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EDITORIAL

Mast cell virus infection and inflammatory cytokines

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Mast cells (MCs) are hematopoietic cells developed from bone marrow progenitors in response to the ligand stem cell factor, a trans-membrane tyrosine kinase kit receptor. MCs are located virtually in all vascularized tissues and in proximity to neurons and play a decisive role in both innate and adaptive immune responses. Their activation is involved in oxidative stress correlated with infection and inflammation. Pro-inflammatory cytokines are secreted by MCs after physiologic and psychological stress due to virus infection, including SARS-CoV-2. MCs, along with macrophages and pulmonary alveolar epithelial cells, are the main targets attacked by the coronavirus. COVID-19 induced by SARS-CoV-2 causes inflammatory stress which activates MCs to secrete corticotrophin-releasing hormone (CRH), SP, IL-6, TNF, and IL-1. Toll-like receptor (TLR) virus activation in MCs leads to pro-inflammatory cytokine generation without degranulation, an effect that can be inhibited by IL-10, IL-4, IL-1Ra and IL-37. TLR has the ability to recognize extracellular PAMPs by causing the transcription of NLRP, pro-IL-1, and other pro-inflammatory cytokines. The multi-protein complex, comprising pro-caspase-1, activates caspase-1 which in turn activates pro-IL-1 that is transformed into highly inflammatory mature IL-1. In COVID-19, viral RNA is specifically recognized by TLR, followed by recruiting the signal transfer proteins MyD88, IRAK, IKK and TRAF6 which can activate the NF-κB, resulting in transcription of the pro-inflammatory cytokines IL-1 and TNF, responsible for the “cytokine storm” phenomenon. Meanwhile, a new variant of the coronavirus-19 called C.1.2. has been discovered in the United States in the past few days, the effects of which are unknown, and it is therefore of great concern. Researchers are now testing it on immune cells to see if they react and are comparing it to a delta variant. Thus, from the existing data in biomedical literature, we can conclude that the suppression of pro-inflammatory cytokines in viral infections (including COVID-19) mediated by MCs represents a promising therapy not only in this field of medicine, but also in autoimmune, allergic, and cardiovascular disorders, as well as tumor inflammation where MCs play a key role.

Key words: mast cell; virus; cytokine; inflammation; allergy; COVID-19; SARS-CoV-2; coronavirus; antiinflammatory

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MAST CELLS, VIRUS AND INFLAMMATORY CYTOKINES

Mast cells (MCs) are hematopoietic cells that reside in virtually all vascularized tissues and play an important role in both innate and adaptive immune responses (1). Furthermore, they intervene in infections caused by microorganisms, aggravating the inflammatory process (2). MCs, terminally differentiated, are ubiquitous and try to oppose viral infection by collaborating with other immune cells (3). They are major effector cells in allergic reaction but also, by expressing Toll-like Receptors (TLRs), they can be activated by bacterial and viral antigens (4). In innate immunity, MCs support TH1 responses by mediating resistance to pathogens (including SARS-CoV-2), while anti-inflammatory cytokines, such as IL-10, contribute to TH2 responses by increasing immune activity and contributing to virus and parasite elimination. In chronic fatigue syndrome mediated by SARS-CoV-2 MC activation, cytokine levels are also elevated (2). The increase in pro-inflammatory cytokines in COVID-19 causes the “cytokine storm” with complement activation, excessive recruitment of neutrophils due to the increase of CXCL1,2,3,5,8 chemokines, injuries of the vascular system and pulmonary thrombosis. These effects may result in a high degree of morbidity and potential lethal consequences. IL-1 receptor antagonist (IL-1Ra) a member of the IL-1 family that binds to IL-1 receptors but does not act intracellularly, performs an immunoregulatory function and intervenes in TH2 responses (5). The protective role of T cells from the pathogenesis of SARS-CoV-2 infection has not yet been established. However, memory SARS-CoV2-specific T-cell response occurs as result of virus infection. CD8⁺ T cells, after infection, are very important in clearing the virus from lung, thus preventing or decreasing lung complications. CD4⁺ T cells polarize to Th1 profile with IFN γ , TNF, and IL-1 which can be detected in infected SARS-CoV-2.

Furthermore, coronavirus-19 with its RNA is specifically recognized by TLR; this reaction leads to recruit signal transfer proteins MyD88, IRAK, IKK and TRAF6 which can activate the NF- κ B

that is followed by the transcription of the pro-inflammatory cytokines such as IL-1 and TNF.

MCs with their TLRs respond to a wide variety of cytokines, microbes and microbial products, producing pro-inflammatory cytokines without degranulation. In viral infections, cytokines secreted by immune cells can trigger the activation of MCs which can also be activated by IL-1, IL-6, IL-33 and other cytokines, proving their effectiveness in inflammation, while they can be inhibited by IL-10, IL-4, IL-1Ra and IL-37 (6). The production of cytokines by MCs is an inflammatory reaction tending to stem and inhibit viral replication, but in some cases this reaction can become very harmful and sometimes lethal.

