difference in the average age range of each group ($P > 0.05$).

Exclusion criteria: heart, lung, kidney and other organ dysfunction; recent severe trauma, mental irritation, or extensive burns; systemic infectious diseases or

Key words: elderly gastritis patients; Hp infection; precancerous lesions; hs-CRP

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those who had recently taken proton pump inhibitors, glucocorticoids, antibiotics and other drugs; malignant tumors or blood diseases. The informed consent forms were voluntarily signed by all the patients, and the study was approved by the ethics committee of Guizhou Second Provincial People's Hospital.

Experimental methods

After admission, all patients underwent the relevant examinations, including assessment of physical condition. Then, all patients received the ¹⁴C-urea breath test, and they were divided into *H. pylori* positive and *H. pylori* negative according to the results. Five ml of fasting venous blood was collected from patients in the morning, and centrifuged at 3000r/min for 10 min at -4°C. Then, the serum was stored at -80°C for further use. The expression levels of IL-8, IL-10, IL-32 and hs-CRP were measured using ELISA kit (Boshide Bioengineering Co., Ltd, Wuhan, China) according to the manufacturer's instructions. The ELISA kits were as follows: IL-8 Rat ELISA Kit (Invitrogen, USA), IL-10 Rat ELISA Kit (Invitrogen, USA), IL-32 Rat ELISA Kit (Invitrogen, USA) and hs-CRP Rat ELISA Kit (Invitrogen, USA).

Statistical methods

The data were shown as the mean $\pm$ SD from at least three independent experiments. Statistical analysis was performed by SPSS18.0 software. χ^2 or *t*-test was used for the intergroup differences. The correlation analysis was performed by Spearman's Rank correlation coefficient test. $P < 0.05$ was considered as a statistically significant.

RESULTS

Analysis of the expression levels of inflammatory cytokines and hs-CRP

As shown in Table I, the levels of IL-8, IL-10, IL-32 and hs-CRP in the gastritis patients were higher than those in the control group ($P < 0.05$). More importantly, the levels of IL-8, IL-10, IL-32 and hs-CRP in patients with superficial gastritis were higher than those with atrophic gastritis and precancerous lesions ($P < 0.05$). The levels of IL-8, IL-10, IL-32 and hs-CRP in atrophic gastritis were higher than those in precancerous lesions ($P < 0.05$).

Comparison of inflammatory cytokines and hs-CRP levels in different H. pylori infections in patients with chronic gastritis

The levels of IL-8, IL-10, IL-32 and hs-CRP in precancerous lesion patients with *H. pylori*-positive infection were significantly higher than those in *H. pylori*-negative group ($P < 0.05$). However, there were no significant differences between *H. pylori*-positive and *H. pylori*-negative group in superficial gastritis and atrophic gastritis patients (Table II).

Correlation analysis of H. pylori infection, inflammatory factors and hs-CRP expression in elderly gastritis patients

Spearman correlation analysis results showed that the *H. pylori* infection was positively correlated with IL-8, IL-10, IL-32 and hs-CRP levels in elderly

Table I. Comparison of inflammatory cytokines and hs-CRP levels in each group. Statistical analyses were performed by the χ^2 test. * $P < 0.05$ was considered significant.

Group	Cases	IL-8	IL-10	IL-32	hs-CRP
Control	70	6.37 $\pm$ 2.91	5.17 $\pm$ 3.55	11.52 $\pm$ 5.19	2.39 $\pm$ 1.01
Superficial gastritis	27	15.49 $\pm$ 3.15*	10.98 $\pm$ 2.95*	49.58 $\pm$ 8.96*	5.71 $\pm$ 2.94*
Atrophic gastritis	35	19.39 $\pm$ 3.71*	12.03 $\pm$ 2.09*	60.07 $\pm$ 7.88*	6.42 $\pm$ 3.09*
Precancerous lesions	24	30.04 $\pm$ 5.26*	19.92 $\pm$ 4.11*	79.03 $\pm$ 9.09*	15.83 $\pm$ 4.75*

Table II. Comparison of inflammatory cytokines and hs-CRP levels in observation group

Group	<i>H. pylori</i> infection	IL-8	IL-10	IL-32	hs-CRP
Superficial gastritis	<i>H. pylori</i> -	16.55±3.47 [#]	13.19±4.22 [#]	53.11±7.92 [#]	6.58±3.75 [#]
	positive	14.79±3.26	11.03±2.46	48.91±8.44	4.73±3.41
	<i>H. pylori</i> - negative				
Atrophic gastritis	<i>H. pylori</i> -	18.49±4.05 [#]	13.97±3.68 [#]	63.57±9.07 [#]	8.15±4.37 [#]
	positive	16.03±3.39	11.55±2.49	59.41±7.90	6.06±2.93
	<i>H. pylori</i> - negative				
Precancerous lesions	<i>H. pylori</i> -	37.38±6.71*	25.89±5.36*	84.82±10.79*	20.61±6.27*
	positive	26.03±3.36	17.25±3.41	76.10±7.68	13.29±3.88
	<i>H. pylori</i> - negative				

Statistical analyses were performed by the χ^2 test. * $P < 0.05$ was considered significant.

patients with chronic gastritis in precancerous stage ($r = 0.679, 0.614, 0.731, 0.595$; $P = 0.007, 0.000, 0.0003, 0.0009$).

DISCUSSION

Recently, studies have shown that the incidence of chronic gastritis has gradually increased, and is closely related to *H. pylori* infection (6, 7). In the present study, it was found that the expression levels of IL-8, IL-10, IL-32 and serum hs-CRP in *H. pylori*-positive patients with precancerous lesions were significantly increased compared with *H. pylori* negative patients. However, there was no significant difference between the superficial gastritis group and the chronic atrophic gastritis group.

IL-8 is a cytokine of the chemokine family, and plays an pro-inflammatory role (8). IL-10, an important cytokine in the human body, is a factor

capable of inhibiting inflammation, which can regulate the human immune system (9). Moreover, IL-32, as a new proinflammatory factor of the interleukin family, is significantly higher in *H. pylori*-infected gastric mucosa tissues (10), which is consistent with the results of our study. hs-CRP is the only inflammatory indicator with WHO standard at present, which belongs to acute time-type reactive protein, and is used as a sensitive indicator of inflammation and trauma generated by various causes (11, 12). In this study, we found that in patients with precancerous lesions, hs-CRP was significantly higher than in patients with superficial gastritis or atrophic gastritis. Taken together, in elderly patients with chronic gastritis, *H. pylori* infection is closely related to the expression levels of IL-8, IL-10, IL-32 and hs-CRP, and the degree of *H. pylori* infection directly affects their expression levels, which has important clinical significance for the evaluation of efficacy and prognosis.

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LETTER TO THE EDITOR

Long noncoding RNA KCNQ1OT1 contributes to the development of nephroblastoma via modulating miR-21/PTEN axisXH. Liu¹, CM. Zhang², PQ. Pan³, DQ. Zhang⁴, X. Huang⁵ and ZH. Ji⁶

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To the Editor,

Nephroblastoma, also known as Wilms' tumor, is the most common childhood kidney cancer (1). Although the prognosis for nephroblastoma patients is good, 13% of patients showed a two-year relapse after tumor diagnosis (2). A better understanding of nephroblastoma progression would therefore be a substantial advancement for identifying the molecular biomarkers to predict high risk patients and guide treatment.

Previous studies have identified that lncRNAs play important roles in the development of nephroblastoma by regulating cell proliferation, migration, invasion and apoptosis, such as lncRNA SOX21-AS1, lncRNA CRNDE, lncRNA HOXA11-AS, lncRNA MIAT (3-6). KCNQ1OT1 was found to target the corresponding miRNA to modulate the pathological processes of various cancers including hepatocellular carcinoma, tongue cancer and gastric cancer (7-9). Nevertheless, it is unclear whether KCNQ1OT1 is related to the biological function and molecular mechanism of nephroblastoma.

In the present study, we investigated KCNQ1OT1 expression in nephroblastoma samples and cells. Furthermore, we investigated the effects and molecular mechanism of KCNQ1OT1 on nephroblastoma cell

migration and invasion. The results demonstrated that KCNQ1OT1 was up-regulated in nephroblastoma tissues and cells. Moreover, the findings clarified that KCNQ1OT1 enhanced nephroblastoma metastasis by regulating miR-21/PTEN axis, which accelerated the understanding of nephroblastoma pathogenesis and development of effective therapies.

MATERIALS AND METHODS

Clinical samples

Nephroblastoma tumor specimens (n=26) and adjacent non-tumor specimens (n=26) were obtained from nephroblastoma patients at Qingdao Hospital of Traditional Chinese Medicine. The collected samples were then frozen and stored at -80°C. None of the patients enrolled in this study had received any preoperative chemotherapy or radiotherapy before surgery. All patients signed written informed consents, and the protocols of this study were approved by the Clinical Research Ethics Committee of Qingdao Hospital of Traditional Chinese Medicine.

Cell lines and cell culture

Human embryonic kidney normal cell line CCC-HEK-1 and two human nephroblastoma cell lines (HFWT,

Key words: KCNQ1OT1; nephroblastoma; miR-21; PTEN

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WTCLS1) were purchased from American type culture collection (ATCC, Manassas, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 mg/mL streptomycin, and 100 U/mL penicillin in a humidified air with 5% CO₂ at 37°C.

Cell transfection

HFWT cells were transfected with overexpressed KCNQ1OT1 (oe-KCNQ1OT1), small hairpin KCNQ1OT1 (sh-KCNQ1OT1) or negative controls (oe-NC, sh-NC). They were designed and constructed by GenePharma (Shanghai, China). The miR-21 mimic, negative control (NC) mimic, miR-21 inhibitor, and NC inhibitor, designed and synthesized by Shanghai R&S Biotechnology Co. (Shanghai, China), were also introduced in HFWT cells. The transfections used Lipofectamine® 2000 (Invitrogen, CA, USA) in accordance with the manufacturer's protocol. After transfection for 48 h, the cells were harvested for further experiments.

RNA extraction and quantitative real-time PCR

Total RNAs were extracted from nephroblastoma by TRIzol Reagent (Invitrogen, Grand Island, USA). cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen) and qRT-PCR was performed with StepOne Plus Real-time PCR System (Applied Biosystems, CA, USA) system. U6 or GAPDH was applied as internal control. The expression level of genes was calculated by $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: KCNQ1OT1 forward: 5'-GCACTCTGGGTCTGTCTC-3', reverse: 5'-CACTTCCCTGCCTCCTACAC-3'; miRNA-21 forward: 5'-GCACCGT CAAGGCTGAGAAC-3', reverse: 5'-CAGCCCATCGACTGGTG-3'; PTEN forward: 5'-TTTGAGAGTTGAGCCGCTGT-3', reverse: 5'-ATGCTTTGAATCCAAAAACCT TACT-3'. U6: forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5'-CGCTTCAGAATTTGCGTGTTCAT-3'; GAPDH: forward: 5'-CGCTCTCTGCTCCTC CTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western blot

For Western blotting assay, HFWT cells were lysed with a cell lysis for collecting total proteins. Equal amounts of protein lysates (50 µg) were separated by 8-12% SDS-PAGE and transferred to PVDF membranes.

The membranes were blocked with 5% skim milk for 1 h at 37°C and then incubated with specific primary antibody against PTEN (Abcam, ab32199, 1:1000) overnight at 4°C. After incubating with horseradish peroxidase-labeled secondary antibody for 1 h at 37°C, the protein bands were visualized by an Enhanced Chemiluminescence Kit (Thermo Fisher Scientific). GAPDH was used as the loading control.

Transwell assays

The cell migration and invasion were performed by Transwell chamber (Corning Costar). HFWT cells (1×10^5) were seeded in upper chambers, and the lower chambers were fixed with DMEM containing 10% FBS. After being incubated for 24 h, the cells were fixed with methanol for 15 min, and stained with 0.2% crystal violet for 20 min. Cells on the upper surface of chambers were carefully wiped off with the cotton swabs. The cells were observed under a microscope and photographs taken

RNA immunoprecipitation (RIP) assay

HFWT cells were transfected with miR-21 mimic or inhibitor for 48 h, then they were lysed with RIP lysis buffer following manufacturer's instructions and incubated with RIP immunoprecipitation buffer mixed with magnetic beads conjugating human anti-Argonaute2 (Ago2) antibody or negative control mouse IgG (Millipore, MA, USA). Acquired RNAs were analyzed by qRT-PCR. Input and IgG served as controls in RIP assay.

Luciferase reporter assay

The wild-type and mutant binding sites of KCNQ1OT1 or PTEN to miR-21 was sub-cloned to pmirGLO dual-luciferase vector to construct plasmids, called KCNQ1OT1-WT or KCNQ1OT1-MuT, PTEN-WT or PTEN-MuT. HFWT cells were co-transfected with luciferase reporter vectors and miR-21 mimic, inhibitor or negative controls using Lipofectamine 2000. After transfection for 48 h, the luciferase activity was measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, USA).

Statistical analysis

Data were manifested as the mean $\pm$ SD from at least three independent experiments. The differences between two groups or among multiple groups were analyzed using

two-tailed Student's *t*-tests or Turkey's post-hoc tests in one-way ANOVA. Statistical analysis was performed with the use of SPSS Statistics 20.0 software (IBM, NY, USA). $P < 0.05$ was considered as a statistically significant.

RESULTS

KCNQ1OT1 enhanced cell metastasis in nephroblastoma

The expression level of *KCNQ1OT1* in 26 matched pairs of nephroblastoma and adjacent non-tumor samples were detected by qRT-PCR. The

results showed that *KCNQ1OT1* was significantly up-regulated in nephroblastoma samples compared to adjacent non-tumor samples (Fig. 1A). Consistent with *KCNQ1OT1* expression in nephroblastoma samples, *KCNQ1OT1* was also obviously increased in nephroblastoma cell lines in comparison with the normal human embryonic kidney cell line CCC-HEK-1 (Fig. 1B). Next, the functional significance of *KCNQ1OT1* was explored in nephroblastoma cells. *KCNQ1OT1* level was increased or decreased significantly by transfection with oe-*KCNQ1OT1* or

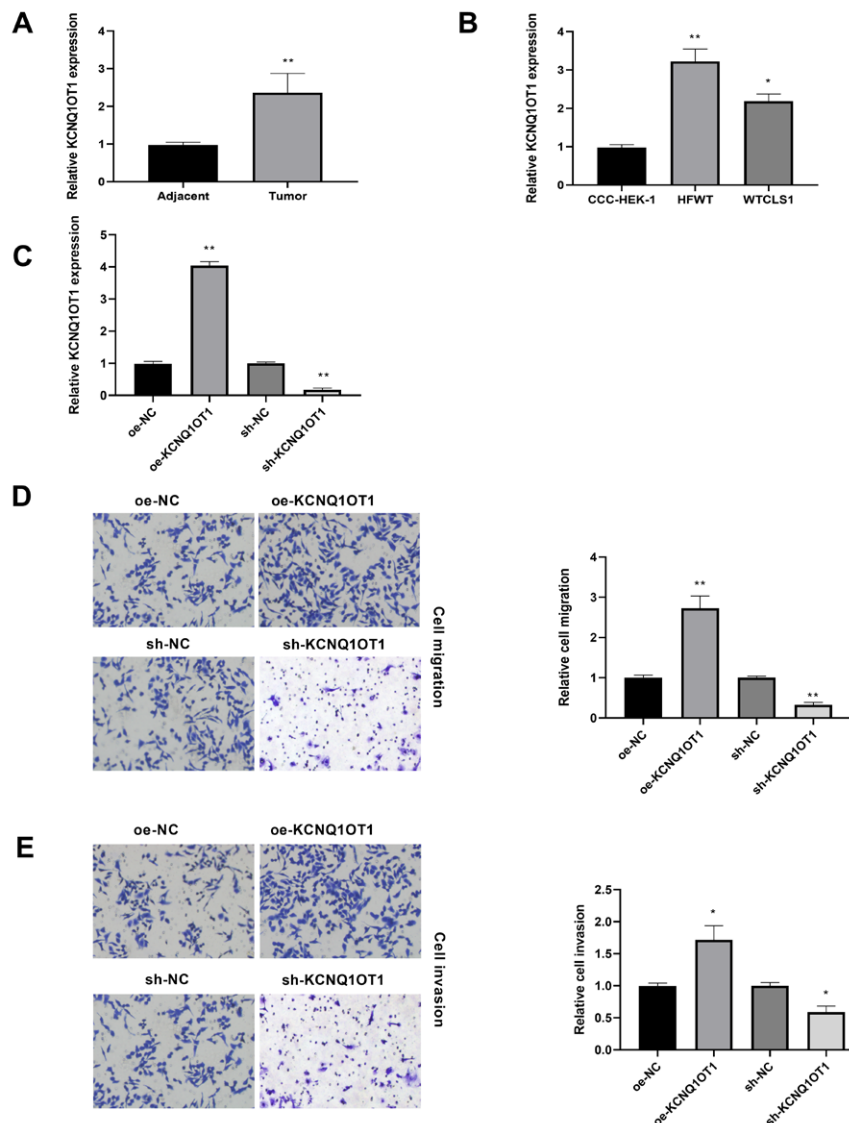


Fig. 1. *KCNQ1OT1* enhanced cell metastasis in nephroblastoma. **A)** RT-qPCR was used to detect the expression of *KCNQ1OT1* in tumor tissues and non-tumor tissues. **B)** RT-qPCR was used to detect the expression level of *KCNQ1OT1* in osteosarcoma cell lines. **C)** *KCNQ1OT1* over-expression or knockdown was achieved by oe-*KCNQ1OT1* or sh-*KCNQ1OT1*, and the transfection efficiency was verified by qRT-PCR. **D)** Cell migration was measured by Transwell migration assay. **E)** Cell invasion was measured by Transwell invasion assay * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

sh-KCNQ1OT1 in HFWT (Fig. 1C). Transwell assays manifested that cell migration and invasion were significantly elevated in oe-KCNQ1OT1 transfected cells, while inhibited in sh-KCNQ1OT1 transfected cells compared with that in control cells (Fig. 1 D-E). All these results indicated that KCNQ1OT1 facilitated HFWT cell migration and invasion.

KCNQ1OT1 directly interacted with miR-21

The underlying molecular mechanism by which KCNQ1OT1 exerted its functional effects on nephroblastoma cells was further studied. Starbase prediction algorithm validated that miR-21 was a potential binding target of KCNQ1OT1 (Fig. 2A). MiR-21 expression was increased or decreased by

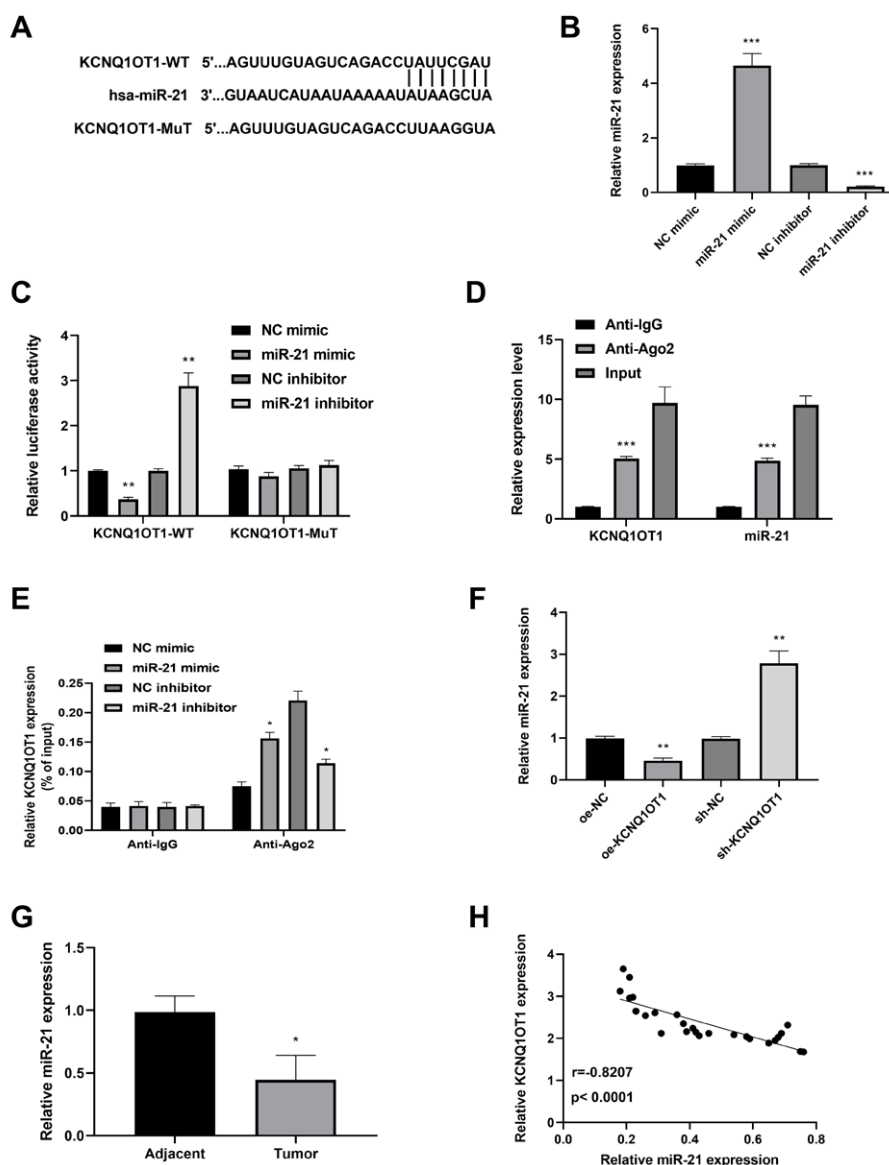


Fig. 2. *KCNQ1OT1 directly interacted with miR-21.* **A)** Bioinformatics analysis of the predicted binding site between *KCNQ1OT1* and *miR-21*. **B)** qRT-PCR detection of the expression of *miR-21*. **C)** Luciferase reporter analysis of the luciferase activity of the *KCNQ1OT1*-WT or *KCNQ1OT1*-MuT. **D)** RIP assay displayed the enrichment of *KCNQ1OT1* or *miR-21* expression in anti-Ago2 group. **E)** The enrichment of *KCNQ1OT1* in anti-Ago2 group can be increased by *miR-21* mimic and decreased by *miR-21* inhibitor. **F)** *KCNQ1OT1* negatively regulated *miR-21* expression. **G)** *miR-21* expression was significantly down-regulated in nephroblastoma tumor tissues (n=26). **H)** The inverse correlation between *KCNQ1OT1* and *miR-21* expression in nephroblastoma tissues (n=26). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

transfection with miR-21 mimic or inhibitor in HFWT cells, and the successful transfection efficiency was confirmed by qRT-PCR (Fig. 2B). Luciferase reporter assay and RIP assay were subsequently carried out to verify the interaction between KCNQ1OT1 and miR-21. Luciferase reporter assay showed that miR-21 over-expression considerably reduced, while miR-21 suppression increased the luciferase activity of KCNQ1OT1-WT. However, neither miR-21 over-expression nor suppression showed any impact on the luciferase activity of KCNQ1OT1-MuT (Fig. 2C). RIP assay revealed that KCNQ1OT1 and miR-21 expression were elevated in anti-Ago2 group compared to that in the control group (Fig. 2D). Moreover, the increased KCNQ1OT1 in anti-Ago2 group was facilitated by miR-21 mimic and inhibited by miR-21 inhibitor (Fig. 2E). QRT-PCR results showed that over-expression of KCNQ1OT1 significantly decreased

miR-21, while knockdown of KCNQ1OT1 markedly increased miR-21 expression (Fig. 2F), implying a negative interaction between KCNQ1OT1 and miR-21. Moreover, miR-21 expression was detected in nephroblastoma tissues and the results demonstrated that miR-21 was significantly down-regulated in nephroblastoma tissues compared with that in non-tumor tissues (Fig. 2G). Pearson's correlation analysis revealed that KCNQ1OT1 was inversely associated with miR-21 in nephroblastoma tissues (Fig. 2H). These findings strongly suggested that KCNQ1OT1 directly interacted with miR-21 in nephroblastoma.