We have previously reported that the MC reaction against SARS-CoV-2 viral infection is mediated by cytokines and other extracellular stimuli, including stress (7). Coronavirus-19 seems to increase the risk of psychological factors which have been shown to influence COVID-19 severity. MCs activated by stress release corticotrophin-releasing hormone (CRH), an important central nervous system mediator of the stress response that is also elevated in the brain and cerebrospinal fluid (8). CRH can stimulate the release of vascular-endothelial growth factors from MCs and can increase vascular permeability. In addition, CRH provokes blood brain barrier damage through MC activation. Infection with pathogenic viruses can cause stress and fatigue syndrome, where levels of substance P (SP) are increased and induce MC activation with production of CRH implicated in brain inflammation and pathophysiology of pain.

In the past 20 years, considerable progress has been made in the field of IL-1 stimulation and inhibition, influencing therapeutic interventions. Cytokine IL-1 released by macrophages, MCs and other immune and non-immune cells, mediates almost all physiological and pathological processes including microorganism infections. Moreover, IL-1 stimulates itself in an autocrine manner as well as other cytokines that intervene on the efficacy of the vaccine and on the allergic response by influencing inflammatory and immunological processes (9). Cytokines, including IL-1, with the NALP3 (Nod-like receptor pyrin3) gene mutation, are known to impact COVID-19 pathogenesis, lymphocyte

trafficking and inflammation. The TLR, which is similar to that of IL-1, recognizes microbial products, mediating the host response to SARS-CoV-2 infection. Mature IL-1 beta derives from its immature precursor IL-1 which is activated by the active caspase-1 generated by the pro-caspase-1, which in turn is activated by the inflammasome, a complex of interacting intracellular proteins (5). Viral genomes encode molecules homologous to cytokines to prevent the host's immune response against viral infection. Blocking these genes inhibits viral infection with improved immune response. There are some viral genes that can code for anti-inflammatory cytokines, such as IL-10 which is a regulatory cytokine produced by almost all immune cells and is expressed to suppress the high IFN- γ that is generated in the infectious process as an immune response. Corticosteroids also reduce IL-1 transcription and translation by inhibiting allergic inflammation, decreasing the ability of blood immune cells to synthesize this potent pro-inflammatory cytokine, but can block the synthesis of antibodies useful against the virus. Thus, by neutralizing pro-inflammatory cytokines, such as IL-1, significant therapeutic progress can be achieved.

During harmful inflammatory SARS-CoV-2 lung infection, treatment with IL-1 inhibitors such as IL-37 could cause suppression of NLRP3 with a reduction in the number of inflammatory granulocytes that in COVID-19 prevail over other immune cells (3).

SARS-CoV-2 INDUCES INFLAMMATION IN COVID-19

Coronaviruses (CoVs) are a large family of zoonotic RNA viruses commonly circulating globally among humans. As has already been seen, CoVs can mutate and recombine rapidly to form new CoVs that can spread between individuals. SARS-CoV-2 (severe acute respiratory syndrome coronavirus-19) pandemic, spread around the world, has killed thousands of people every day, causing a severe outbreak of disease crisis that has never been seen before (2). Now, we hope that this unpleasant event is teaching us to better defend ourselves against another possible pandemic.

Most viruses, including those that cause flu and COVID-19, do not travel only in the air but can be contained at higher concentrations in the so-called respiratory droplets that the people emit when sneezing or coughing. Masks can block most droplets, especially the larger ones. If both the person sneezing and the people nearby are wearing masks the likelihood of transmission is much lower. Without the shadow of a doubt, vaccinations have supported the general well-being of the world population and have been recognized as an important means in public health.

The spreading of the SARS-CoV-2 pandemic has accelerated the rapid production of virus-neutralizing vaccines in the scientific world, a process that in the past took several years. The most widely used vaccines today are those with mRNA with lipid nanoparticle technology (mRNA1273 Moderna and mRNA BNT162b2 Pfizer-BionTech) and the viral vector one (AstraZeneca). mRNA vaccines give the body's cells instructions to mount a strong defense against a virus. To create the new mRNA in the laboratory, a large library for different pathogens was built. This system could also be used to create an HIV vaccine.

mRNA vaccine consists in encapsulating this molecule in nanoparticles and injecting them into the body by stimulating the cells to produce antigenic proteins which activate the production of antibodies. The high concentration of certain natural antibodies in the blood can mean that patients have encountered the virus and could potentially face COVID-19.

Anti-SARS-CoV-2 antibodies bind to the receptor-binding domain (RBD) and neutralize the virus. After infection the immune system takes time to build a sufficient number of neutralizing antibodies and by that time the coronavirus has done considerable damage. The mRNA vaccine represents the first generation of lifesaver vaccines that work by introducing spike protein information into the human body in order to instruct the immune system to recognize the spike protein receptor RBD. But this receptor often gets hidden by other parts of the spike protein, preventing antibodies from binding to it. The spike protein of the vaccine, not being the whole virus, does not stimulate the immune system

as it does with SARS-CoV-2; therefore to have an effective reaction, a large dose of the vaccine must be administered. When the dose of the vaccine is too high, strong side effects can be created with harmful consequences for the host. To avoid this, a vaccine with the RBD receptor alone should be created, even if it would be too small to induce an immune response and also technically difficult to implement.