KCNQ1OT1 sponged miR-21 to modulate cell metastasis in nephroblastoma

Next, whether miR-21 was involved in the effect of KCNQ1OT1 on cell migration and invasion was investigated in HFWT cells. HFWT cells were

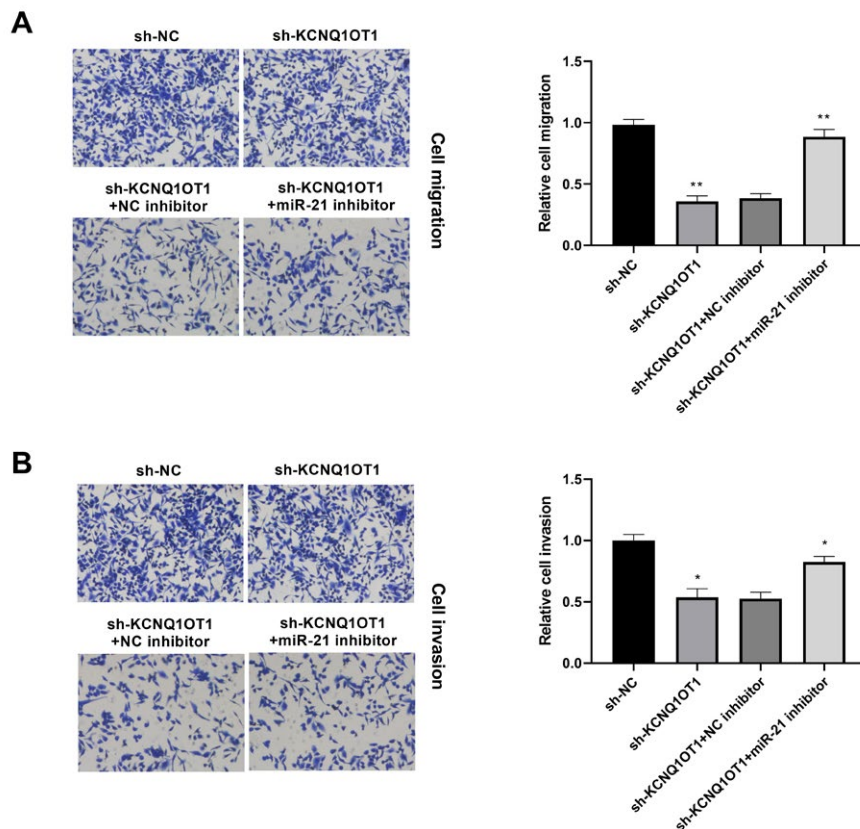


Fig. 3. *KCNQ1OT1 sponged miR-21 to modulate KCNQ1OT1 cell migration and invasion. A)* The reduced cell migration induced by sh-KCNQ1OT1 was restored by miR-21 inhibitor. **B)** Transwell assay displayed that cell invasion was declined by sh- KCNQ1OT1, and rescued by miR-21 inhibitor. * $P < 0.05$, ** $P < 0.01$

transfected with sh-KCNQ1OT1 or combined with miR-21 inhibitor. Transwell migration results showed that the migration of HFWT cells reduced by sh-KCNQ1OT1 could be partly rescued by the incorporation of miR-21 inhibitor (Fig. 3A).

Furthermore, Transwell invasion assay showed that the inhibitory effect of sh-KCNQ1OT1 on nephroblastoma cell invasion was also partly overturned by being combined with miR-21 inhibitor (Fig. 3B). These results indicated that KCNQ1OT1

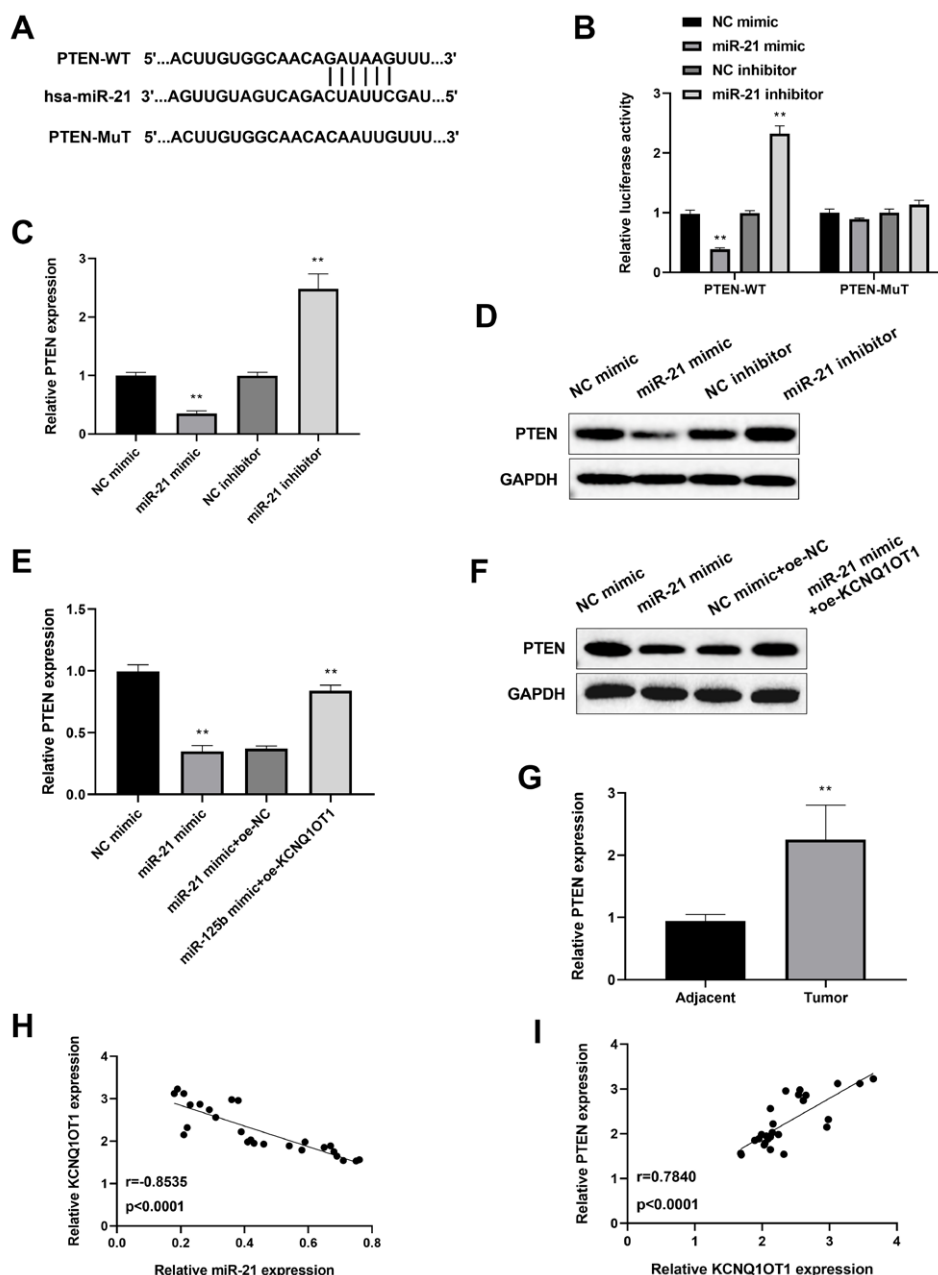


Fig. 4. KCNQ1OT1 regulated PTEN expression through miR-21. **A)** Bioinformatic analysis displayed a putative PTEN binding site with miR-21. **B)** Luciferase activities of PTEN-WT or PTEN-MuT was detected after transfection with miR-21 mimic or inhibitor. PTEN mRNA expression (**C**) and protein level (**D**) were detected in HFWT cells after treatment with miR-21 mimic or miR-21 inhibitor. **E, F)** The effect of KCNQ1OT1 on miR-21 modulated PTEN expression. **G)** PTEN expression was examined by qRT-PCR in nephroblastoma tumor tissues ($n=26$). **H)** Negative relationship was found between PTEN and miR-21 expression, (**I**) while positive correlation between KCNQ1OT1 and PTEN in nephroblastoma tissues ($n=26$). ** $P < 0.01$

modulated nephroblastoma cell migration and invasion by sponging to miR-21.

PTEN was the target of miR-21 and KCNQ1OT1 regulated PTEN through miR-21 in nephroblastoma

The potential target genes of miR-21 were then predicted by Targetscan, and PTEN was discovered to own a binding site for miR-21 (Fig. 4A). Then, luciferase assay was carried out to further confirm this prediction, and the results showed that the luciferase activity of PTEN-WT was reduced significantly by miR-21 mimic in HFWT cells, but not in PTEN-MuT (Fig. 4B), indicating that PTEN was a downstream target gene of miR-21. More importantly, qRT-PCR and Western blot results discovered that miR-21 mimic inhibited PTEN expression, while miR-21 inhibitor facilitated PTEN expression (Fig. 4 C, D). Subsequently, the relationship between KCNQ1OT1, miR-21 and PTEN was explored, and results showed that PTEN expression reduced by miR-21 mimic could rescue through the incorporation of oe-KCNQ1OT1 (Fig. 4 E, F), indicating that KCNQ1OT1 regulated PTEN expression through targeting miR-21. PTEN expression in nephroblastoma samples was higher than that in non-tumor samples (Fig. 4G). Spearman's correlation analysis displayed that the relationship between PTEN and miR-21 was negative, whereas the correlation between PTEN and KCNQ1OT1 was positive in nephroblastoma tissues (Fig. 4 H, I). Collectively, these data demonstrated that PTEN was a target of miR-21, and KCNQ1OT1 regulated PTEN expression by sponging to miR-21.

DISCUSSION

In recent years, the relationship between lncRNAs and malignant tumors has gradually become clear. Previous studies have found that lncRNA KCNQ1OT1 is highly expressed in nephroblastoma and facilitates cell proliferation (10). In the present study, we found that KCNQ1OT1 was remarkably higher in nephroblastoma tissues than in those of adjacent normal tissues. More importantly, down-regulation of KCNQ1OT1 remarkably inhibited cell metastasis, while KCNQ1OT1 overexpression

promoted metastasis abilities in nephroblastoma cells.

LncRNAs can be used as miRNA sponges to bind miRNAs competitively and inhibit the function and activity of miRNAs (11, 12). Bioinformatics analysis predicted that miR-21 may interact with KCNQ1OT1 and PTEN was the downstream target of miR-21. Based on the findings, we found that KCNQ1OT1 expression was negatively correlated with miRNA-21 expression, while positively with PTEN expression. Given that only a few lncRNAs have been identified as exerting important biological functions in nephroblastoma, the direction of future studies is to search for specific lncRNA molecules and to explore their potential function and signal mechanisms. This will not only help to understand the molecular mechanism of the occurrence and development of nephroblastoma, but also to provide a new direction for nephroblastoma diagnosis and treatment through targeted lncRNA.

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LETTER TO THE EDITOR

Effects of sevoflurane postconditioning on the expression of AKT/mTOR and apoptosis of myocardial cells in myocardial ischemia-reperfusion ratsF-Q. Liu¹, C-P. Liu², M-H. Xu³, J. Zhu⁴, W. Yu⁵ and L-L. Wang^{6*}¹Operation Room, The People's Hospital of Zhangqiu area, Jinan, Shandong Province, China;²Department of Cardiology, Jiyang People's Hospital, Jinan, Shandong Province, China; ³Department of Clinical Laboratory, Yantai Central Blood Station, Yantai, Shandong Province, China; ⁴Respiratory Department, The People's Hospital of Zhangqiu Area, Jinan, Shandong Province, China; ⁵Department of Cardiology, The People's Hospital of Zhangqiu Area, Jinan, Shandong Province, China; ⁶Department of Anesthesiology, Jining No.1 People's Hospital, Affiliated Jining No.1 People's Hospital of Jining Medical University, Jining Medical University, Jining, Shandong Province, China*Received July 6, 2020 – Accepted October 2, 2020*

To the Editor,

Myocardial ischemia-reperfusion (MIR) is a common complication of ischemic heart disease, which refers to the phenomenon that the damage of ischemic myocardial tissue is further aggravated after the restoration of blood perfusion. Apoptosis is one of the important forms of cardiomyocyte death in IR injury (1). The Bcl-2 family proteins play a key role in the mitochondrial-mediated apoptosis pathway. They are divided into anti-apoptotic regulatory proteins (Bcl-2 and Bcl-XL) and pro-apoptotic regulatory proteins (Bax and Bak) (2). Therefore, we can analyze cell apoptosis by detecting changes in Bcl-2/Bax protein expression.

With the extensive clinical application of sevoflurane in the field of anesthesia and the rapid development of cardiac surgery and interventional techniques, a large number of basic and clinical studies have found that pre/post-treatment of sevoflurane has a significant protective effect on myocardial I/R injury (3, 4). Compared with sevoflurane pretreatment, the timing of sevoflurane postconditioning has the same significant myocardial

protective effect, and it is easier to directly use in clinical anesthesia work (5). Previous studies have shown that the PI3K/AKT signaling pathway is one of the important signaling pathways for the protection of sevoflurane postconditioning (6). The mTOR is a downstream target of PI3K/AKT signaling pathway, and is involved in the protective effect of myocardial I/R injury. Therefore, this study explored the changes of Bcl-2/Bax and AKT/mTOR protein expression in rats with myocardial I/R injury after sevoflurane postconditioning.

MATERIALS AND METHODS

The animal experiments in this study were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" and were approved by the Animal Ethics Committee of Jining No.1 People's Hospital. Forty-eight adult male SD rats were selected and purchased from the Laboratory Animal Center of Medical College of Nanchang University. The rats were 2-3 months old, weighed 250-300 g, and were healthy and clean. In order to establish an *in vivo* model of myocardial I/R injury in

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rats, we first injected 1% pentobarbital sodium into the abdominal cavity of the rats. After anesthesia, each rat was placed in a supine position and fixed, and mechanically ventilated with tracheal intubation and a small animal ventilator. The electrocardiogram was connected. The chest cavity was opened in the 4th intercostal space on the left side of the rat to expose the heart and blood vessels. Then, the suture passed through the left anterior descending coronary artery (LAD) to induce myocardial ischemia. The successful construction of rat myocardial ischemia is that the T wave of the rat is high and the ST segment is significantly elevated. The ligature was loosened and reperfused for 6 h. The indication for the successful construction of myocardial I/R injury model is the gradual recovery of the rat's T wave and the decrease of the ST segment.

At the end of reperfusion, cardiac function was assessed, including ejection fraction (EF), short axis shortening rate (FS), stroke volume (SV), left ventricular end-diastolic diameter (LVIDd), left ventricular end-systolic diameter (LVIDs), interventricular septal diastolic thickness (IVSd), interventricular septal systolic thickness (IVSs), left ventricular posterior wall end-diastolic thickness (LVPWd) and left ventricular posterior wall end-systolic thickness (LVPWs).

Experimental group

The 48 rats successfully modeled were divided into three groups (n=16 in each). Sham group: threading without blocking LAD; IR group: blocking LAD ischemia for 30 min after threading was stable for 30 min and reperfusion for 2 h; SPC group: Sevoflurane (2.4%) at end-tidal 1 min before reperfusion (number: H 20130614). 2.4% sevoflurane was continuously inhaled at the end of ischemia for 15 min from the start of reperfusion. And reperfusion was performed for 105 min.

Determination of percentage of myocardial infarction area

Triphenyltetrachloroazole chloride 1% (number: T 8877, Sigma, USA) was used to determine the percentage of myocardial infarction area. After 2 h of reperfusion, the heart was quickly removed and frozen at -80°C. The heart was cut into 6 thick tissue slices (2 mm) from the apex of the heart to the bottom of the heat source. The heart tissues were put in 1% triphenyltetrachloroazole chloride and

incubated for 20 min at 37°C in the dark after phosphate buffer (pH-7.4). Next, the tissues were washed and fixed in 10% formaldehyde overnight. The infarcted myocardium was stained off-white, and the survival area was stained brick-red. Alpha View gel image software was used to analyze the percentage of myocardial infarction area. The percentage of myocardial infarction area is the percentage of the left myocardial infarction area to the total area of the left myocardium.

Western blot analysis

At the end of reperfusion, the left ventricular myocardial tissue was incised and immediately placed in liquid nitrogen for storage. The total protein of myocardial tissue was extracted and lysed with tissue lysate. Then, 30 µg of the sample was used for electrophoresis through the SDS-PAGE gel system and transferred into the membrane. Blocking with skimmed milk at 37°C for 2 h, the protein was incubated with p-AKT (rabbit monoclonal; dilution, 1:1,000; cat. no. ab81283; Abcam), AKT (rabbit polyclonal; dilution, 1:1,000; cat. no. ab8805; Abcam), p-mTOR (rabbit monoclonal; dilution, 1:1,000; cat. no. ab137133; Abcam), mTOR (rabbit monoclonal; dilution, 1:1,000; cat. no. ab32028; Abcam), Bcl-2 (rabbit monoclonal; dilution, 1:1,000; cat. no. ab185002; Abcam), Bax (mouse monoclonal; dilution, 1:1,000; cat. no. ab77566; Abcam) and GAPDH (rabbit monoclonal; dilution, 1:1,000; cat. no. ab181602; Abcam) primary antibodies at 4°C overnight. After rinsing the buffer, secondary antibodies (dilution, 1:5,000; cat. no. ab7090; Abcam) were added to incubate the protein sample for 2 h at room temperature. ECL was used to detect protein bands, and the Quantity One image analysis system was used to analyze the gray value of the target protein band.

Detection of cardiomyocyte apoptosis

The extracted rat tissues were fixed with 5% paraformaldehyde at 25°C for 24 h. The sample was permeated with 0.1% Triton-X 100 and sodium citrate for 15 min, and then incubated with 100 mL terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL, 100 mg/mL) solution in the dark at 37°C for 1 h. DAPI was used to stain cell nuclei. The fluorescence of TUNEL stained cells was analyzed by flow cytometry. The percentage of apoptotic cells in the total number of cells was the cardiomyocyte apoptosis rate.

Statistical analysis

All experiments were repeated three times. The data was analyzed by SPSS 23.0 statistical software and shown as mean $\pm$ standard deviation. The independent sample *t*-test was used to compare the differences between the two groups. One-way analysis of variance was used for multiple group comparisons. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Compared with the Sham group, the EF, FS, SV, IVSd, IVSs, LVPWd and LVPWs of the IR group at the end of reperfusion were significantly reduced, while LVIDd and LVIDs significantly increased ($P < 0.05$, Table I). In addition, compared with the IR group, EF, FS, SV, IVSd, IVSs, LVPWd and

LVPWs in the SPC group increased significantly, while LVIDd and LVIDs decreased significantly ($P < 0.05$, Table I). However, the heart function of the SPC group still did not reach the level of the Sham group, and the heart function index was still lower than that of the Sham group.

Compared with the Sham group, the area of myocardial infarction in the IR group increased significantly at the end of reperfusion. Compared with the IR group, the myocardial infarction area of the SPC group was significantly reduced at the end of reperfusion, but it was still higher than that of the Sham group ($P < 0.01$, Fig. 1A). In addition, TUNEL assay indicated that apoptotic nuclei showed green fluorescence, while normal nuclei showed blue fluorescence. There were only a few apoptotic cells

Table I. Comparison of cardiac function indexes at the end of reperfusion in three groups

Index	Sham Group (n=16)	IR Group (n=16)	SPC Group (n=16)
EF (%)	65.12 $\pm$ 2.32	34.03 $\pm$ 3.75 ^a	45.88 $\pm$ 5.21 ^{ab}
FS (%)	36.56 $\pm$ 1.38	17.15 $\pm$ 2.02 ^a	23.88 $\pm$ 3.14 ^{ab}
SV (ml)	172.45 $\pm$ 23.15	106.2 $\pm$ 14.5 ^a	132.05 $\pm$ 10.06 ^{ab}
LVIDd (mm)	6.68 $\pm$ 0.71	7.54 $\pm$ 0.58 ^a	6.52 $\pm$ 0.23 ^b
LVIDs (mm)	4.32 $\pm$ 0.33	6.04 $\pm$ 0.49 ^a	5.02 $\pm$ 0.12 ^{ab}
IVSd (mm)	1.61 $\pm$ 0.31	1.02 $\pm$ 0.18 ^a	1.44 $\pm$ 0.21 ^b
IVSs (mm)	2.16 $\pm$ 0.33	1.38 $\pm$ 0.31 ^a	1.78 $\pm$ 0.38 ^{ab}
LVPWd (mm)	1.57 $\pm$ 0.18	1.31 $\pm$ 0.41	1.42 $\pm$ 0.20 ^b
LVPWs (mm)	2.03 $\pm$ 0.21	1.39 $\pm$ 0.26 ^a	1.66 $\pm$ 0.41 ^{ab}

^aCompared with Sham group $P < 0.05$; ^bCompared with IR group $P < 0.05$

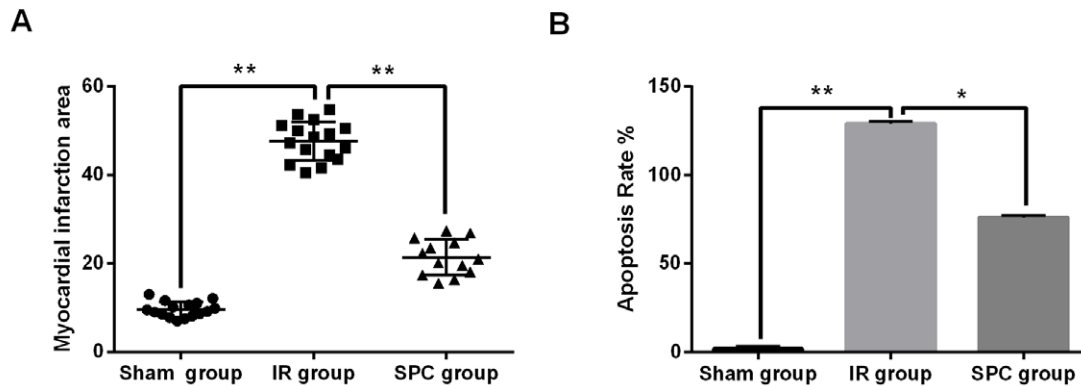


Fig. 1. Comparison of myocardial infarction area and myocardial cell apoptosis in 3 groups. **A)** The myocardial infarction area in Sham group, IR group and SPC group. **B)** Number of cardiomyocyte apoptosis in Sham group, IR group and SPC group. * $P < 0.05$, ** $P < 0.01$

in the Sham group. Compared with the Sham group, the number of cardiomyocyte apoptosis in the IR group was significantly increased ($P < 0.01$, Fig. 1B). The number of apoptotic cells in the SPC group was significantly lower than that in the IR group, but still higher than that in the Sham group ($P < 0.05$, Fig. 1B).

Compared with the Sham group, the protein expression of p-AKT and p-mTOR was significantly up-regulated in the IR group at the end of reperfusion. Compared with the IR group, the protein expression of p-AKT and p-mTOR was increased in the SPC group ($P < 0.05$, Fig. 2A, Table II). Compared with the Sham group, the total AKT and mTOR proteins of the IR group and SPC group was up-regulated, but the difference was not significant ($P < 0.05$, Fig.

2A, Table II). In addition, compared with the Sham group, Bcl-2 expression was significantly down-regulated at the end of reperfusion in the IR group, while Bax expression was significantly up-regulated in at the end of reperfusion in the IR group ($P < 0.05$, Fig. 2B, Table II). Compared with the IR group, Bcl-2 expression was increased, while Bax expression was decreased at the end of reperfusion in the SPC group ($P < 0.05$, Fig. 2B, Table II).

DISCUSSION

Research on the prevention and treatment of MIR has become a hot topic in the medical field. Due to ethical considerations, this study used the rat MIR

Table II. Protein expression of AKT/mTOR and Bcl-2/Bax in three groups

Group	p-AKT	p-mTOR	Bcl-2	Bax
Sham group	1.00±0.31	1.00±0.03	0.97±0.06	1.00±0.15
IR group	1.91±0.28 ^a	2.32±0.52 ^a	0.31±0.04 ^a	6.26±0.45 ^a
SPC group	3.61±0.18 ^b	5.54±0.56 ^b	0.68±0.05 ^b	3.26±0.25 ^b

^aCompared with Sham group, $P < 0.05$; ^bCompared with IR group, $P < 0.05$

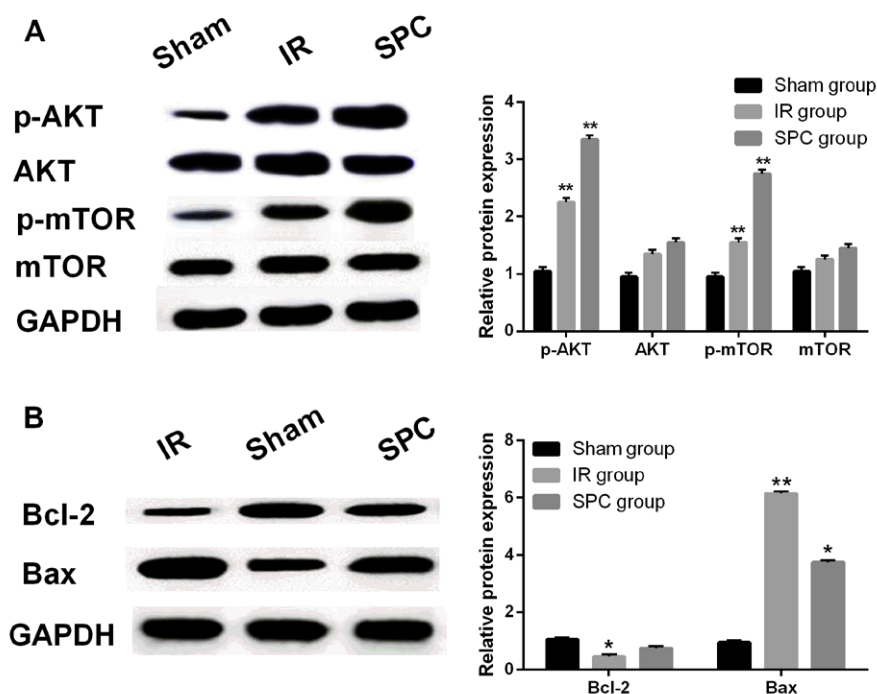


Fig. 2. Expression of AKT/mTOR and apoptotic related proteins in myocardial I/R rats. **A)** Protein expression of p-AKT/AKT and p-mTOR/mTOR detected in the Sham group, IR group and SPC group by Western blot analysis. **B)** Protein expression of Bcl-2/Bax detected in the Sham group, IR group and SPC group by Western blot analysis. * $P < 0.05$, ** $P < 0.01$

model to evaluate the protective effect of sevoflurane. Although the protective effect of sevoflurane has been reported in previous studies (7), there are still many issues that need to be explored and clarified. Similar to previous studies, this study also showed that the SPC group had a better cardiac function index and a lower myocardial infarction area than the IR group. These results indicate that sevoflurane post-treatment can improve left ventricular myocardial remodeling after MIR injury and play a protective role in MIR injury.

In addition, it has been reported that the PI3K/AKT signaling pathway is involved in the protective effect of sevoflurane post-treatment (8). The mTOR has been found to regulate cardiomyocyte apoptosis under conditions of oxidative stress caused by AKT activation (9). The study also found that 2.4% sevoflurane significantly increased the protein expression of p-AKT and p-mTOR. We also found that sevoflurane post-treatment enhanced Bcl-2 protein expression and reduced Bax protein expression in myocardial tissues. Correspondingly, it was found that the apoptotic cells in the SPC group were significantly less than in the IR group. However, the effect of sevoflurane post-treatment on human myocardial ischemia-reperfusion cells has not been investigated in this study and needs to be clarified in the future. In summary, sevoflurane post-treatment can inhibit the apoptosis of MIR cardiomyocytes by regulating the expression of Bcl-2/Bax protein. More importantly, sevoflurane post-treatment can also activate the AKT/mTOR signaling pathway to reduce myocardial damage.

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LETTER TO THE EDITOR

Effects of long non-coding RNA ANRIL on the biological function of human osteosarcoma cellsWB. Xu¹ and N. Zhang²

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To the Editor,

Osteosarcoma (OS), the most common type of primary malignant bone tumor, is defined by the presence of malignant mesenchymal cells producing osteoid or immature bone (1). It has been reported that long non-coding RNAs (lncRNAs) participate in various types of cancer (2). These carcinogenic or tumor-suppressive lncRNAs regulate pathogenesis of OS, including cell growth, proliferation, invasion, metastasis and apoptosis, functioning as an independent prognostic biomarker (3). Accumulating evidence suggests that ANRIL may function as an oncogene and act as a key player in diverse types of cancer. Several known potent oncogenes regulate ANRIL expression in cancer cells (4). LncRNA ANRIL is up-regulated in many human tumors, and downregulation of lncRNA ANRIL can inhibit the proliferation, invasion and migration of cervical cancer cells (5). However, the role of LncRNA ANRIL in OS has still remained elusive. Small interfering RNA (siRNA) can be used to silence lncRNA ANRIL, and reduced expression of lncRNA ANRIL can influence cell growth and metastasis. The present study aimed to assess the effects of LncRNA ANRIL on the proliferation, apoptosis, invasion, and migration of human osteosarcoma (HOS). In addition, its possible mechanisms were explored.

MATERIALS AND METHODS

Cell culture and transfection

HOS cells were purchased from Shanghai Fuxiang Biotech Co., Ltd. (Shanghai, China). After the routine recovery of HOS cells, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Xi'an Yike Biotechnology Co., Ltd., China) containing 10% fetal bovine serum (FBS) in an incubator (Sanyo, Japan) at 37°C with 5% CO₂. HOS cells of logarithmic stage were inoculated into a 6-well plate with a density of 5×10⁵ cells/well, and transfected when the cells reached a confluency of 30~50%. The experiment involved the three following groups: a) an experimental group (siRNA-ANRIL group): HOS cells were transfected with siRNA-ANRIL, and the expression of lncRNA ANRIL was silenced; b) a negative control group (siRNA-NC group): lncRNA ANRIL-Negative control was transfected; c) a blank group (control group): only liposomes were transfected. A mixture containing an effective transfection complex was prepared according to the instructions provided. The mixed solution was added to the cells and incubated at 37°C for 4-6 h in presence of 5% CO₂. The cells were further cultured in a fresh medium for 2-3 days. After stabilization, the cells were digested and transferred to the cell culture bottle for subsequent cultivation. The cells were observed regularly. When the fusion degree of

Key words: lncRNA ANRIL; human osteosarcoma; proliferating cell nuclear antigen; matrix metalloproteinases-2; mechanism

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cells in the culture bottle reached 75-85%, the cells were digested with trypsin (Beijing Wobison Technology Co., Ltd., China).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA in each group was extracted using Trizol reagent (TRIZOL one-step RNA extraction kit; Beijing Saiyintan Technology Co., Ltd., China) according to the manufacturer's instructions. The extracted RNA was reversely transcribed into cDNA. RT-qPCR was conducted using Maxima SYBR Green qPCR Master Mix (2x) according to the manufacturer's instructions (Thermo Fisher Scientific, USA). The relative expression of lncRNA ANRIL was calculated according to the $2^{-\Delta\Delta C_t}$ method: $\Delta\Delta C_t = \Delta C_t \text{ target gene} - \Delta C_t \text{ control gene}$. The PCR conditions were as follows: at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C, and extension for 30 s at 60°C. Primers were designed using Premier Primer 5 software. The full-length primers of mRNA sequence were designed as shown in Table I.

Cell proliferation assay

HOS cells with stable growth after transfection were selected from each group. After cell count, cells of each group were seeded into a 96-well plate with a density of 2×10^4 cells/well. After that, 10 μ L CCK-8 solution was added to each well, and the cells were incubated in a humid incubator at 37°C with 5%CO₂. The optical density (OD) values were detected at 24, 48, 72, and 96 h, respectively. All experiments were repeated at least three times in duplicate.

Cell apoptosis

Cells in good growth condition were selected from the 3 groups at 48 h after transfection. After phosphate-buffered saline (PBS) washing for three times, the cells were centrifuged at 800 g for 10 min. After the supernatant was discarded, the cells were mixed with a binding buffer solution at a concentration of 1×10^6 cells/ml. Then, 100 μ L of the cell culture medium was transferred to a centrifuge tube (5 mL), where it was added, mixed, and incubated with 5 μ L of (fluorescein isothiocyanate) FITC Annexin V and propidium iodide (PI) solution (BD, USA) for 15 min. After 400 μ L of binding buffer was added, the changes in apoptosis rates were detected by flow cytometry (Beckman Coulter FC500 flow cytometer; Beckman, USA).

Cell cycle

After 48 h of transfection, trypsin was used to digest the adherent cells to make cell suspension. After PBS washing, the cells were transferred to a centrifuge tube for centrifugation (120g, 5 min). The supernatant was carefully removed, 1 mL of precooled PBS was added to re-suspend the cells, and the cells were moved to the flow tube. The cells were centrifuged again and flicked at the bottom of the tube to allow them to be properly dispersed; 1 mL of precooled 75% ethanol was added for cell fixation and incubated overnight at 4°C. The supernatant was carefully removed, 1 mL of precooled PBS was added to wash the recast cells once, and the cells were centrifuged again at 120 g, for 5 min. The resuscitated cells were treated with $1 \times \text{PBS } 800 \text{ uL} + 1\% \text{ BSA solution, } 100 \text{ uL}$ PI solution, incubated at 37°C for 30 min in the dark, and detected and analyzed by flow cytometry.

Table I. Primer sequences used for PCR

Primer		Sequence
siRNA-ANRIL	Positive	5'-GGUCAUCUCAUUGCUCUAUTT-3'
	Reverse	5'-AUAGAGCAAUGAGAUGACCTT-3'
NC	Positive	5'-UUCUCCGAACGUGUCACGUTT-3'
	Reverse	5'-UCGTGUCUCGTTTCGGUGUUTT-3'
LncRNA ANRIL	Positive	5'-TCATGATGGAATTGGAGCCTT-3'
	Reverse	5'-CTCTTCCTGGCTTGCAGATTG-3';
GAPDH	Positive	5'-AACAGGGTGGTGGACCTCAT-3'
	Reverse	5'-GGAGGGGAGATTTCAGTGTGG-3'

Cell invasion assay

The HOS cells were inoculated into 24-well plates at a density of 10^4 cells/well. Subsequently, the cells were added to the upper and lower chambers, respectively. Serum-free medium with a volume of 100 μ L was added to the lower chamber, and serum-containing medium with a volume of 500 μ L was added to the upper chamber. The cells were placed in a CO₂ incubator for 24 h. Then, the cells on the upper surface of the Transwell-96 well were swabbed, and the cells on the lower surface were fixed with alcohol for 20 min and stained with hematoxylin for 5 min. The cells were observed and photographed under an inverted microscope (Olympus, Japan).

In vitro scratch assay

The cell monolayer in a straight line was scraped to create a “scratch” with a p200 pipette tip. The debris was removed, and the edge of the scratch was smoothed by washing the cells once with 1 ml of the growth medium and then replacing with 5 ml of medium specific for the *in vitro* scratch assay. After the reference points were defined, the dish was placed under a phase-contrast microscope, and the reference mark was left outside the capture image field but within the eye-piece field of view. The dish was placed in a tissue culture incubator at 37°C for 8–18 h. After the incubation, the dish was placed under a phase-contrast microscope, and matched with the reference point. The photographed region acquired in Step 6 was aligned and a second image was acquired. The images acquired were further analyzed quantitatively by using a software.

Western blot assay

After the culture medium in the cells was discarded, the cells were washed with ice-precooled PBS, added with protein lysate, and collected. After the cells were lysed with ultrasonic crushing apparatus (Guangzhou Yuhua Instrument Co., Ltd., China), they were stored at -20°C until further analysis. Electrophoresis (Electrophoresis apparatus; BIO-RAD Company) was carried out with 8%

separated gel and 5% concentrated gel. First, electrophoresis was conducted with a voltage of 80 V for 30 min and then with a voltage of 110 V. The nitrocellulose membrane was sealed in 5% BFS for 2 h, and then washed three times with TBST (T: Tris; B: Buffer; S: Solution; T: Tween). Then, the nitrocellulose membrane was placed in the reaction solution overnight at 4°C, washed with TBST, and incubated at room temperature with a 1:300 diluted horseradish peroxidase (HRP)-labeled anti-antibody on a shaker for 2 h. Then, NC film was washed with TBST, and its color was developed by enhanced chemiluminescence (ECL). The gray value of protein strips was analyzed by Quantity-One software. The relative expression levels of the target proteins were calculated as the ratio of the gray value of the target protein band to that of the internal reference band (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The experiment was repeated for at least 3 times.

Statistical analysis

All experimental data were analyzed using SPSS 19.0 software (IBM, USA), and measured data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Multi-group comparison was carried out by one-way analysis of variance. The intergroup comparison was conducted with Student–Newman–Keuls (SNK) test. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of lncRNA ANRIL expression levels by RT-qPCR

After 48 h of transfection, the cell growth was stable. The results of RT-qPCR showed that the expression level of lncRNA ANRIL in the siRNA-ANRIL group after transfection was significantly lower than that in the siRNA-NC group and the control group ($P < 0.05$), and there was no significant difference in the expression level of lncRNA ANRIL between the siRNA-ANRIL group and the control group ($P > 0.05$; Table II).

Table II. Expression levels of lncRNA ANRIL among the three groups

Group	Control group	siRNA-NC group	siRNA-ANRIL group
lncRNA ANRIL	0.983	0.991	0.328 ^{*#}

^{*} $P < 0.05$, compared with the control group; [#] $P < 0.05$, compared with the siRNA-NC group.

Cell proliferation

After 48 h of transfection, the OD values of HOS cells in the siRNA-ANRIL group significantly decreased from 24 h compared with the control group and the siRNA-NC group ($P < 0.05$; Fig. 1). The OD values of HOS cells in the siRNA-NC group and the control group were similar, and the difference was not statistically significant ($P > 0.05$).

The inhibitory effects of lncRNA ANRIL expression on HOS cell cycle

Flow cytometry revealed that there was no

significant difference in the percentage of G1 phase cells in each group, indicating that HOS cells had no obvious cycle arrest and failed to regulate the cell cycle to inhibit the expression of lncRNA ANRIL (Fig. 2A).

Cell apoptosis

The results of flow cytometry revealed that the apoptosis rate of HOS cells in the siRNA-ANRIL group was significantly higher than that in the siRNA-NC group and the control group ($P < 0.05$) (Fig. 2B). There was no significant difference between the siRNA-NC group and the control group ($P > 0.05$).

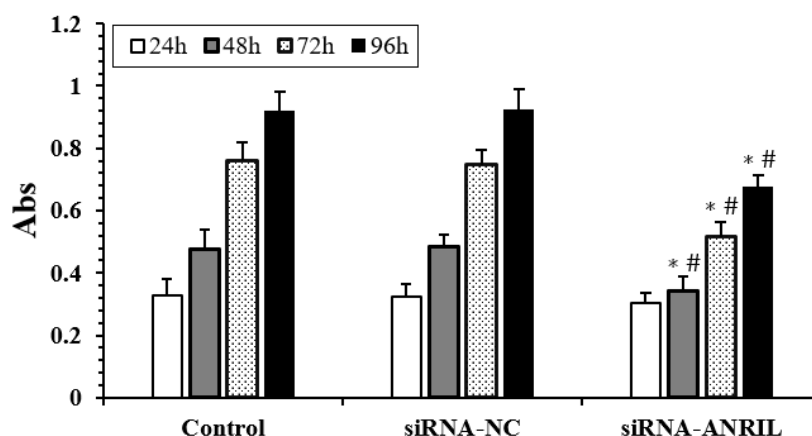


Fig. 1. OD values of HOS cells among the three groups. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the siRNA-NC group.

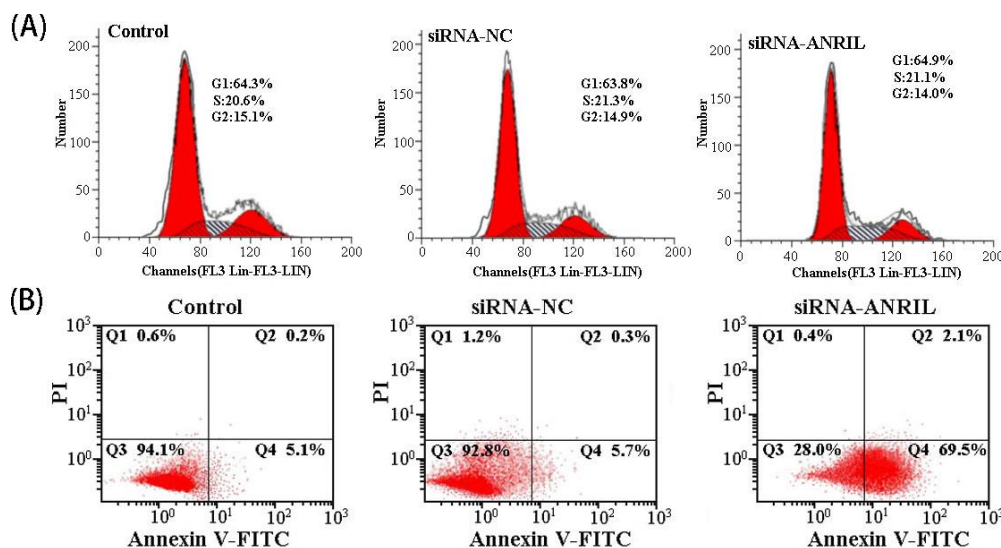


Fig. 2. Inhibitory effects of lncRNA ANRIL expression on HOS cell cycle (A) and apoptosis (B) detected by flow cytometry.

Transwell invasion assay

As shown in Fig. 3 A and B, after 24 h of culture, the results of Transwell invasion assay indicated that the number of HOS cells penetrating basement membrane in the siRNA-ANRIL group was markedly smaller than that in the siRNA-NC group and the control group ($P<0.05$), while there was no significant difference in the number of HOS cells penetrating basement membrane between the siRNA-ANRIL group and the control group ($P>0.05$).

Cell migration assay

Fig. 3C shows that the migratory ability of HOS

cells in the siRNA-ANRIL group significantly decreased compared with that in the siRNA-NC group and the control group ($P<0.05$). No significant difference was found between the siRNA-NC group and the control group ($P>0.05$).

Protein expression detected by Western blotting

After the expression of lncRNA ANRIL was silenced, results of Western blotting showed that compared with the siRNA-NC group and the control group, the expression levels of Bcl-2, proliferating cell nuclear antigen (PCNA), and matrix metalloproteinases-2 (MMP-2) in the siRNA-ANRIL group notably decreased, while the expression level

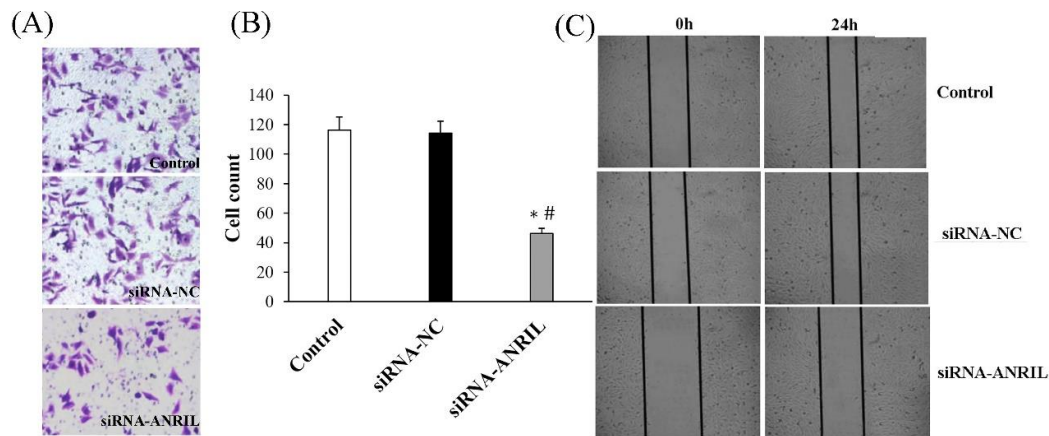


Fig. 3. Results of Transwell invasion assay. **A)** cells invaded after undergoing Transwell invasion assay, $\times 200$; **B)** the number of cells invaded. $^*P<0.05$, compared with the control group; $^{\#}P<0.05$, compared with the siRNA-NC group; **C)** The migration effect of cells in each group after 24 hours of scratch treatment detected by scratch test.

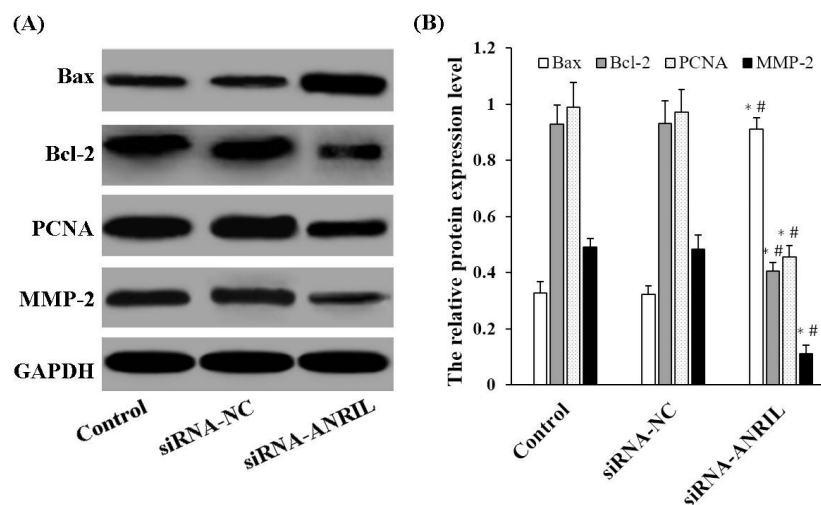


Fig. 4. Expression levels of Bax, Bcl-2, PCNA, and MMP-2 proteins among different groups. **A)** Western blotting results; **B)** Expression levels among the groups. $^*P<0.05$, compared with the control group; $^{\#}P<0.05$, compared with the siRNA-NC group.

of Bax markedly increased ($P < 0.05$). However, the expression levels of each protein in the siRNA-NC group and the control group were similar, with no significant difference ($P > 0.05$; Fig.4).

DISCUSSION

lncRNA ANRIL has been proven to play a carcinogenic role in various types of cancer (6), in addition to controlling the proliferation, apoptosis, invasion, and migration of cancer cells *in vitro*. The results of the present study demonstrated that the inhibited expression of lncRNA ANRIL could significantly inhibit the proliferation, invasion, and migration of HOS cells, and promote apoptosis, while it had no significant effect on cell cycle. Huang et al. pointed out that ANRIL was also up-regulated in cancer tissues (7, 8). This was consistent with the results of the present research.

Studies (9, 10) confirmed that OS patients with a high expression level of PCNA had poor prognosis. Matrix metalloproteinase (MMP) is an important protease that is closely associated with tumor growth, invasion, and metastasis. Researchers (11) reported that the expression of MMP-2 was increased in a variety of tumor cells, e.g., OS, which could promote the invasion and metastasis of tumor cells. A number of scholars (12) demonstrated that up-regulation of Bax expression promoted the apoptosis of OS cells. Our findings revealed that expression levels of PCNA, Bcl-2, and MMP-2 were significantly decreased, and the Bax expression level was markedly increased in HOS cells. The results suggested that the inhibitory effect of lncRNA ANRIL on HOS cell proliferation, invasion, migration, and apoptosis was related to the regulation of expression levels of PCNA, MMP-2, Bcl-2, and Bax.

In summary, lncRNA ANRIL could inhibit the expression of lncRNA ANRIL in HOS cells by using siRNA-ANRIL. The silencing of lncRNA ANRIL could promote the apoptosis of HOS cells, and inhibit the proliferation, invasion and migration of HOS cells. Silencing of lncRNA ANRIL was found to be closely associated with the proliferation, invasion, and migration of OS cells, rather than cell cycle. The underlying mechanism could be related

to downregulation of PCNA, MMP-2 and Bcl-2 expression levels, as well as upregulation of Bax expression levels.

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LETTER TO THE EDITOR

LIMK1 attenuates sevoflurane-induced neurodevelopmental toxicity through caspase-3/cofilin/PARP-1 pathway

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To the Editor,

Sevoflurane is a new halogenated inhalation anesthetic, which has the advantages of rapid induction, small respiratory tract irritation, low solubility, rapid absorption and clearance, quick recovery, easy adjustment of anesthesia depth, and light circulation inhibition. It has significant advantages in both pediatric and outpatient surgery (1). However, many studies have shown that sevoflurane has neurotoxic effects on the nervous system. Sevoflurane and isoflurane can promote the apoptosis of cultured hippocampal neurons in a dose-dependent and time-dependent manner (2). However, the results of this study are controversial, and some studies used anesthetics in excess of clinical doses (3). It has been reported that cognitive dysfunction can be induced only when the exposure time of anesthetics and the degree and location of histological damage (such as Hippocampus) reach a certain threshold, and the results of animal experiments cannot be simply applied to humans (4). At present, large-scale clinical trials are still in progress, and there are no relevant studies showing that inhalation anesthetics have neurotoxic effects on the human body.

LIMKs are serine/threonine and tyrosine double

active protein kinases. They are downstream molecules of Rho, Cdc42 and Rac1, and partake in regulating various cell functions (5). Previous studies have shown that LIMKs also play an important role in cytoskeleton reorganization, cell migration, cell cycle and cell pathology as well as in the function of the nervous system. (6). However, the role and mechanism of LIMK1 in neurodevelopmental toxicity remain unclear. The effect of LIMK1 on sevoflurane neurotoxicity remains to be studied. In this study, Morris water maze was used to evaluate the cognitive function of rats and to investigate the role and mechanism of LIMK1 in sevoflurane neurotoxicity.

MATERIALS AND METHODS

Animals and grouping

Seventy-two male, 7-day-old SD rats were purchased from Allcells Biotechnology Co., Ltd. (Shanghai, China). Rats were housed at 24-26°C with a humidity of 55-65%. During the 12/12 hour light/dark cycle, rats had free access to chow and water. All animal experiments were approved by the ethics committee of Affiliated Hongqi Hospital of Mudanjiang Medical University and were carried out in accordance with relevant regulations.

The rats were randomly divided into four groups (n

Key words: LIMK1; sevoflurane; neurotoxicity; caspase-3; Poly (ADP-ribose) polymerase 1(PARP-1)

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= 18 in each group): control group (inhaled 30% oxygen concentration for 6 h), sevoflurane group (injected into hippocampus 30 min before anesthesia with normal saline, inhaled 2% sevoflurane for 6 h), negative control group [2 μ L (108 Tu/ml) of negative LIMK1 was injected into the hippocampus 30 min before anesthesia, and then 2.0% sevoflurane was used for 6 h] and LIMK1 group [2 μ L (108 Tu/ml) of LIMK1 was injected into the hippocampus 30 min before anesthesia, and 2% sevoflurane was used for 6 h].

The rats in each model group were placed in a transparent plastic anesthesia box, where they inhaled sevoflurane, and the inhaled oxygen concentration of 30% was maintained. The skin color and respiration of the rats were observed during anesthesia to prevent respiratory depression or skin cyanosis.

Hippocampus harvesting and protein level quantification

At the end of anesthesia, five rats in each group were randomly selected and sacrificed by decapitation at 6 h, 1 day, 3 days and 7 days, respectively. The hippocampus was removed and preserved at -80°C . The hippocampus was then split by frozen radioimmunoassay. Radio Immunoprecipitation Assay (RIPA) buffer used was 50 mM Tris HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L Ethylene Diamine Tetraacetic Acid (EDTA), 0.5% nonidet P-40, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptide, 1 $\mu\text{g/ml}$ pepstatin, 1mM Na_3VO_4 and 1mM NaF. The lysates were then collected and centrifuged at 4°C for 30 min, and the amount of protein was quantified with a double bicinchoninic acid (BCA) protein kit.

Western blots

Using 12.5% SDS-PAGE gel and a half dry sucking instrument (BIO-RAD, USA), the proteins were transferred to nitrocellulose membrane (millipore, Eschborn, Germany) by electricity and incubated overnight at 4°C with primary antibody. The second antibody combined with horseradish peroxidase was added for 2 h and washed for 3 times. Amersham enhanced chemiluminescence was used to detect the reaction bands. Amersham scanning imprinted image 600 scanner (GE, USA) and protein band density were used to quantify.

Cell viability assay

Cell-lumiTMPlus Biyuntian cell viability detection kit was used to detect neuronal cell viability. The cell culture

plate was removed and equilibrated at room temperature for 10 min. 100 μL CellTiter-LumiTM + luminescence detection reagent was added to the 96-well plate, and shaken at room temperature for 2 min to promote cell lysis, then incubated at room temperature for 10 min to stabilize the luminescence signal. Chemiluminescence detection was performed using a multi-functional microplate reader for chemiluminescence detection. The corresponding parameters were set according to the requirements of the instrument. The detection time of each well was generally 0.25-1 s or longer, and the specific adjustment was adjusted according to the detection sensitivity of the instrument. The relative viability of cells was calculated based on chemiluminescence readings.

Morris water maze (MWM)

In order to interfere with the nervous system, especially the function of learning and memory, after 28 days 24 rats from four groups were tested with a water maze: round pool (diameter 1.6 m, height 60 cm), water temperature $23\pm 1^{\circ}\text{C}$. The escape latency test was conducted once a day for five consecutive days. In the experiment, the rats were trained to swim to a hidden platform and locate it. After each trial, each rat was placed in a cage under a hairdryer for five min to dry before returning to the normal cage. After the escape latency test, the platform was removed and the mice were allowed to swim freely for 90 s; the whole behavior test was recorded and analyzed using Noldus. Each mouse was placed on a platform in the center of the MWM for 30 s, then released into the water from a designated release point. The mouse was allowed to swim for 90 s or until it landed on the platform. If the mouse failed to reach the platform within 90 s, it was put on the platform for another 10 s. The records needed for swimming distance and time to reach the platform were tracked by video and analyzed by MWM software.

Statistical analysis

All data were processed by SPSS 23.0. The measured data is expressed in the form of mean $\pm$ standard deviation. The *t*-test was used for comparison between the two groups and analysis of variance for comparison of three or more groups. $P < 0.05$ was considered statistically significant.

RESULTS

Sevoflurane reduces the expression of LIMK1, caspase-3, poly (ADP-ribose) polymerase 1 (PARP-1) and protein kinase A (PKA) in hippocampus of rats

As shown in Fig. 1, compared with the control group, the expression of LIMK1, caspase-3, PARP-1 and PKA in the sevoflurane exposed group decreased significantly, while the expression of cofilin increased significantly.

Sevoflurane exposure induced neuronal apoptosis in rat hippocampus

Fig. 2 shows the effect of sevoflurane on the apoptosis of hippocampal neurons in anesthetized rats. The results show that sevoflurane inhalation anesthesia could increase the apoptosis of hippocampal neurons,

and the overexpression of LIMK1 could inhibit the apoptosis induced by sevoflurane.

LIMK1 regulates neurotoxicity of sevoflurane through caspase-3/cofilin/PARP-1 pathway

After primary culture of hippocampal neurons in the model group and control group, it was found that the cell viability of hippocampal neurons in the model group decreased (Fig. 3A). LIMK1 overexpressed hippocampal neurons were constructed. Compared with the control group, after LIMK1 overexpression and sevoflurane exposure, LIMK1 promoted caspase-3 protein expression, reduced cofilin protein expression, and promoted PARP-1 protein expression, but had no significant effect on PKA protein expression (Fig. 3B).

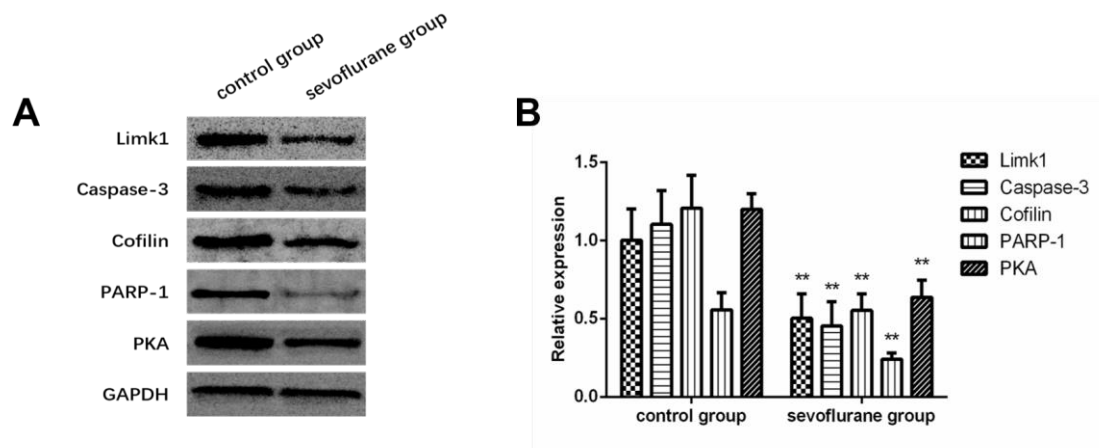


Fig. 1. Expression of LIMK1, Caspase-3, Cofilin, PARP-1 and PKA protein in the control group and sevoflurane group.

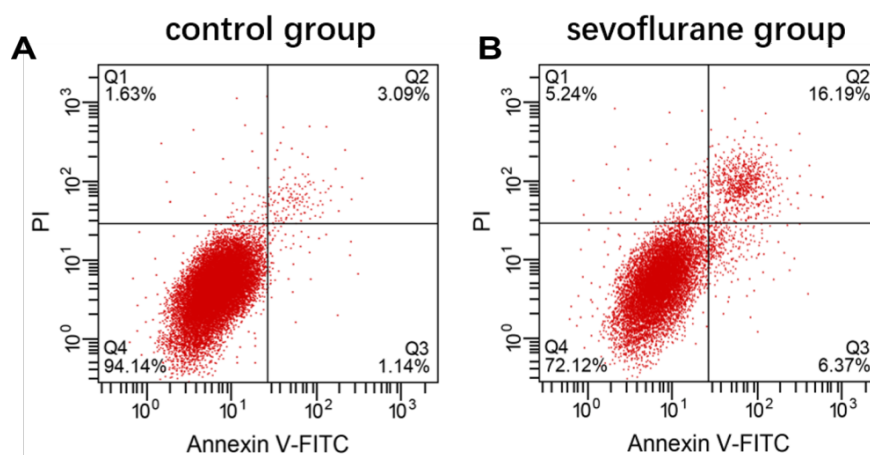


Fig. 2. Results of apoptosis in the control group (A) and sevoflurane group (B).

Sevoflurane exposure reduces learning and memory

The learning performance of rats in each group was improved 6 days after modeling and the escape latency was shortened after training. The results showed that sevoflurane-anesthetized rats had delayed escape, and the rats in the LIMK1 overexpression group had decreased escape delay. The results showed that sevoflurane inhalation anesthesia may lead to spatial learning and memory disorders in rats. The up-regulation of LIMK1 expression can improve the spatial learning and memory disorders induced by sevoflurane anesthesia (Fig. 4).

DISCUSSION

One of the most important functions of LIMKs

is to phosphorylate ser3 sites of three different substrates: cofilin1 expressed in non-muscle tissues, cofilin2 and Destin expressed in muscle tissues, also known as actin depolymerization factor (ADF) (7). This study confirmed that LIMK1 is involved in the mechanism of neurotoxicity and plays a role through caspase-3/cofilin/PARP pathway. LIMK1 plays the most important role in regulating the morphology and neurite growth of neurons (9). Some studies have shown that in LIMK1 knockout mice, the decrease of neurons, the loss of growth cone and the abnormal morphology of dendrites can be observed, which leads to the abnormal synaptic structure and the development of dendritic spines. In cultured hippocampal pyramidal neurons, the expression of early wild-type LIMK1 can induce the formation of

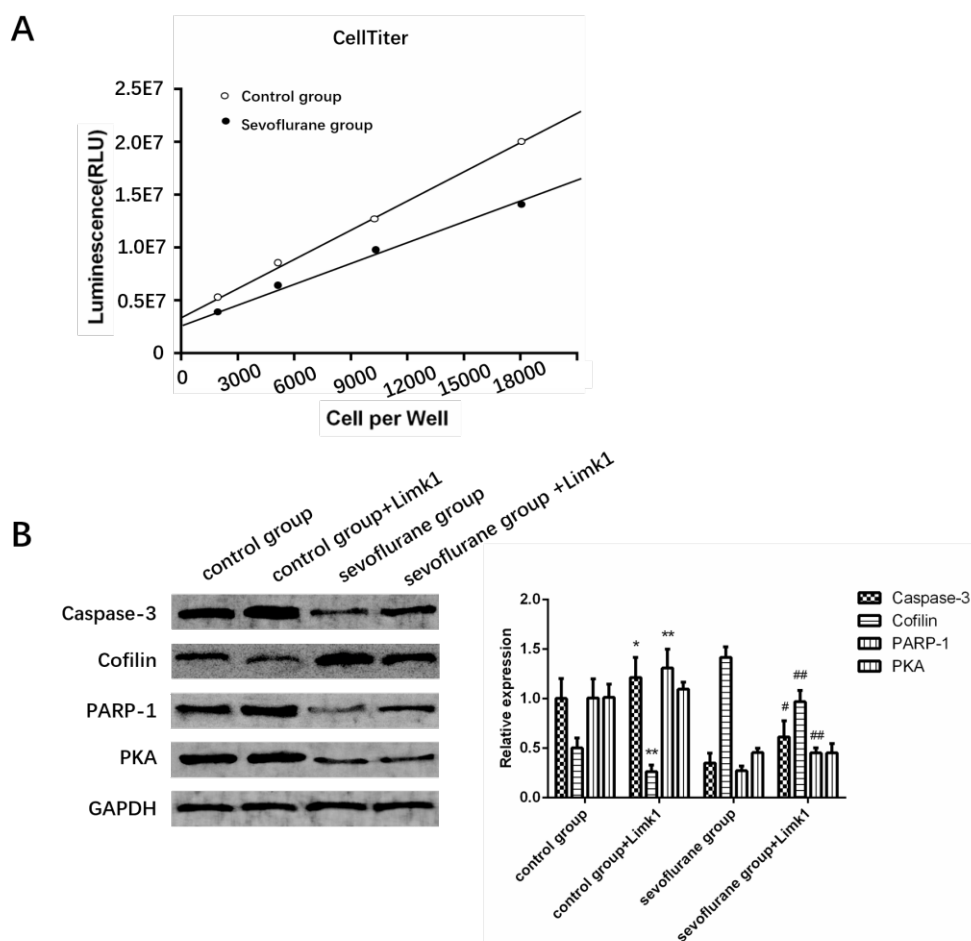


Fig. 3. Cell viability and expression of Caspase-3, Cofilin, PARP-1 and PKA protein in different groups. **A)** Cell viability in control group and sevoflurane group. **B)** Expression of Caspase-3, Cofilin, PARP-1 and PKA protein in control group, control group+ LIMK1, sevoflurane group and sevoflurane group+ LIMK1.

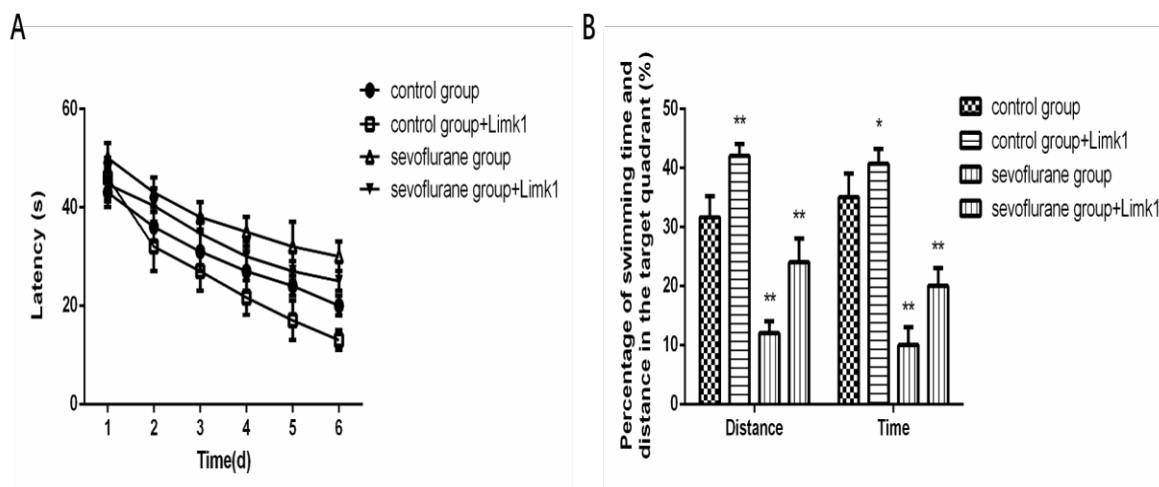


Fig.4. Changes of spatial learning and memory function in each group. **A)** Average escape latency from water to platform in each group. **B)** The swimming time and distance in each group.

growth cones and neurites, while the late expression of LIMK1 can cause the withdrawal of growth cones and neurites, and eventually lead to axonal retraction. It seems that LIMK1 plays an important role in the regulation of neuronal morphology. Recently, LIMKs have been found to be associated with some neurodevelopmental disorders, such as Williams syndrome which is a congenital disease caused by genetic disorder (11). Chromosome analysis showed that Williams syndrome was associated with LIMK1 deletion. The LIMK1 knockout mice exhibited reduced behavioral spatial positioning capabilities, similar to the symptoms observed in human Williams syndrome patients. Inhibition of caffeine phosphorylation prevents neuronal degeneration, which supports this hypothesis. Inactivation of cofilin/ADF by LIMKs phosphorylation can affect actin polymerization, thus regulating cell migration, differentiation, apoptosis and metastasis.

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LETTER TO THE EDITOR

Modulation of gut microbiota in patients with IBS and systemic nickel allergy after diet and probiotic supplementation: a pilot study

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To the Editor,

The gastrointestinal tract (GIT) has a very important immune function in developing either effector or tolerant responses to different antigens by balancing the activities of Th1 and Th2 cells as well as regulating Th17 and T regulatory (Treg) cells in the lamina propria (1). Immune dysfunction has been associated with lifestyle changes, such as dietary modifications and increased antibiotic use. These habit changes have profoundly altered the co-evolved relationship between host and microbiota, depleting bacterial populations critical for the maintenance of mucosal homeostasis (2). The gut microbiota can be defined as the trillions of microbes that collectively inhabit the gut lumen, and increasing evidence shows that altered patterns of microbial exposure (dysbiosis) in an early stage of life can lead to the development of adverse food reactions (AFR) by negatively influencing immune system development (3).

The gut microbiota could be considered as a potential target for preventive and therapeutic

intervention against AFR. Recent studies have reported the efficacy of intervention in the gut microbiota against AFR. In recent years, strong evidence of a possible relationship between modifications of the gut microbiota composition and development of irritable bowel syndrome (IBS) has been collected (4). The currently reported general population prevalence of nickel (Ni) allergy is 8-18% in the US and Europe; in southern European countries, such as Italy, it is estimated to be 16% (5). In sensitized subjects, ingested Ni may induce gastrointestinal symptoms similar to IBS, in addition to typical systemic cutaneous lesions [systemic nickel allergy syndrome (SNAS)] (6). A low-nickel diet could reduce the systemic manifestations (7).

We report here the results of a pilot observational study to evaluate the effects of probiotic supplementation in patients with IBS and SNAS, that had been previously treated only with dietary intervention without a complete success, in terms of

Key words: AFR; IBS; SNAS; microbiota; probiotics

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modulation of faecal microbiota population, reduction of GI symptoms, increase of the patient's quality of life and modification of gut dysbiosis (8).

MATERIALS AND METHODS

Between March and September 2017 40 consecutive patients, admitted to the Allergy Unit reporting nonspecific gastrointestinal symptoms, were evaluated on the presence of abdominal pain or distension, meteorism, diarrhea and/or constipation (Roma criteria III). Exclusion criteria were: age <18 years; organic gastrointestinal diseases, such as peptic ulcer, inflammatory bowel diseases, celiac disease, gastrointestinal infections, and small intestinal bacterial overgrowth; diabetes mellitus, hepatic, renal or cardiac dysfunction, and thyroid disease or tumor; intake of non-steroidal anti-inflammatory agents and pregnancy, lactation, abuse of alcohol, coffee, tea, and cola intake.

After gastroenterological and allergological evaluation, 14 patients were diagnosed as IBS, 24 as Ni sensitization and SNAS and two patients showed non-specific lipid transfer protein (LTP) sensitization. Age was between 18

and 73 years (median age 40 years). The F/M ratio was 33/7. The median BMI at baseline was 21 (Table I). All patients signed informed consent to participate in the study that was approved by the ethics committee.

All patients had previously been prescribed different diet protocols, without reporting a full improvement of GI symptoms. Eligible participants were adults who i) had a positive Ni-patch test, ii) reported symptoms suggesting SNAS, and iii) improved at least 70% from baseline after one month on a Ni-poor diet (severity of symptoms rated on a visual analog scale - VAS) (6). All patients were evaluated for gut dysbiosis (9) at baseline. DNA extraction method (Next Generation Sequencing) with a commercial kit was performed on stool samples. Direct sequencing of 16S rRNA amplicons is a fast and useful method to determine gut bacterial population (10). Twenty-five out of the 40 patients repeated dysbiosis evaluation also after three months (T1). Quality of life was assessed through IBS QoL (11).

Dietary intervention and probiotic supplementation

IBS patients were arbitrarily divided into two groups, according to a gluten-free diet prescription (11 pts) or a

Table I. Patient characteristics at baseline (T0) and after treatment (T1).

	T0	T1
n° of patient	40	
Age in years (median, range)	40 (18-73)	
F/M ratio	33/7	
IBS (n°)	14	
Low fodmap diet	10	
Gluten-free diet	4	
SNAS (n°)	24	
LTP sens (n°)	2	
Dysbiosis (n°/%)	31/40 (77.5)	7/25 (28)
IBS	14	3
SNAS	16	3
LTP sens	1	1
BMI (median)	21	21.7

low Fermentable Oligo-, Di- and Mono-saccharides And Polyols (FODMAP) diet prescription for three months (10 pts). FODMAP are a group of carbohydrates that are poorly absorbed in the small intestine and subsequently fermented in the small or large intestine. Dietary advice on a low FODMAP diet was mostly delivered by a dietitian. Table II is a representative example of common foods that are known to have a high FODMAP content.

Similarly, SNAS patients were prescribed a low-Ni diet (100 µg/kg nickel content during the first four weeks and then up to 200 µg/kg for up to three months). The absolute removal of Ni from the diet is impractical because of its ubiquitous presence in almost all foods; patients were asked to avoid the use of stainless-steel utensils to reduce Ni contamination during cooking.

Two non-specific LTP sensitized patients underwent an

Table II. *Foods divided according to the content of FODMAP.*

Types of sugars	High FODMAP foods	Low FODMAP alternatives
Oligosaccharides	FOS-Grains: wheat-, rye-, and barley-based products Vegetables: onion, garlic, artichokes, leeks, beetroot, and savoy cabbage	Fruit: banana, most berries (except boysenberries and blackberries), grapes, lemon, lime, mandarin, orange, kiwi fruit, pineapple, passion fruit, and rhubarb
Disaccharides	Fruits: watermelon, peaches, persimmon, prunes, nectarines and most dried fruit	Vegetables: capsicum, bok choy, green beans, parsnip, silverbeet, cucumber, carrots, celery, eggplant, lettuce, potatoes, yams, tomatoes, and zucchini
Monosaccharides	GOS-Legumes: red kidney beans, baked beans, and soya beans Vegetables: beetroot and peas	Grains: wheat-free grains/flour, gluten-free bread or cereal products, and quinoa
polyols	Lactose-Dairy products: cows/goat milk, and yoghurt	Dairy products: lactose-free, almond or rice-based milk, yoghurt and ice cream, hard cheese, feta and cottage cheese
	Fructose (in excess of glucose) Fruits: apples, pears, watermelon, mango, cherries, boysenberries and fruit juice from high-fructose foods	Fruit: banana, grapes, honeydew, melon, kiwifruit, lemon, lime, mandarin, orange, passionfruit, paw paw, and most berries (except boysenberries and blackberries)
	Honey-Sweeteners: high-fructose corn syrup	Sweeteners: maple syrup and golden syrup
	Vegetable: asparagus and snap peas	Sweeteners: Maple syrup, and sugar (sucrose) Fruits: banana, grape, honeydew, melon, kiwifruit, lemon, mandarin, orange, passionfruit, and paw paw
	Sorbitol fruit: apples, pears, avocado, apricots, blackberries, nectarines, peaches, plums, prunes, and watermelon Mannitol Vegetables: sweet potato, mushrooms, cauliflower, and snow peas	

LTP-free diet; this food allergy is by far the main cause of primary food allergy in Italian adults and generally in the Mediterranean European countries, and is also responsible for the largest number of food-induced anaphylactic reactions (12).

A fixed probiotic supplementation was prescribed along with diet: *Enterococcus faecium*, *Saccharomyces boulardii*, *Lactobacillus acidophilus* (Enterelle®) for the first 12 days, *Lactobacillus salivarius*, *Lactobacillus acidophilus*,

Bifidobacterium bifidum, *Bifidobacterium longum*, *Bifidobacterium lactis*, *Lactobacillus rhamnosus* for up to three months (Acronelle® and Serobioma®).

One study involved two outpatient scheduled visits [baseline (T0) and 3 months after the prescription of diet and probiotic supplementation (T1)] and a phone contact 2 weeks after the conclusion of treatment (T2). Gastrointestinal symptoms were evaluated using VAS before and after treatment. We used VAS-IBS to assess

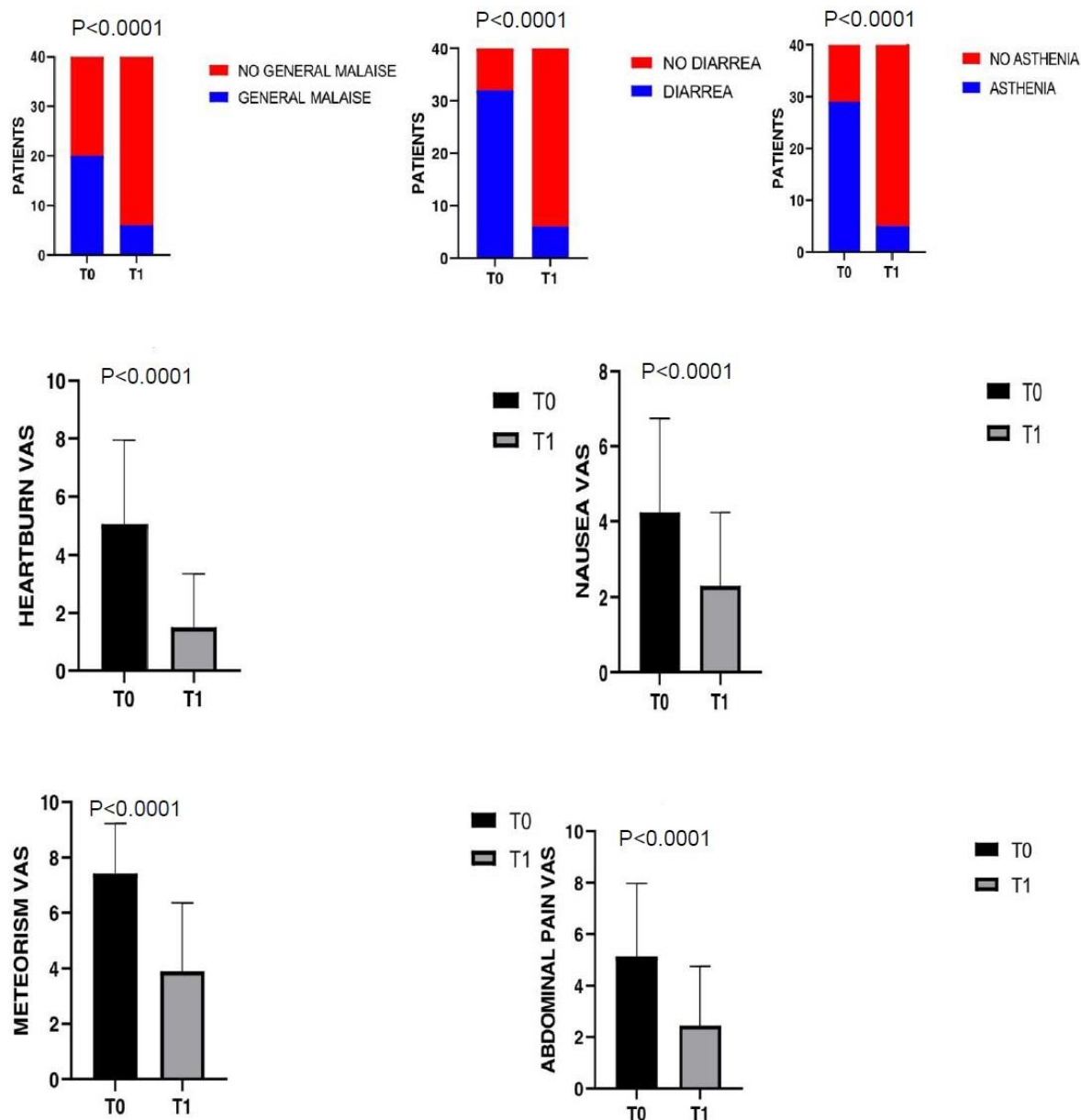


Fig. 1. Improvement of the major items of IBS-VAS at T1 (after diet and probiotic supplementation).

the presence and severity of symptoms related to IBS and the improvements achieved through dietary and probiotic treatment, assigning a score from 0 to 10 at T0, T1 and T2. The most outstanding physical symptoms identified were divided into six main groups; abdominal pain, diarrhoea, constipation, bloating and flatulence, abnormal bowel passage and vomiting and nausea. All symptoms except vomiting and nausea also support the diagnosis of IBS (13).

In 25 pts that accepted to repeat the test, gut dysbiosis was re-assessed at T1 in order to underline any relevant modification. Two weeks after the conclusion of the prescribed treatment, during a telephone follow-up visit, patients were re-questioned about any variation of clinical symptoms.

Statistical analysis

Data analysis was carried out by McNemar's *Chi*-squared test with continuity correction (comparison of proportions) and Paired Sample *t*-test (comparison of means). Probability levels <5% were considered statistically significant.

RESULTS

At baseline, 31 out of 40 patients had an abnormal dysbiosis index (n.v. 0.070, 0.326). It was not possible to find any significant difference in gut bacteria populations among the three groups of patients, but patients with a higher value of dysbiosis showed a lack of lactate production. Dysbiosis values returned to a normal range after three months of treatment in 18 out of 25 patients that underwent re-assessment at T1.

Comparison of VAS scales means three months after the beginning of the therapy showed significant improvement of gastrointestinal symptoms, in terms of nausea (4.24 ± 2.50 vs 2.31 ± 1.93 ; $P < 0.0001$), heartburn (5.07 ± 2.88 vs 1.52 ± 1.84 ; $P < 0.0001$), meteorism (7.41 ± 1.80 vs 3.90 ± 2.47 ; $P < 0.0001$) and abdominal pain (5.14 ± 2.84 vs 2.45 ± 2.31 ; $P < 0.0001$). After three months there was a significant reduction of patients with diarrhoea (32/40 (80%) vs 6/40 (15%); $P < 0.0001$), asthenia (T0 29/40 (72.5%) vs 5/40 (12.5%); $P < 0.0001$) and general malaise (20/40 (50%) vs 6/40 (15%); $P < 0.0005$) (Fig. 1).

The clinical improvement was confirmed also after

the cessation of probiotic supplementation, in fact, VAS score remained unmodified at T2 evaluation, thus indicating the persistence of clinical benefits even after the end of the intervention. No significant difference was found among the groups in terms of improvement of VAS scores: IBS patients (regardless of the diet) as well as Ni-sensitized patients showed similar benefits of clinical symptoms after the intervention. We could speculate that the clinical improvement is mostly due to the probiotic supplementation rather than to different diet protocols, but the size of groups is too small for definitive considerations. Moreover, further studies in a double-blind placebo-controlled setting are needed. No statistical consideration could be made for the two LTP patients. Notably, all patients experienced an improvement of quality of life measured by IBS QoL (11) after the intervention, while no meaningful change of BMI was observed at T1 (Table I).

DISCUSSION

It has been widely observed that probiotics might be able to exert a beneficial influence on several pathogenetic pathways of IBS, including the restoration of altered gut microbiota, by increasing the number of beneficial bacteria and reducing the number of pathogens because of their reaction to the presence of the coexisting ones, and consequently the decrease of inflammation associated with the proliferation of pathogenic bacteria (9). This way, probiotic supplementation combined with diet could be useful to reduce GI symptoms and improve the quality of life in IBS patients.

In this pilot study, the gut microbial community of SNAS patients was investigated with DNA extraction method on stool samples. Previous findings suggested that the low-Ni diet is useful to improve the symptoms and the quality of life of patients with SNAS (6), however, our preliminary findings hypothesize that its association with probiotics might allow to rapidly decrease the frequency and the severity of symptoms related to meals, likely due to the increase of intestinal gut microbial diversity.

In the present study, for the first time, the effectiveness of probiotic administration coupled to a low-Ni diet in SNAS patients was revealed. Moreover, the

observation that, before the study, all these patients had partially improved with the diet alone may support the idea that probiotic implementation could represent not only a useful but probably a crucial mean for the treatment of these conditions. This data suggests that, although different from an etiopathological point of view, an altered intestinal homeostasis may have a pivotal role in both conditions. However further studies on a larger population are needed to support this hypothesis.

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LETTER TO THE EDITOR

Electromyographic evaluation during orthodontic therapy: comparison of two elastodontic devices

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To the Editor,

Neuromuscular orthodontics is assuming increasingly significant clinical and scientific roles in dentistry. Numerous studies have shown that the use of surface electromyography (sEMG) and kinesiography can be of fundamental help in all orthodontic cases (1). Initially employed for the treatment of temporomandibular disorders (TMDs), these modalities have proven to aid the planning, control of the therapy, and remote control of orthodontic treatments (2-5). Correct occlusion, as well as being aesthetically appreciable, must be able to interact perfectly with the neuromuscular balance of the stomatognathic system. The evaluation of muscle activity can support the determination of the mandibular and cranial growth vectors in the treatment of TMDs and in follow-up management to reduce the risk of recurrence after orthodontic therapy. Stomatognathic and extra-stomatognathic tension at the level of the trigeminal system should be eliminated before the start of treatment, to enable the procedure to be performed correctly also from a medicolegal point of view. Therefore, electromyographic (EMG) examination is used in standard practice to verify the resolution of neuromuscular tension (with physiotherapeutic support in some cases) before the initiation of orthodontic treatment (6). Nowadays, orthodontic therapies with elastodontic appliances are spreading a lot. There is a complete array of activators

for every type of mouth, according to the skull conformation, body features, and dental arch shape. Proper employment of this activator in association with physical exercises will allow patients to obtain benefits in the entire neuromyofascial system. This device improves the chewing function, aligns the teeth, re-educates the tongue due to stimulation toward the retroincisal papilla spot, and modulates the muscular tone in occlusal-postural syndrome; moreover, it is ideal for treating obstructive sleep apnea syndrome. Activators balance the skeletal bases through two double matched planes, upper and lower, where teeth are positioned with effects of propulsion, retropulsion, and expansion. After employing the positioners, the orthodontist will require minor tooth movement after functional treatment because of the elastomeric material (7). The aim of this study was to verify the clinical validity of assessments performed with two of these devices (EQ OSA; Eptamed and Occlus-o-Guide; Sweden & Martina) by comparing EMG activity before treatment and 6 months after treatment. The hypothesis was that soft appliances should be more comfortable for the patient.

MATERIALS AND METHODS

Study sample

This study was carried out in accordance with the fundamental principles of the Declaration of Helsinki,

Key words: EMG (electromyography); EQ (equilibrator) OSA device; Occlus-o-Guide; orthodontics

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and was approved prior to commencement by the Ethics Committee of the University of L'Aquila (no. 16137/2016). Eighty-two patients aged 7–11 years were clinically examined at the Dental Clinic of the University of L'Aquila, Italy. The same clinician performed all examinations, which included the acquisition of dental panoramic radiographs, extraoral and intraoral photographs, and alginate impressions of both dental arches. Based on these data, the orthodontist created a treatment plan specific to each patient according to the index of orthodontic treatment needs described by Brook and Shaw (8). The following exclusion criteria were applied: presence of epilepsy, systemic disease, TMD, or periodontal disease; and lack of written informed consent from a parent or legal guardian. The inclusion criteria were skeletal and dental class II malocclusion (divisions 1 and 2) and deep bite. Ultimately, 66 patients aged 7–11 years were enrolled in the study and divided into the test and control groups. The test group comprised 36 patients (18 male, 18 female; mean age, 9.19 ± 1.43 years) treated with the EQ OSA device. The control group comprised 30 patients (15 male, 15 female; mean age, 9.16 ± 1.41 years) treated with the Occlus-o-Guide. The two groups exhibited the same orthodontic features. All patients underwent sEMG analysis, with muscle tone evaluated with the eyes closed, at baseline (T0; prior to the initiation of orthodontic therapy) and 6 months after treatment initiation (T1). Registration of Scan 9, which shows the activity of the muscles (masseters, anterior digastrics, sternocleidomastoid, and anterior temporalis) in the resting position, was performed at both timepoints (1).

Protocol

Each patient in the test group received a soft, white, natural rubber EQ OSA device suitable for his or her dentition phase (7). This device, which has a shape similar to a mouthguard, embraces both dental arches, reaching distally to cover the last molars present in the arch. There are several measures, based on the distance between the palatal cusps of the first premolars. The patient fits the upper and lower splints over his/her teeth. The device is activated by biting, through soft elastic forces led by muscle energy. The activator is worn overnight. The EQ O.S.A. is a type of orthodontic appliance that stimulates growth and, through the input of muscle movements, elicits tissue development toward a suitable chewing function. Biting this elastomeric device balances tension up to the

sphenobasilar synchondrosis, according to osteopathic medicine and philosophy (7).

Each patient in the control group received a G-type (for mixed dentition) Occlus-o-Guide device, according to his or her dentition phase. Constructed from an elastomeric material, these preformed activators are ideal for use in the early through late mixed dentition. There are also different sizes of this appliance, the most appropriate is chosen by measuring the distance between the distal wall of the upper left lateral incisor and the distal wall of the upper right lateral incisor. It is also possible to measure through the lower lateral incisors. Everything is measured through a special ruler provided by the manufacturer. Along with their activator properties, ideal for correction of class II malocclusion, being based on tooth size, these appliances [aptly coined EGAs (Eruptive Guidance Appliances)] also function as a positioner along with correcting overbite and mild-to-moderate crowding. The appliance provides depressive forces to the front teeth and simultaneously encourages the posterior elements to erupt in their optimal vertical position. It is necessary, however, to intervene when they are erupting, before the periodontal fibers stabilize the definitive vertical level of the posterior elements. It is also a myofunctional regulator that tends to properly rebalance muscle forces, rehabilitates the posture of the tongue, re-educates atypical swallowing and stimulates correct breathing. The patients were instructed to use the device overnight (2, 9). The orthodontist checked patients every 30 days to evaluate eventual modifications to carry out on the device. The EQ OSA device and the Occlus-o-Guide device are shown in Fig. 1.

EMG assessment

EMG activity was recorded using an eight-channel Myotronics K7 Evaluation System (Myotronics Inc., Seattle, WA, USA) equipped with bipolar electrodes, with an inter-electrode distance of 20 mm. The patient's skin was cleaned thoroughly with alcohol, and electrodes were positioned on the left and right masseter and anterior temporal muscles, as described by Castroflorio et al. (10, 11). Electrodes were also placed on the left and right anterior digastric muscles (12) and on the left and right sternocleidomastoid muscles, parallel to the muscle fibers and over the lower portion of the muscle to avoid the innervation point, as described by Falla et al. (13). Electrical

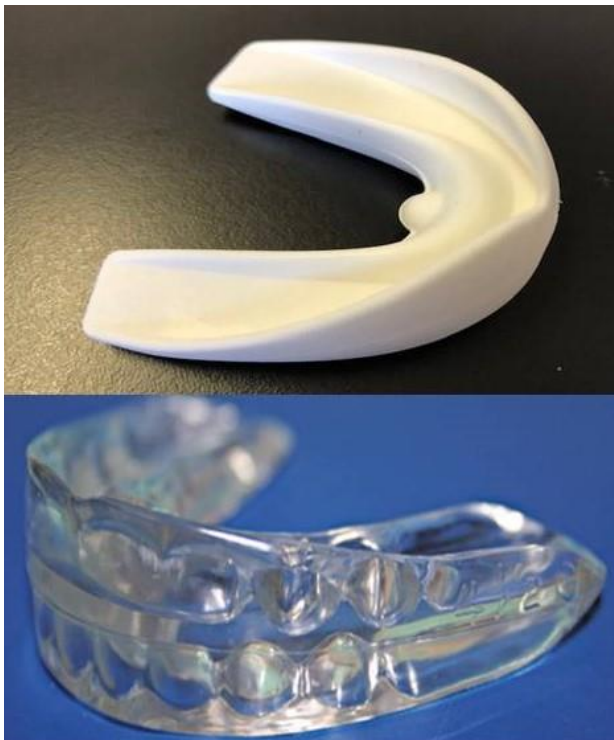


Fig. 1. EQ OSA device and Occlus-o-Guide device.

signals were amplified, recorded, and digitized with the K7 clinical software package (Myotronics Inc.). Root mean square values (in microvolts) were used as indices of signal amplitudes (1). This study involved the evaluation of scan 9, performed with the eyes closed and open, to assess muscle tone in the resting position. Scan 9 shows sEMG muscular activity at basal condition in resting position. The resting position is the correct relationship between the jaw and the upper dental arch in resting condition, when the teeth are not in contact.

RESULTS

The unpaired *t*-test was used for within-group comparisons. Statistical significance was set at $p < 0.05$. The results are shown in Table I. Data are presented as mean (standard deviation). Eptamed vs Occlus-o-Guide, unpaired *t*-test. Although values for the right and left sides showed improvement in patients treated with the EQ OSA relative to those treated with the Occlus-o-Guide, no difference was significant at T0 or T1. From T0 to T1, the sum of sEMG values changed from 15.1 to 14.9 μV in the

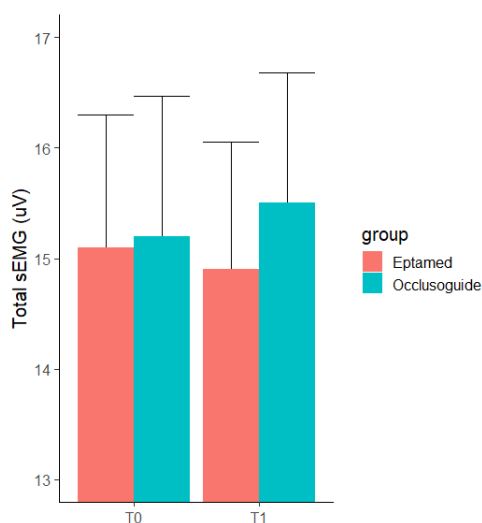
test group and from 15.2 to 15.5 in the control group; these differences were not significant. The mean sEMG value improved in the test group (changing from 1.9 at T0 to 1.7 at T1) relative to the control group (changing from 1.8 to 2.1), but this difference was not significant, as shown in Table I and in Fig. 2.

DISCUSSION

Many studies have examined muscle changes during orthodontic therapy. The achievement of lower levels of resting activity for the mandibular and postural muscles is a desirable outcome of orthodontic treatment. The sum of resting electrical activities of the eight mandibular and postural muscles serves as a primary marker of orthodontic treatment efficacy. Conventional orthodontic treatment aims to achieve morphological and aesthetic norms based on static assessments, however, the functioning of the cranial-mandibular muscles and joints involved in occlusion occurs through interactions with the nervous system. Farronato et al. (2) reported that EMG values obtained with two devices for the advancement of the jaw (the Occlus-o-Guide and Andresen devices) *in situ* were comparable. Another recent study demonstrated that neuromuscular orthodontic treatment resulted in greater relaxation of the masticatory muscles and better function than with traditional orthodontic treatment. In the present study, EMG values of patients treated with EQ OSA and Occlus-o-Guide devices did not differ significantly, but patients who wore the EQ OSA device showed greater muscle relaxation. In fact, with the devices *in situ*, electromyographic values were similar in the two groups (no significative differences), but in the test group the muscles were more relaxed. The use of the activator, in association with exercises and techniques for muscular balance, allows the patient to gain benefits throughout the neuromyofascial system – with fewer concerns for the orthodontist. In fact, this device can help the patient to educate the tongue posture, educate the nasal breathing, align the teeth and to advance the jaw. Biting these elastomeric devices balances tension up to the sphenobasilar synchondrosis, according to osteopathic medicine and philosophy. The teeth's positions are determined by this new skull harmony

Table I. Electromyographic values (microvolts) obtained before treatment initiation (T0) and 6 months thereafter (T1).

	T0			T1		
	Eptamed	Occlusoguide	p-	Eptamed	Occlusoguide	p-
	n=36	n=30	value	n=36	n=30	value
RTA	2.2 (1.2)	2 (1.0)	0.4703	2.3 (1.2)	2.2 (1.3)	0.7466
LTA	2.2 (1.1)	2.3 (1.2)	0.7254	2.0 (1.0)	2.5 (1.2)	0.0694
RMM	1.9 (0.9)	1.7 (0.8)	0.3482	1.7 (0.8)	1.9 (0.9)	0.3430
LMM	1.8 (1.0)	2 (1.1)	0.4423	1.8 (0.9)	2.0 (0.8)	0.3482
RDA	1.4 (0.9)	1.5 (0.6)	0.6051	1.5 (1.1)	1.9 (0.9)	0.1156
LDA	1.3 (0.8)	1.6 (1.1)	0.2050	1.6 (1.2)	2.0 (1.2)	0.1823
RTP	1.9 (1.1)	2.0 (1.5)	0.7561	1.8 (1.4)	2.3 (1.4)	0.1534
LTP	1.9 (1.2)	1.8 (0.9)	0.7078	2 (1.4)	2.1 (1.1)	0.7517
Sum	15.1					
sEMG	(7.2)	15.2(7.6)	0.9565	14.9(6.9)	15.5(7.1)	0.7296
Mean						
sEMG	1.9 (0.8)	1.8(1.0)	0.6532	1.7(0.5)	2.1 (1.3)	0.0934

**Fig. 2.** Bar graph of total sEMG values stratified by timing according to group “Eptamed” and “Occlusoguide”. No statistical differences between groups were shown.

through an osteopathic effect; benefits are also in the cervical posture (6).

The limitations of this study include the small sample and relatively short (6-month) orthodontic evaluation period. In the future, it would be highly desirable to repeat this study with a larger, and possibly more homogeneous, cohort. The results of this study indicate that the use of these types of elastodontic devices, that must be worn only during the night, is effective in aligning the teeth, advancing the jaw, nasal re-education and tongue posture, as well as having cervical benefits. Therefore, wearing a device for less hours a day than traditional ones, having a more relaxed muscle activity detected by EMG, and having greater functional and neuromuscular results and in less time can be the future of interceptive orthodontics.

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LETTER TO THE EDITOR

Is the success of treatment of obstructive sleep apnea related to operator experience?

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To the Editor,

The use of Mandibular Advancement Device (MAD) in the treatment of Obstructive sleep apnea syndrome (OSAS) and snoring has been known for many years, and many authors have discussed its limitations and indications in the treatment of sleep respiratory disorders. At present, the treatment of OSAS with an MAD is indicated only for snoring and mild-to-moderate OSAS. The MAD is used as support in severe OSAS in association with continuous positive air pressure (C-PAP) to reduce the airflow pressure needed (1-2).

The limitations are often advocated to the experience of the operator, patient compliance, variability in the setup of the device, presence of severe TMJ disorders, and periodontal disease (3-4). For this reason the authors decided to check the effectiveness of the therapy with MAD in patients with different OSAS problems who were treated, under supervision, by operators who had no experience with MAD treatment who were instructed to follow a step-by-step setting protocol. The aim of the study was to evaluate whether the therapeutic

success rate is related to operator experience or is intrinsic in the proposed therapeutic method.

MATERIALS AND METHODS

This is a retrospective study derived from the analysis of the results obtained during a master hands-on course entitled "Treatment of snoring and OSAS with an oral device: from theory to practice" which was held at the Department of Dentistry and Maxillofacial Surgery of the "Policlinico A. Gemelli" Foundation between April and July 2017. For the hands-on part of the course, 21 OSAS patients were enrolled and treated by 21 dentists taking part in the course (ratio patients/dentists was 1:1). All the patients were treated with a "dorsal" MAD manufactured by OrthoApnea. All patients were informed regarding the therapy protocol and gave their signed approval for study participation.

Patient selection criteria

All patients were enrolled with spontaneous candidature through an announcement published on social media (Facebook), which reported the course specifications. Patients referred by specialists who already

Key words: OSAS; obstructive sleep apnea; Mandibular Advancement Device; AHI; oral device; snoring; respiratory disorders; snore

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gave therapeutic indications for treatment with an oral device were excluded. This was decided in order to have a sample as heterogeneous as possible and to evaluate more accurately the therapeutic capacity of an MAD.

Inclusion criteria were: age between 18 and 80 years, good periodontal health (bleeding index < 10%, general probing pocket depth < 3 mm and grade 1 of tooth mobility in at least 60% of each arch (5), absence of TMJ disorder, and with a cardio-pulmonary disease blank history. All patients underwent functional endoscopy and those without positive response of the soft tissues of the upper airways to the spontaneous mandibular advancement were excluded. The severity of OSAS was determined according to the manual scoring method proposed by the American Academy of Sleep Medicine (AASM) (6).

All patients were required to undergo a polysomnographic examination for OSAS severity evaluation, orthopantomography to evaluate the occlusal conditions, and a tele-radiography to evaluate cephalometric parameters.

Parameters considered for the evaluation of the effectiveness of this study:

To evaluate the rate of success of the MAD therapy, 3 polysomnography parameters with or without MAD were considered:

- the snore index or the snoring time in total hours of sleep,
- the Apnea Hypopnea Index (AHI)
- the nadir of the O₂ levels.

Patient study protocol and oral device implementation protocol

During the first visit, two self-evaluation tests were administered to the patients: the Obstructive Airway Adult Test (OAAT) and Epworth Sleepiness Scale (7). Information about sleep quality was collected and the presence of concomitant diseases, including diabetes, cardiovascular or pulmonary diseases, arterial hypertension, thyroid diseases and gastrointestinal disorders were recorded.

The patient was examined, and the condition of the dental elements was evaluated through mobility assessment of each dental elements and orthopantomography X-ray evaluation. The clinical conditions regarding symptomatic or functional problems of the TMJ were also investigated.

The data considered for the evaluation of temporomandibular dysfunctions (TMD) were: presence of joint pain, presence of joint noises, and joint mobility. The data were stratified on a scale from 1 to 5. For pain, patients were asked to report “1” as no pain and “5” as the maximum pain; for articular noise, we assigned “1” to no noise, “2” to early articular click, “3” to intermediate joint click, “4” to late click, and “5” to closed lock; for the articular motility parameter, we considered a value of “1” for opening of over 35 mm, “2” for opening between 30 and 35 mm, “3” for opening between 25 and 29 mm, “4” for opening between 24 and 15 mm and “5” for opening less than 15 mm.

The data for the occlusal evaluation were found through evaluation of the orthopantomography and the clinical inspection. Inclusion parameters were stable dental elements, presence of dentition of 60% in each arch, good periodontal condition.

For the evaluation of polysomnography, the following were considered:

- the snore index, as the percentage of snoring time compared to the sleep time,
- the AHI,
- the minimum level of saturation reached during sleep.

All patients underwent functional endoscopy of the upper airway. Through this examination, the obstruction was assessed in the sites described by the nose oropharynx hypopharynx and larynx (NOHL) classification. The easiness of passage of the endoscopic instrument at the nose level was also evaluated (8).

At the palatal level, the level of obstruction was assessed at basal conditions and after Muller’s maneuver (9) and the movements of the soft tissues following the maximal protrusion of the mandible were evaluated (10). The posterior lingual space was also evaluated at basal conditions and after mandibular protrusion.

Cephalometric analysis was performed on the lateral cephalometric radiograph. Four values were considered: the SNA (Sella – Nasion – A), the SNB (Sella – Nasion – B), the PAS (Posterior Airway Space) and the MP-H (Mandibular plane – Hyoid bone) (11).

To take precision dental casts, we adopted the double impression technique similar to that used to make a fixed prosthesis.

For the evaluation of mandibular advancement, a George Gauge Kit was used and was stabilized at 50% of the possible excursion (12).

Table I. Means for One-way ANOVA for decrease in Snore Index, AHI, and nadir by severity

SNORE INDEX						
Level	Number	Mean	Std Error	Lower 95%	Upper 95%	Prob > F
mild	5	27.1600	8.0207	10.309	44.011	0.4979
moderate	5	38.7400	8.0207	21.889	55.591	
severe	11	27.9455	5.4076	16.585	39.306	
AHI						
Level	Number	Mean	Std Error	Lower 95%	Upper 95%	Prob > F
mild	5	6.3200	5.5563	-5.35	17.993	0.0015*
moderate	5	11.7600	5.5563	0.0866	23.433	
severe	11	32.4636	3.7461	24.59	40.334	
NADIR						
Level	Number	Mean	Std Error	Lower 95%	Upper 95%	Prob > F
mild	5	4.0000	3.6901	-3.753	11.753	0.1187
moderate	5	8.2000	3.6901	0.447	15.953	
severe	11	13.4545	2.4879	8.228	18.681	

Table II. Values of correlation matrices between the pre-therapy and post-therapy variables

PRE-THERAPY			
	Snore-pre	AHI-pre	Nadir-pre
Snore-pre	1.0000	0.4926	-0.7227
AHI-pre	0.4926	1.0000	-0.6520
Nadir-pre	-0.7227	-0.6520	1.0000
POST-THERAPY			
	Snore-post	AHI-post	Nadir-post
Snore-post	1.0000	0.7320	-0.6246
AHI-post	0.7320	1.0000	-0.5457
Nadir-post	-0.6246	-0.5457	1.0000

Realization of the oral device

To keep the result as homogeneous as possible, only one type of oral device was used for this study: a “dorsal” MAD with an anterior respiratory canal (OrthoApnea).

First assessment. After the patients wore the MADs for the first time, the eventual discomfort on the dental elements was immediately addressed along with possible interferences or difficulties in insertion, the interface between the slipping planes of the upper and lower bites and articular discomfort under conditions of basal advancement.

Serial clinical controls. Patients were followed-up after 15 days, 1 month, 3 months, 6 months and 12 months after delivering the MADs. During the visit, the comfort, the state of the OSAS using anamnestic criteria and the periodontal and TMJ situations were evaluated.

Controls with polysomnography. The examination of the quality of sleep and OSAS were assessed in the following steps:

- first check with polysomnography 1 month after implementation of the MAD. If the response to

mandibular advancement therapy was positive:

- Follow-up after 6 months
- Follow-up after 12 months

In cases where the response was insufficient, the MAD protrusion was increased by 1 mm, and the follow-up flow-chart was restarted as previously described until optimal results were obtained.

RESULTS

The sample consisted of 21 patients, 16 males and 5 females; the patient ages ranged between 38 and 67 years, with an average of 51 years. In total, 1 patient was affected by simple snore; 4 patients, by mild OSAS; 5 patients, by moderate OSAS; and 11 patients, by severe OSAS.

Statistical analysis

Data considered in the statistical analysis are those related to the polysomnography exam obtained in pre-treatment and those related to the 12-month follow-up.

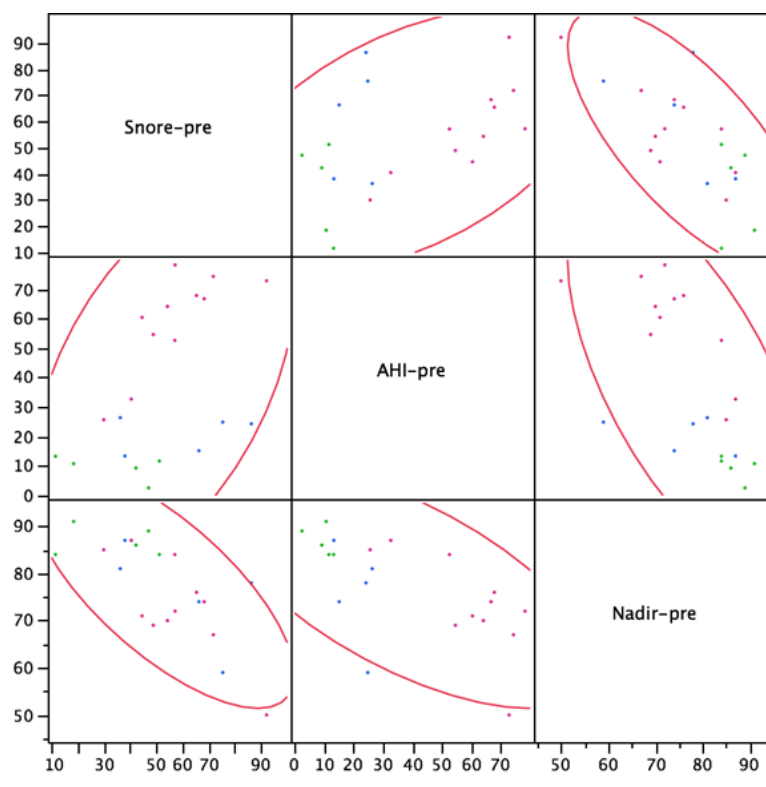


Fig. 1. Correlation matrices between the pre-therapy variables

Through analysis of variance and Student's *t*-test, with a level of significance equal to 5% applied to the three single variables, it was concluded that oral device therapy had significantly positive effects on each patient. Using the method of one-way ANOVA (Analysis of Variance) applied to the differences in the measured values before and after therapy, the effects of therapy on mild, moderate and severe OSAS patients were investigated.

Snore Index

The snore index results showed a decrease of approximately 30% regardless of the severity of the disease (as seen in Table I, with *p*-value = 0.4979, it can be deduced that the averages of the three groups are not significantly different).

AHI

The effects of the therapy varied depending on the severity of the disease; in fact, the test was significant, with the *p*-value = 0.0015, and was more

effective for serious OSAS patients than for other patients. These patients showed a reduction in the index of 32.4%; for moderate OSAS patients, the decrease was of 11.7% and for mild OSAS patients, the decrease was of 6.3% (Table I). However, even though the other percentages were lower than those for severe OSAS patients, an improvement was recorded.

Nadir

The improvement in the nadir index seemed to be related to the severity of the disease, as shown in Table I: 13.4% for severe OSAS, 8.2% for moderate OSAS and 4% for mild OSAS. However, since the *p*-value was not lower than 0.05, the level of significance of the test is not very reliable.

Correlation matrices have been designed in order to better evaluate eventual relationships between the pre-therapy and the post-therapy variables and the treatment effects (Table II, Figs. 1 and 2). The effects of the therapy have been also correlated with

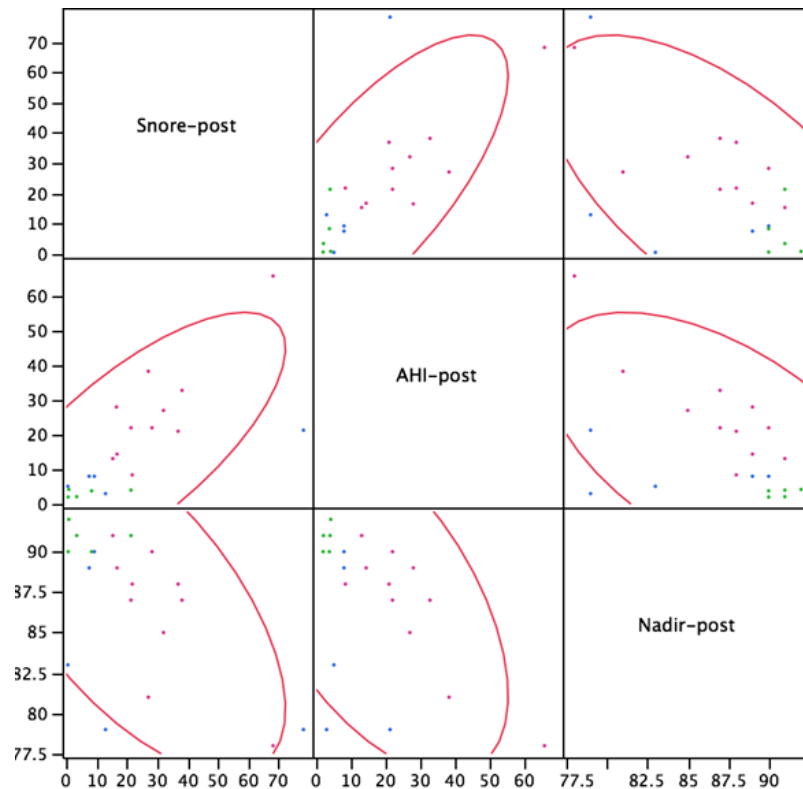


Fig. 2. Correlation matrices between the post-therapy variables

the three indexes; among all indexes the strongest association was found between the effects on the AHI and the nadir indices (Table III, Fig. 3).

DISCUSSION

From the results of this study, the efficacy of MAD therapy in treating snoring and OSAS is clear. Through the correlation matrices it is obvious that

the severity of the three symptoms is positively correlated: if the patient improves with regard to an index, he/she will most likely also see improvement in the other indexes.

It has also been shown that the link between the effects on the AHI and nadir indices is much stronger than the one between the snore index and the other two values. Therefore, if the patient shows improvement in the AHI, it can be expected that the effects of the

Table III. *Correlation matrices between the effects of the therapy on the three indexes*

	Decrease snore	Decrease AHI	Increase Nadir
Decrease snore	1.0000	0.2008	0.3068
Decrease AHI	0.2008	1.0000	0.6452
Increase Nadir	0.3068	0.6452	1.0000

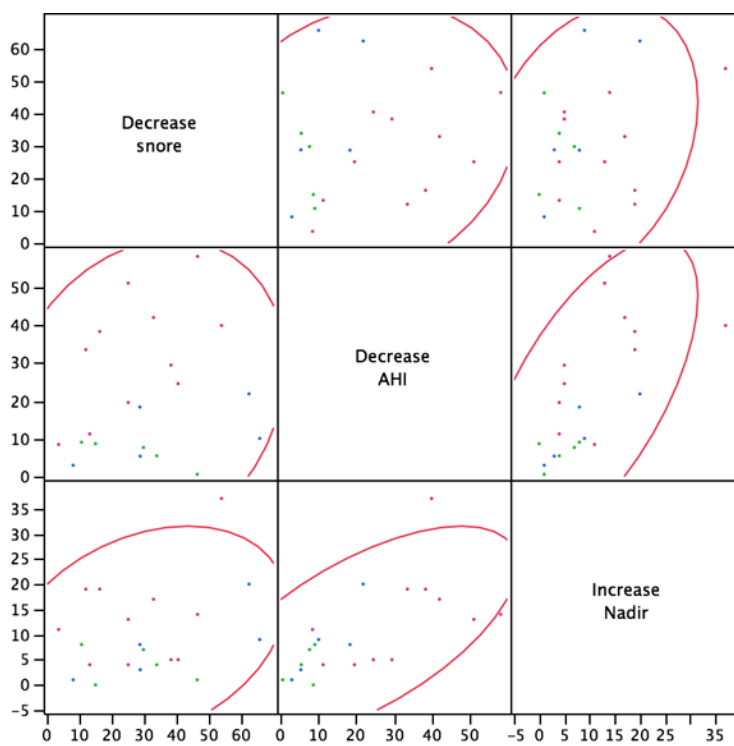


Fig. 3. *Correlation matrices between the effects of the therapy on the three indexes*

therapy on the nadir index will be more evident than those on the snore index, which, although improving in all patients, does not always normalize.

It is interesting to note that there is better improvement in severe OSAS compared to medium or mild OSAS for all parameters. The average improvement of 32.4% of the AHI in severe OSAS is a very satisfactory result; even if it does not overcome the need to use C-PAP or surgical interventions in many cases, it can significantly lower the risk of serious events for the patient. To further support this hypothesis, an increase in the O_2 nadir, which was 13.4% in these patients, was noticed and such increase brought the values of O_2 in blood to less dangerous levels for serious cerebrovascular accidents.

In patients with mild OSAS, the reduction in AHI was 6.3%, while the increase in nadir was 4%. These results were very good, and for all patients, the values returned to normal.

The results of this study confirm that in severe OSAS, MAD cannot be considered a primary therapeutic choice but it must absolutely be accounted in patients who refuse other therapies. This approach ensures substantial improvement in the apnea pathology, allowing a drastic reduction in the risk of serious diseases. Even in patients with mild and moderate OSA, the results were very good, and in this case, the use of an MAD can replace C-PAP therapy.

Even if not statistically supported because of the small number of patients enrolled in this study, an important issue to be highlighted is the “no response to therapy”: in the reported sample there was no evidence of this situation, but further studies should be carried out to assess whether this is an actual possibility and its eventual incidence.

From the experience gained with this study, it can be affirmed that the use of a MAD in the treatment of OSAS is a valid and reliable method not subordinate to the experience of the operator, as long as the standard rules for the realization and management of the device are observed.

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LETTER TO THE EDITOR

Intra-articular treatment of knee and ankle osteoarthritis with polynucleotides: prospective case record cohort vs historical controlsM. Guelfi¹, R. Fabbrini² and MG. Guelfi¹¹*Casa di Cura “Villa Montallegro”, Genoa, Italy;* ²*Istituto Clinico Ortopedico Traumatologico, Latina, Italy**Received April 23, 2020 – Accepted September 1, 2020*

To the Editor,

The most recent guidelines and evidence-based consensus reports still cast doubts about the real value of hyaluronic acid (HA) infiltrative management of knee osteoarthritis (OA) in addition to accepted core OA treatments — arthritis education, structured land-based exercise programs, dietary weight management, topical non-steroidal anti-inflammatory drugs (NSAID) (1). The situation is quite similar in ankle osteoarthritis: there is evidence from the few published studies that viscosupplementation with HA might be effective in the short term, but benefits are less clear even after six months due to the lack of well-designed dosing studies and the overall low quality of clinical investigations (1-4).

An alternative, or at least a complementary option, to intra-articular HA should aim to relieve pain and inflammation and slow joint damage (2, 3). The strong viscosupplementation efficacy, similar to high-molecular weight HA, and the trophic properties on mesenchymal cells, including chondrocytes, of intra-articular, highly purified, natural-origin polynucleotides from trout gonads candidate them for this role (4, 5). Reduction of pain, as shown in several studies with a proprietary intra-articular polynucleotide gel formulation (Condrotide[®], Mastelli Srl, Sanremo, Italy), might be another plus (5-7).

Over several years, the authors built two large case

record databases of patients with knee and ankle OA treated with polynucleotides. Their long experience in treating knee and ankle OA with a short three-week protocol of intra-articular polynucleotides may help to define their benefits in everyday knee and ankle OA management. The authors herein illustrate their outcomes. A small group of patients of the two knee and ankle OA cohorts, previously treated with HA, was selected to act as historical controls for an exploratory, short-term, intra-patient comparison of short-term outcomes between intra-articular polynucleotides and HA. Such comparison, however preliminary and waiting for well-designed randomized clinical trials to confirm all results, might be useful to better define the benefits of polynucleotides as an alternative or a complement to HA in the management of knee and ankle OA.

MATERIALS AND METHODS

Patients

The medical records of 146 prospective ambulatory patients with knee OA (85 women, 61 men; mean age, 73 years, range, 47-82) and 56 ankle OA patients (24 women, 32 men; mean age, 62 years, range 40-70) were collected over, respectively, nine and eight years (2010-2018 and 2011-2018). The overall knee OA cohort was divided into two sub-cohorts according to the 2016 Kellgren-Lawrence

Key words: ankle osteoarthritis; knee osteoarthritis; intra-articular polynucleotides; PN-HPT[™]; FAOS; WOMAC

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Classification of Osteoarthritis (8) — “Knee OA grade 1-2” (121 patients, 74 women, 47 men; mean age, 66 years) and “Knee OA grade 3-4” (25 patients, 16 women, 9 men; mean age, 77 years).

Eleven patients of the “Knee OA grade 1-2” sub-cohort (6 women and 5 men; mean age, 65 years) had in the past received several intra-articular infiltration cycles with a gel of medium molecular-weight HA; they underwent their first polynucleotide gel infiltration one year, on average, after the last HA injection. These 11 patients acted as historical controls for a short-term, within-patient, exploratory comparison between the two infiltrative strategies one month after the last intra-articular injection of HA and, after one year, the polynucleotide gel.

The overall ankle OA cohort was also divided into two sub-cohorts: “Ankle OA grade 1-2” (50 patients, 29 women, 21 men; mean age 58 years, range 40-68) and “Ankle OA grade 3-4” (6 patients, 4 women, 2 men; mean age 71 years, range 58-70). Nine patients (5 women and 4 men; mean age, 56 years) of the “Ankle OA grade 1-2” sub-cohort had already received several intra-articular injections of a medium molecular-weight HA gel the previous year and were selected as historical controls for a within-patient, short-term comparison between the two infiltrative strategies one month after the first month of the respective follow-up.

All patients of the two case series, when treated, had provided consent for their data to be possibly used for research in the future.

Intra-articular injections of polynucleotides and evaluation of efficacy

All knee and ankle OA patients received 3 weekly infiltrations of highly purified polynucleotides derived from trout gonads (acronym henceforth, PNs-HPT™); in ankle OA patients, injections were performed into the tibiotarsal joint space by the commonly used anteromedial portal (Fig. 1). The Condrotide® gel formulation injected in the patients of the two case series is a transparent, colorless, viscoelastic solution of polynucleotides (concentration: 40 mg in 2 mL) in single-use, apyrogenic, 2-mL glass vial-syringes. According to instructions, all the content of one vial-syringe is usually injected. The indication for prosthesis implantation could not be fulfilled in the patients of the “Knee OA grade 3-4” and “Ankle OA grade 3-4” sub-cohorts, and PNs-HPT™ were a fallback therapy for those patients.

The final assessment was performed after, on average, a

further month of follow-up in all knee and ankle OA patients. Due to the exploratory nature of the study with a focus on rapid control of OA symptoms, a short-term, 1-month follow-up period was deemed adequate. Medium-term outcomes (3 and 6 months) were also available for PNs-HPT™ for the two subgroups of 11 and 9 knee and ankle OA patients who had been previously also treated with HA and who acted as historical controls.

In the two knee OA sub-cohorts and the two ankle OA cohorts, assessments and qualitative comparison of baseline vs final mean scores after treatment with PNs-HPT™ were based on the validated Italian versions of, respectively, the 24-item Western Ontario and McMaster Universities (WOMAC) questionnaire (total score ranging between 0 and 96) (9) and the 42-item Foot and Ankle Outcome Score (FAOS) questionnaire (total score ranging between 0 and 100) (10). Short-term PNs-HPT™ vs HA comparisons of, respectively, WOMAC and FAOS mean scores (baseline mean scores vs mean scores one month after the last infiltration of either PNs-HPT™ or HA) were also performed on the patients of the “Knee OA grade 1-2” and the “Ankle OA Grade 1-2” sub-cohorts treated in the past with HA and acting as historical controls.

Statistical analysis

Due to the exploratory and descriptive nature of the study, performed on two case series of unselected prospective patients treated with PNs-HPT™, no formal inferential statistics were planned for the two case series. Only descriptive tables of disease severity (WOAMC and FAOS scores) at baseline and at the end of the 1-month follow-up period were planned to summarize the outcomes of intra-articular PNs-HPT™ treatment in knee and ankle OA.

Conversely, based on the hypothesis-generating nature of the comparisons with historic controls, Fisher’s exact tests were planned for the knee and ankle historical controls treated, one year apart, with both HA and PNs-HPT™, with the aim of tentatively looking for differences between HA and PNs-HPT™ intra-articular injections after 1, 3 and 6 months.

RESULTS

Knee OA patients

PNs-HPT™ intra-articular injections were

well tolerated, with 4 patients developing knee tenderness and intra-articular effusions. Symptoms resolved uneventfully in 4 days with rest and ice packs. The WOMAC score decreased from a mean baseline value of 49 to a mean of 12 at the end of the follow-up period (−75.5%) in the patients with mild to moderate disease (“Knee OA Grade 1-2” sub-cohort), and from 61 to a mean of 38 (−37.7%) in the patients with more severe or disabling disease (“Knee OA Grade 3-4” sub-cohort) with no gender differences. Independently of baseline severity, all patients subjectively referred less pain together with an objective reduction of knee friction noises and an improvement of Range Of Motion (ROM).

Table I summarizes descriptively the short-term within-patient explorative comparison of

efficacy between PNs-HPT™ and HA in the 11 patients previously infiltrated with HA and acting as historical controls. WOMAC scores fell by 75.5% with HA and by 76.6% with PNs-HPT™ between baseline and the first month of follow-up (no statistical difference); Table I also illustrates the outcomes of PNs-HPT™ infiltrations in these 11 patients after 3 and 6 months.

Ankle OA patients

Three patients developed tenderness and intra-articular effusions after the procedure; symptoms resolved in 5 days with rest and ice packs. FAOS scores increased from a mean baseline value of 35 to a mean of 68 (+94.3%) at the end of the 1-month follow-up period in the patients with mild to

Table I. Within-patient explorative PNs-HPT™ gel vs HA comparison of WOMAC scores in the subgroup of patients of the “Knee OA Grade 1-2” sub-cohort acting as historical controls (last HA infiltration at least one year before the first PNs-HPT™ intra-articular infiltration); score assessment one month after the last HA or PNs-HPT™ intra-articular injection. For these patients, longer-term PNs-HPT™ efficacy outcomes (after 3 and 6 months) are also illustrated (shaded area).

	FAOS scores			
	Baseline	After 1 month	After 3 months	After 6 months
HA	49	12	//	//
PNs	47	11	15	15

Table II. Within-patient explorative comparison of FAOS scores (PNs-HPT™ gel vs HA) in the subgroup of patients of the “Ankle OA Grade 1-2” sub-cohort acting as historical controls (last HA infiltrations at least one year before the first PNs-HPT™ intra-articular infiltration); score assessment one month after the last HA or PNs-HPT™ intra-articular injection. For these patients, longer-term PNs-HPT™ efficacy outcomes (after 3 and 6 months) are also shown (shaded area); * $p < 0.05$ vs HA.

	FAOS scores			
	Baseline	After 1 month	After 3 months	After 6 months
HA	37	61	//	//
PNs	35	66*	68	64

moderate disease ("Ankle OA degree 1-2") and from a mean value of 13 to 55 (+323.1%) in the patients with more severe disease ("Ankle OA Grade 3-4" sub-cohort). Subjectively and regardless of baseline



Fig. 1. Anteromedial portal to the ankle joint space: the tibial (medial) malleolus, the tendon of the tibialis anterior muscle, and the greater saphenous vein are used as anatomical landmarks, with the portal just medial to the tibialis anterior tendon and about 5 mm proximal to the tibial malleolus. Great care to avoid vulnerable anatomical structures is paramount, with special reference to the greater saphenous vein and nerve, both lying 7-9 mm medial to the portal and to the tibialis anterior nerve. With the patient in supine position, the injection is performed at the junction between the lower tibial margin and the external margin of the tibialis anterior muscle tendon after identification of the tibio-astragalic joint space and the medial margin of the tibialis anterior muscle. The needle is inserted deeply, with an anteroposterior oblique direction and parallel to the tibial joint surface.

OA severity, many patients referred to experience less pain and friction noises, as well as a Range Of Motion (ROM) improvement.

Table II summarizes the within-patient comparison between PNs-HPT™ and HA in the 9 patients acting as historical controls. FAOS scores fell by 67.8% with HA and by 80.7% with PN ($p < 0.05$ vs HA, Fisher's exact test) between baseline and the first month of follow-up; Table II also shows the longer-term outcomes of PN infiltrations in those 9 patients after 3 and 6 months of follow-up.

DISCUSSION

A guiding principle led to the development of the PNs-HPT™ formulation used in the study: maximizing the trophic activity on knee and ankle cartilages without renouncing the viscoelastic properties of HA. As polar molecules, highly hydrophilic PNs-HPT™ have long-term moisturizing and viscoelastic efficacy (7); by steadily supplying nucleotides and other precursors to chondrocytes, PNs-HPT™ also support their metabolic efficiency and the repair activities of cartilage (7).

PNs-HPT™ appear a reliable viscoelastic option to substitute or complement HA in knee and ankle OA. At the end of the 1-month follow-up period, the knee and ankle OA patients with mild to moderate severity showed strong decreases in WOMAC and FAOS scores (on average, -75.5% and -76.4%, respectively, vs baseline), with still substantial score decreases (-37.7% and -48.6%) in the grade 3-4 patients who would normally candidate for joint substitution. As regards the explorative comparisons in the small subsets of grade 1-2 patients who acted as historical controls, short-term efficacy seems fairly similar between PNs-HPT™ and medium-molecular-weight HA, with mean baseline WOMAC and FAOS scores of -76.6% and -75.5% vs baseline, respectively. Even with all due caution, the intra-patient design of the comparisons confers reliability to those suggestions. Mean WOMAC and FAOS scores increased only marginally over 6 months of follow-up in historical controls, supporting the hypothesis that PNs-HPT™ might behave like high-molecular-weight HA, with the benefit of less

injection difficulties.

Summarizing, with all the limitations of a retrospective non-controlled study, PNs-HPT™ confirmed to be an alternative, and possibly a complement, to HA for the relief of symptoms and improvement of quality of life in knee and ankle OA. Speculatively, PNs-HPT™ might leverage their demonstrated chondrocyte activation, which is stronger than HA (11, 12), to act as primers of cartilage reactivation, with their preliminary action then completed by HA.

CONFLICT OF INTEREST: Mastelli Srl, Sanremo, Italy, patent holder and producer of Condrotide®, the intra-articular polynucleotide formulation used by the authors, helped to support the publication of this retrospective analysis of their archived data.

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LETTER TO THE EDITOR

Thin metallic thread in the gastrointestinal tract: an uncommon complication after coil embolization of a visceral pseudoaneurysm

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To the Editor,

Endovascular coil embolization of visceral pseudoaneurysms and aneurysms is largely safe and effective (1). More common complications include arterial bleeding, pseudoaneurysm reperfusion, organ infarction and subsequent infection. However, gastroenteric coil migration is a rare but well-described complication that we should keep in mind before endovascular treatment of pseudoaneurysm with enteric fistula (2).

In this letter, the Authors describe the case of a 75-years-old man affected by melena and severe anemia occurring two weeks after cardiac surgery (coronary artery bypass surgery). Abdomen CT showed a large inferior pancreaticoduodenal artery (IPDA) pseudoaneurysm (9 x 4 mm) with active bleeding in the duodenal lumen. An angiography was performed, showing a large IPDA pseudoaneurysm with active contrast agent extravasation (Fig. 1). As a consequence, the interventional radiologist embolized the IPDA pseudoaneurysm with deployment of two coils (Concerto Ev3 4 mm x 6 cm and Packing coil soft 30 cm) distal and proximal to the pseudoaneurysm with the “sandwich technique”. During coil deployment, loops of the Packing coil herniated in the

pseudoaneurysm sac. Post-embolization angiogram confirmed complete IPDA exclusion and the patient was discharged five days later.

Eight months later, the patient was admitted to our medical Center for crampy abdominal pain. CT scan revealed the presence of a very thin and long metallic thread in the gastroenteric tract, extending from the duodenum to the ascending colon. It is important to underline that the metallic thread was a fragment of one of the coils previously positioned in the IPDA and was still connected with the coil pack in the pseudoaneurysm sac (Fig. 2).

After multidisciplinary evaluation, we decided to perform a treatment combining percutaneous and surgical strategies. At preliminary fluoroscopy, the coil pack in the pseudo-aneurysm sac was less represented and connected with the metallic thread in the intestinal lumen. First of all, in the angiographic suite the interventional radiologist completed pseudoaneurysm embolization deploying two more coils (two Penumbra Ruby coils 3 mm x 5 cm) in the afferent vessel in order to prevent new bleeding. Then, the surgeon removed the metallic thread from the small bowel through a mini-invasive approach with multiple enterotomies in the surgical theatre (Fig.

Key words: pseudoaneurysm; embolization; coil

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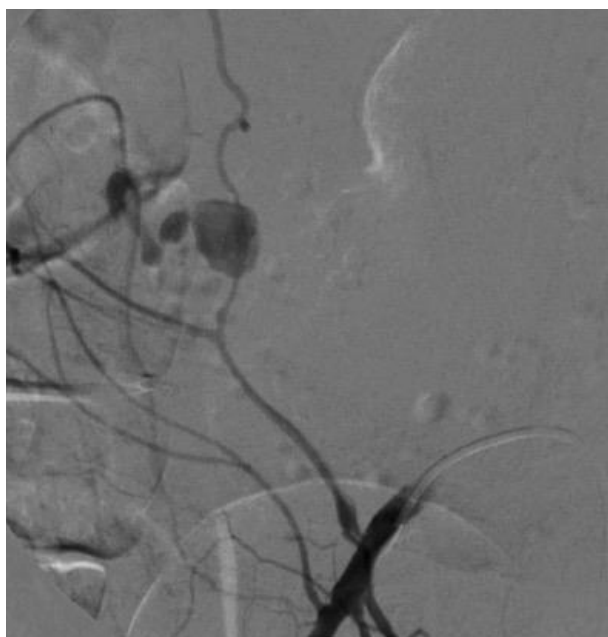


Fig. 1. Contrast injection fluoroscopy from a 5 F catheter in the superior mesenteric artery showing a large visceral pseudo-aneurysm (9 x 4 mm) in the inferior pancreaticoduodenal artery (IPDA) with irregular profile.



Fig. 2. Abdominal CT. Coronal plane MPR and MIP image showing the presence of a very long thread (yellow arrows) originating from coil (red arrow) positioned in the pseudoaneurysm sac.

3 A, B, C). The patient was discharged two weeks later with no pain and in good health conditions. Six months later, a follow up CT was performed and confirmed the absence of the metallic threads with a correct treatment of the visceral pseudo-aneurysm.

We would like to focus the attention of the interventional radiologists on a very rare complication that can occur after an endovascular coil embolization of visceral pseudoaneurysm. This is the first case reported in the literature where the interventional radiologist, together with the surgeon, faced the presence of a metallic thread (a portion of a coil previously positioned) in the enteric lumen, still connected with the coil pack in the pseudoaneurysm sac. Coil migration cases reported in literature have usually occurred after treatment of visceral pseudoaneurysms with a direct fistula to air-filled structures (3, 4). In our opinion, the interventional radiologist should consider this uncommon complication during pretreatment planning and the choice of embolic agent. We believe that in case of injury in close proximity to the bowel, the use of an embolizing liquid or glue could be a safer choice as it allows a quick embolization because of its rapid polymerization time, without embolic agent migration risks (5).

All the procedures performed in the study were in accordance with the ethical standards of the

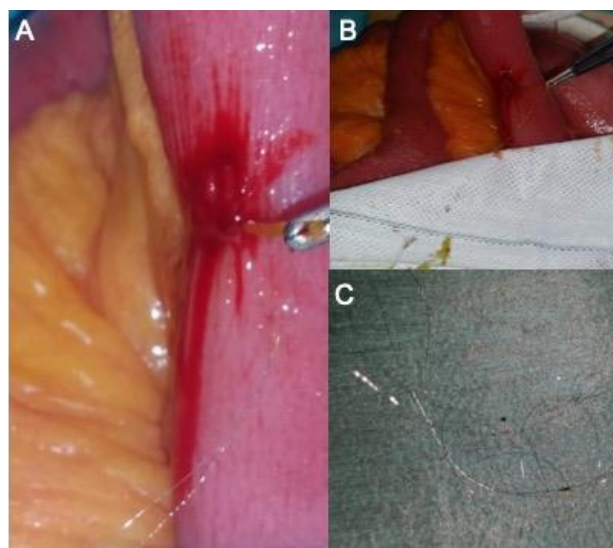


Fig. 3. Images from the surgical theatre. Through multiple enterotomies (A, B, C), the surgeon was able to remove the metallic thread from the small bowel.

institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The subject gave informed, signed consent to participate in the study.

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LETTER TO THE EDITOR

Anti-Müllerian hormone as marker of ovarian reserve in patients with long-standing type 1 diabetes

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To the Editor,

Type 1 diabetes (T1D) is a chronic disease caused by β -cell destruction, which leads to insulin deficiency. T1D can develop serious complications, as well as other autoimmune and gynaecological disorders (1, 2). An increased risk of infertility, mainly related to uncontrolled hyperglycaemia, has been postulated in women with T1D compared to women without diabetes, even independently of menstrual irregularities. Specifically, in animal models of T1D, uncontrolled diabetes led to reduced circulating levels of gonadotropins and sex hormones, as well as impaired LH pulsatility (3). Similarly, hypogonadotropic hypogonadism was observed even in clinical studies on women with T1D. Furthermore, several ovarian abnormalities, such as reduced survival of follicular and granulosa cells, alterations in oocyte maturation and follicular development, and impairment of ovarian steroidogenesis have been described in animal models of T1D (4). Besides hyperglycaemia, exogenous hyperinsulinemia that might ensue from chronic administration of insulin is believed to play a role in T1D ovarian dysfunction, possibly acting as a gonadotropin-like hormone, stimulating the recruitment and growth of large follicles (5). Furthermore, endothelial dysfunction

might accelerate ovarian ageing, conceivably leading to a sharp decrease in ovarian reserve.

Endometriosis is an inflammatory disease and infertility is a major concern related to this condition (6). There is consistent evidence about the impaired ovarian reserve in patients with endometriosis, compared to healthy women.

Anti-Müllerian hormone (AMH) is a glycoprotein belonging to the transforming growth factor- β family and is secreted by small ovarian follicles. As AMH serum levels strongly correlate with the antral follicle count (AFC), this hormone has emerged as a biomarker of ovarian reserve and reproductive function, with diagnostic and prognostic value in clinical practice (7).

The aim of this cross-sectional study was to comparatively evaluate AMH serum levels and AFC in long-standing T1D patients, in patients with endometriosis-related infertility and in healthy control women, to ascertain the effects of a long duration of T1D on ovarian reserve.

MATERIALS AND METHODS

One-hundred-forty-five (n.145) women of reproductive age, namely n.49 with long-standing T1D in

Key words: type 1 diabetes; diabetes; anti-Müllerian hormone; AMH; infertility; ovarian reserve; endometriosis

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basal-bolus treatment (mean age 36.0 ± 4.0 years; diabetes duration 13.4 ± 5.0 years), n.50 with endometriosis-related infertility (mean age 32.0 ± 5.0 years) and n.46 healthy control subjects (mean age 28.6 ± 6.0 years), were enrolled in the outpatient clinics of Policlinico Umberto I, "Sapienza" University Hospital of Rome. T1D was diagnosed according to the American Diabetes Association criteria (8). Diagnosis of endometriosis was performed in accordance with current recommendations (9). The exclusion criteria were as follows: recent diagnosis of T1D (diabetes duration < 5 years); current use of estrogenic contraceptives; menstrual cycle alterations; previous or current malignancies, chemotherapy or radiotherapy; history of surgery of the pelvic region; any other active diseases or chronic medications. The protocol was approved by the hospital ethics committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all participants. A detailed medical history was recorded and the relevant anthropometric parameters were assessed (height, weight, body mass index [BMI]). A venous peripheral blood sample in the early follicular phase of the menstrual cycle (within the third day from the beginning of menstruation) was collected to assess AMH levels. All subjects, at enrolment, underwent a transvaginal ultrasound scan (Samsung Electronics, Seoul, South Korea), performed independently by two experienced gynaecologists, to determine the number of antral follicles. AFC was defined as the number of follicles between 2 and 10 mm in diameter, observed on each side.

AMH assay

Blood collection was performed following a standard protocol. Samples were collected in a red top Vacutainer (Becton, Dickinson and Company, Plymouth, UK), clotted 60–90 min and centrifuged for 10 min at $1300 \times g$ at room temperature. The obtained serum fractions were subsequently aliquoted in 1.5 mL Eppendorf tubes (Eppendorf srl, Milano, Italy) and frozen at -80°C until testing.

AMH serum values were measured by ELISA method (Ansh Labs Ultra-Sensitive AMH/MIS ELISA, Webster, USA), a quantitative solid-phase three-step sandwich type non-competitive immunoassay, including two anti-AMH monoclonal

antibodies specific for different epitopes of the AMH. The first monoclonal antibody was coated into microtiter wells for capturing the antigen with the other biotinylated antibody; a streptavidin horseradish peroxidase conjugate (SHRP) was utilized to bind the biotinylated antibody complex. The antibody-antigen biotin conjugate-SHRP complex bound to the well was detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate was determined spectrophotometrically by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as a reference filter. The absorbance measured was directly proportional to the concentration of AMH/MIS in the samples and calibrators. The analytical sensitivity of AMH assay was 0.02 ng/mL. The specificity was 95%. The intra-assay coefficient of variation was 5.6%. According to manufacturer's instructions, the value of 0.9 ng/mL was considered as the AMH cut-off to identify reduced ovarian reserve.

Statistical analysis

Continuous variables are expressed as mean $\pm$ standard deviation (SD). Categorical data are expressed as percentages. Continuous variables were tested for normality with Kolmogorov-Smirnov test. Differences between groups were tested with the one-way analysis of variance (ANOVA), for normally distributed continuous variables, or with the Kruskal-Wallis H test, for not normally distributed variables. As appropriate, a post hoc test was subsequently performed on each group pair. Categorical variables between groups were compared by Fisher's exact test. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed with IBM SPSS Statistics software version 23 (Chicago, IL, USA).

RESULTS

The clinical characteristics of the enrolled population are shown in Table I. Low age-specific serum levels of AMH, indicating a reduced ovarian reserve, were observed in 12% of long-standing T1D patients, 10% of endometriosis infertile patients and 4% of healthy women. The prevalence of low circulating AMH levels was very similar between

Table I. *Clinical characteristics of the enrolled population*

	T1D N.49	Endometriosis N.50	Healthy women N.46
Age (years)	36.0±4.0	32.0±5.0	28.6±6.0
BMI (Kg/m²)	26.8±4.0	25.7±4.0	26.1±5.0
Diabetes duration (years)	13.4±5.0	-	-

T1D and endometriosis infertile patients (12% vs 10%, $p>0.05$, respectively), whereas the prevalence of reduced ovarian reserve was significantly higher in T1D group compared to control group (12% vs 4%, $p<0.05$, respectively) and in endometriosis infertile group compared to control group (10% vs 4% $p<0.02$, respectively) (Fig. 1). AFC did not significantly differ between the study groups.

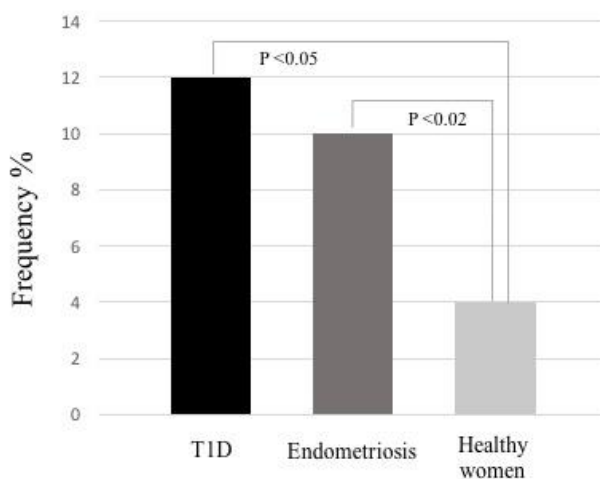
DISCUSSION

In this study, low serum concentration of AMH was a frequent finding in T1D patients with a long course of disease and the prevalence of this phenomenon was significantly higher in long-standing T1D with respect to healthy subjects. This result might mirror a reduced ovarian follicular pool in T1D women of reproductive age compared to age- and BMI-matched control subjects. Kadiroğulları et al. reported similar

findings (10). However, T1D patients were younger and had slightly shorter diabetes duration. Soto et al. showed that AMH levels were significantly lower in T1D patients compared to control subjects of more advanced age, suggesting an earlier decline in ovarian follicle pool in T1D women during the fourth decade of life (11). To date, studies evaluating ovarian reserve in T1D patients between the third and the fourth decades of life are limited, despite the markedly increased risk of infertility reported in this age range. Kim et al. observed a significant association between T1D and lower AMH values. However, AMH concentrations in women with T1D compared to women without diabetes were significantly lower only in the subgroup of those under than 35 years of age, not in older women (12).

To our knowledge, this is the first study comparing ovarian reserve between patients with T1D patients and women affected by endometriosis. Remarkably, the proportion of patients with values of AMH under the cut-off to identify reduced ovarian reserve was comparable between these two groups. This finding suggests that T1D and endometriosis might similarly impair ovarian follicular pool and disturb ovarian function. Even though T1D and endometriosis are entirely different conditions, they might share several pathophysiological features, such as chronic inflammation and altered immune modulation, which have been hypothesized to be involved in the development of follicular damage and dysfunction.

In conclusion, alterations of AMH were common in T1D patients. Although the role of this marker should be further evaluated, the determination of serum AMH might be a useful clinical tool in pregnancy planning and fertility management in patients with T1D.

**Fig. 1.** *Prevalence of low age-specific AMH in the study population*

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LETTER TO THE EDITOR

Reactive perforating collagenosis secondary to mesotherapy: first reported case

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To the editor,

Mesotherapy (MT) is a minimally invasive, local intradermal drug therapy which uses compounds belonging to the common pharmacopoeia at minimal dosage (1).

Despite generally being a safe and minimally invasive procedure (2), MT may also be associated with adverse events (AE), which have been classified into immediate, early and late AE (3).

We hereby describe the first reported case of mesotherapy-induced reactive perforating collagenosis in a patient suffering from long-standing type 2 diabetes mellitus (T2DM). The patient signed the consent form after being informed of the treatment modality and of the possible AE. This report expands the spectrum of known AE associated with MT.

A 49-year-old patient came to the Physical and Rehabilitation Medicine outpatients clinic in Tor Vergata Hospital (Rome, Italy) for chronic neck pain associated with vertigo and tingling-like paresthesia in the upper right limb; the patient had already undergone a cycle of diclofenac intramuscular injections with reportedly a transient benefit. His medical anamnesis was relevant for a history of recurrent kidney stone disease, long-standing T2DM and arterial hypertension; no known allergies to drugs or food were reported. Clinical and

instrumental investigations revealed a functional limitation secondary to cervical spondylarthrosis and discopathy. A therapeutic cycle of bilateral cervical-brachial MT was prescribed. The first session was performed after informing the patient about the treatment modality and the possible AE. The skin was carefully disinfected with Chlorhexidine, and a solution consisting of diclofenac, lidocaine, and saline solution was inoculated using 4 mm Lebel needles.

The patient returned to the hospital after one week to undergo the second session of the treatment cycle, but the physical examination revealed multiple, pruritic papulonecrotic erythematous lesions on the upper back at sites of previous MT injections; skin changes were characterized by an erythematous halo associated with a brown-to-black crusted plug in the center of each lesion. Lesions were seen at different stages of evolution, although at the time of clinical presentation no development of new lesions was observed. MT treatment was suspended. A skin biopsy was taken from a representative nodule for histopathological evaluation (Fig. 1A).

Histologically, prurigo-like changes were observed at the level of the epidermis, with irregular acanthosis, hyperkeratosis, and focal parakeratosis; centrally, a cup-shaped depression of the epidermis was seen,

Key words: mesotherapy, reactive perforating collagenosis, adverse event; musculoskeletal pain; rehabilitation

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A



B



Fig. 1. *Papulonecrotic erythematous lesions one-week (A) and 14 weeks (B) after mesotherapy.*

partially filled by a plug consisting of parakeratotic debris, bluish collagen, and mixed inflammatory cells; basophilic, vertically-oriented collagen fibers were observed in the adjacent epidermis, in the process of being extruded. The underlying dermis was characterized by an accompanying acute and chronic inflammatory infiltrate with neutrophils, lymphocytes, and plasma cells. Based on clinicopathological correlation, a diagnosis of reactive perforating collagenosis (RPC) was made.

No specific treatment for RPC was administered. Approximately 4 weeks after initial presentation, almost complete regression of skin changes was

observed (Fig. 1B). No recurrence of skin lesions has been recorded after 12 months of follow-up.

RPC belongs to the nosology category of so-called “perforating collagenoses”, a group of dermatoses histologically characterized by evidence of transepidermal elimination of altered dermal collagen (4-6). Collagen extrusion is thought to be the critical pathogenetic event defining RPC. Clinically, RPC is characterized by the eruptive appearance of multiple, recurrent, umbilicated papules, which undergo spontaneous regression within 6 to 8 weeks, leaving an area of hypopigmentation (5, 6). A history of superficial trauma (Koebner phenomenon), including a scratch or an insect bite, is frequently reported as the triggering event immediately prior to the eruption (7, 8). Lesions of RPC have been experimentally induced by traumatization of the skin in affected individuals (7). While the usual form of RPC shows an obvious genetic predisposing background (onset in childhood, association with Down syndrome) (8) the acquired clinical variant of RPC (so-called “acquired perforating collagenosis”) is observed in adult patients, especially in those suffering from diabetes mellitus and/or chronic renal failure

To the best of our knowledge, this is the first case of mesotherapy-induced RPC in the existing literature. It appears unlikely that the cutaneous changes observed in our patient had been induced by a specific, immune-mediated reaction against any of the therapeutic principles administered through MT. Indeed, the patient had been taking the same pharmacologic compounds also systemically, without undergoing any adverse reaction. However, it is possible to hypothesize that the traumatic stimulation of the skin, caused by the needle employed for MT, might have unleashed the clinically silent predisposition of the patient to develop RPC. Notwithstanding, further research in this regard is warranted, in order to better establish the basis of the observed link between MT and RPC.

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LETTER TO THE EDITOR

Qualitative evaluation of vomer bone position before and after rapid maxillary expansion: a radiographic studyF. Gazzani¹, C. Pavoni¹, C. Danesi³, G. Gastaldi³ and R. Lione^{1,2}¹*Department of Clinical Sciences and Translational Medicine, University of Rome 'Tor Vergata', Rome, Italy;* ²*Department of Dentistry UNSBC, Tirana;* ³*Department of Dentistry, IRCCS San Raffaele University, Milan, Italy**Received June 25, 2020 – Accepted October 2, 2020*

To the Editor,

Rapid maxillary expansion (RME) is an effective orthopedic procedure commonly used to treat maxillary transverse deficiency in growing patients (1). Transverse dentoskeletal changes are due to a separation of two halves of palatal bones across the median palatal suture. Transversal improvement of maxillary dimensions leads to a widening of nasal cavity and to an enhancement of nasal breathing (2, 3). Monson in 1898 (4) firstly described the improvements in nasal respiration after treatment with RME. Brogan (5) showed a correlation between septal deviation, crossbite, and architecture of the maxilla. In particular, high narrow palate, pointed alveolar arch, and a septal deviation were observed to the side opposite to the crossbite. According to functional matrix theory (3), nasal airflow represents a continuous stimulus for palate development and transverse maxillary growth, indicating a close relationship between nasal breathing and dentofacial morphology. The effects of RME on nasal cavity size and airway have been extensively investigated in literature (6). However, there is a paucity of findings about changes induced by RME on nasal septum (7, 8). More recently, tridimensional (3D) methods of investigation such as multislice Computed

Tomography (CT) and Cone Beam Computed Tomography (CBCT) have been more frequently used to evaluate the transverse effects of RME (1). 3D techniques provide more detailed analysis of sutural areas involved in orthopedic expansion. For this reason, further investigations on CT scans would provide valuable knowledge to the existing literature on RME effects on nasal septum. This osteocartilaginous structure divides the nasal cavity into halves and is delimited by the anterior nasal spine of maxilla and frontal bone anteriorly. Nasal crest of palatine bone determines the posteroinferior region of the septum. Among the bony components, vomer bone is located inferiorly and posteriorly. It articulates with the sphenoid and the nasal crest of the maxilla and the palate. Considering the frequent involvement of vomer bone in nasal septum deformities and the close interconnection with maxillary crest, changes in its position were analyzed before and after suture orthopedic opening. Therefore, the purpose of the present study was to evaluate the effects of RME on vomer bone position by means of low-dose CT.

MATERIALS AND METHODS

A sample of 10 subjects (6 males and 4 females,

*Key words: rapid maxillary expansion; vomer bone; nasal septum; maxillary deficiency; 3D imaging**Corresponding Author:*

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mean age 8.5) treated with a banded RME at the University of Rome "Tor Vergata", was selected for having a moderate NSD (nasal septum deviation) at T0. All patients presented transverse maxillary deficit, variable degree of crowding, and an impacted incisor as assessed by panoramic radiographs. For each subject, the expansion screw was activated twice per day (0.4 mm) for 14 days, until overcorrection of the transverse width was achieved (palatal cusp of the upper posterior teeth approximating the buccal cusp of the lower posterior teeth). After the active phase of expansion, the appliance was left in place six months to allow for the complete ossification of the midpalatal suture. Computed tomography (CT) low dose scans were available at 2 time points for each subject: before appliance placement (T0); and at the end of active expansion (T1). The examinations were performed with the primary indication of evaluating the exact position, the three-dimensional orientation, and the spatial relationship of impacted incisors. Low-dose CT scans were taken with a scanner console (Light-Speed 16, General Electric Medical System, Milwaukee, Wisconsin, USA) with a 1.25 mm slice thickness, 13.7x13.7 cm field of view (FOV) and following a low dose protocol with 80 KV. All exams were performed at the Department of Radiology, University of Rome, "Tor Vergata", Rome with a CT scanner equipped with a Dentascanner reconstruction program used to study the maxillo-facial region

(Light-Speed 16, General Electric Medical System, Milwaukee, Wisconsin, USA). The study was performed under the local ethics committee approval (5, 9) and informed consent was obtained from the parents of the subjects enrolled. In order to evaluate the effects on vomer position, a method to analyze 3D skeletal changes has been proposed. Qualitative evaluation was performed on coronal sections on the scanned images collected. In particular, the coronal scan selected was perpendicular to the bispinal plane passing through ANS-PNS and showed the palatal root and the crown of the maxillary first molars. The scans were chosen due to their ease of identification based on anatomical locations and because they could reasonably show the modality of expansion identifying the position of vomer before and after suture opening (Fig. 1).

RESULTS

The qualitative evaluation identified some common trends in the patients selected. All the subjects showed a pattern of asymmetric suture opening (Fig. 1). It was possible to observe one hemi-maxilla more anchored than the other (Fig. 1). For each patient, the asymmetry in suture opening was also appreciable in the extraoral clinical photos (Fig. 2). The asymmetry in suture opening was marked in all patients selected. In most subjects, the right



Fig. 1. Coronal view of naso-maxillary complex before (A) and after (B) rapid maxillary expansion. B) shows the asymmetric pattern in suture opening. Right maxillary half remained more locked than the left hemi-maxilla that appeared less resistant to orthopedic forces. The vomer bone did not completely detach but remained articulated to the more anchored hemi-maxilla and palatine bones on the right side.

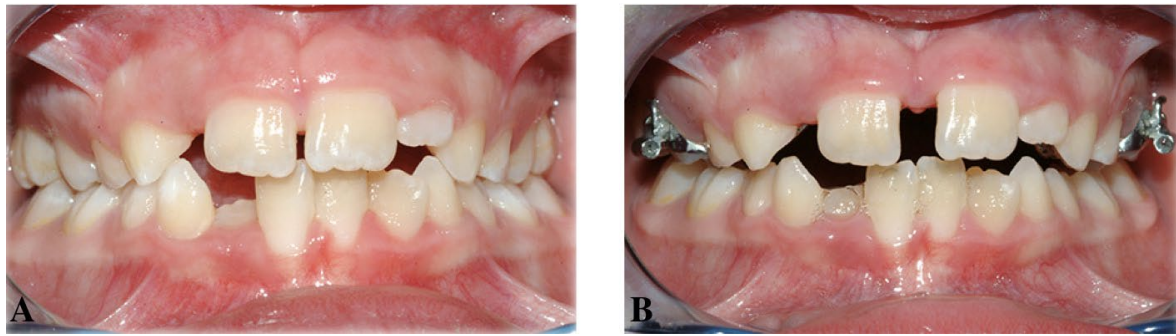


Fig. 2. Intraoral frontal view before (A) and after (B) rapid maxillary expansion. The asymmetry in suture opening was appreciable in the significant diastema appearing between the maxillary central incisors. The transverse displacement of the left side of the upper arch was clearly more evident than the right side.

maxillary side tended to stay locked. The left side showed less resistance during suture opening. The asymmetrical changes were also appreciable in the significant diastema appearing between the maxillary central incisors. No changes of the vomer bone position were observed; it tended to remain anchored to the more resistant side of the hemi-maxilla.

DISCUSSION

Vomer is a small, plow-shape, midline bone included the posterior part of the nasal septum. Because of its anatomical position and connections, it is frequently involved in nasal septum deviation. Positional changes of vomer bone before and after RME were evaluated in order to confirm the possibility of skeletal effects of maxillary expansion on nasal septum deviation. Maxillary structure and nasal cavity have an important anatomical connection from their initial growth phases (5). A deviation of skeletal and cartilaginous septal structures can cause a reduction of nasal airflow and interfere with maxillary growth (3, 7). Monson showed that malformed palatine processes from the top of the vault and floor of nasal cavity were often in close proximity to a distorted vomer (4). Many studies evaluated airway changes after RME, finding significant increases in nasal width and volume, and a decrement of nasal resistance with an improvement of breathing pattern (3, 9-12). However, recent results (2, 3, 9) confirmed the absence of any

influence on the nasal septum. According to the present results, our qualitative evaluation showed no positional changes of vomer bone after opening suture (Fig. 1). In all patients, the vomer bone remained locked to the more resistant hemi-maxilla, remarking an evident asymmetry in suture opening. Schwarz et al. (11) based their study on the undocumented belief that the nasal septum remains attached to one side of the maxilla during expansion. Also, Palaisa et al. (10) concluded that nasal septum, vomer, and ethmoid bone did not detach but remain articulated to one of the separated maxillary and palatine bones. This evidence could suggest a smaller displacement of more posterior circum-maxillary skeletal structures with more anterior aperture width (12). The present 3D analysis could enhance the hypothesis of the absence of influence on orthopedic treatment in improving nasal septum deviation. However, a more detailed analysis of nasal septal deviation, transverse maxillary constrictions and their consequences on nasal airflow should be performed.

The findings of the present study can be summarized as follows: a pattern of asymmetric suture opening was found in all subjects; vomer bone did not change its position after RME, but it remained locked to the more resistant and anchored hemi-maxilla; from the results obtained, the absence of any association between transverse maxillary deficiencies and nasal septum deviations could be hypothesized

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