Recent data published (10) report that the coronavirus-19 vaccine produces reduced but acceptable short-term immunogenicity in patients with rheumatic diseases, demonstrating a good safety profile. The vaccine, in these patients who presented inflammatory rheumatoid arthritis and axial spondyloarthritis, although immunogenic, was however less effective. Coronavirus vaccination does not cause significant adverse events even if it results in reduced but sufficient short-term immunogenicity.

SARS-CoV-2 tends to multiply and therefore genetically mutate, creating variants, as recently happened with the delta variant, which is more contagious, severe and weakens the vaccine response. Therefore, to combat the formation of variants, the majority of individuals must be vaccinated in order to achieve herd immunity. The effectiveness of the variant vaccines is unclear, especially on the new lambda variant which has currently only infected about two thousand individuals in the US.

Meanwhile, the research continues and world-renowned scientists are working hard on a simple, yet scientifically complicated, oral drug that can fight COVID-19 while avoiding hospitalization.

In this article we can conclude that, by targeting pro-inflammatory cytokines produced by MCs such as IL-1, IL-6, IL-33 and TNF after viral activation, it is possible to hope that their inhibition will give a better and novel therapeutic effect, including life expectancy. The concepts reported here are in agreement with the existing biomedical literature which tells us that in pathological states, such as infectious ones caused by pathogenic viruses, blocking pro-inflammatory cytokines generated by various immune cells, including MCs, can prevent, improve and successfully treat inflammatory diseases.

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Overexpression of miR-195-5p reduces osteoporosis through activating BMP-2/SMAD/Akt/RUNX2 pathway via targeting SMURF1

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Osteoporosis (OP) is among the most common frequent chronic metabolic bone diseases in postmenopausal women. Here, the effect and underlying mechanisms of miR-195-5p in OP were investigated both *in vivo* and *in vitro*. In this study, the microgravity (MG) environment was simulated in MC3T3-E1 cells, and miR-195-5p overexpression or SMURF1 knockdown model was constructed to test their effects on the proliferation, apoptosis and osteogenic differentiation of MC3T3-E1 cells. Furthermore, an OVX mouse model was constructed *in vivo*, and adenovirus-loaded miR-195-5p mimics were administered to the mice to overexpress miR-195-5p. HE staining and μ CT were adopted to observe pathological changes of femur. The targeted relationship between miR-195-5p and SMURF1 was predicted by bioinformatics analysis and verified by the dual-luciferase reporter assay and RNA immunoprecipitation (RIP) experiment. The results indicated that miR-195-5p was down-regulated in the head of femur of OP mouse model and MC3T3-E1 cells subjected to MG microenvironment. In addition, overexpression of miR-195-5p promoted MC3T3-E1 cell osteogenic differentiation and inhibited apoptosis. Mechanistically, SMURF1 is identified as a target of miR-195-5p, and overexpressing miR-195-5p activates the BMP-2/SMAD/Akt/RUNX2 signal by inhibiting the SMURF1 expression. Moreover, SMURF1 downregulation accelerated the osteogenic differentiation of MC3T3-E1 cells and attenuated MG-mediated apoptosis. In addition, upregulating miR-195-5p reduced osteoporosis in the OVX mouse model, accompanied with SMURF1 downregulation and BMP-2/SMAD/Akt/RUNX2 pathway activation. Collectively, miR-195-5p enhances osteogenic differentiation of osteoclast and relieve OP progression in the mouse model through activation of the BMP-2/SMAD/Akt/RUNX2 axis by targeting SMURF1.

Osteoporosis (OP) is an age-related bone disorder involving loss of bone mass, microstructural degeneration, and brittle fractures (1). OP is mainly divided into type 1 OP (i.e., postmenopausal OP, which occurs mostly in postmenopausal women) and type 2 OP (namely senile OP, which occurs mostly in people over 70 years old). Its pathogenesis mainly

includes the imbalance of osteoclasts and osteoblasts, resulting in uncontrolled bone remodeling, which is manifested as pain, fractures, and deformities (2). In postmenopausal elderly women, the increase of bone fragility has been considered as the main reason for the high morbidity and mortality of OP patients (3). Preceding works have corroborated that microRNAs

Key words: miR-195-5p; osteoporosis; SMURF1; BMP-2; pathway

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(miRNAs) participate in the evolvement of OP (4). However, the effects and mechanisms of most miRNAs in OP have been revealed, therefore, we to explored the miRNA mechanisms in OP.

MicroRNAs (miRNAs), a class of endogenous single-stranded non-coding small RNA, are 20-24 nucleotides in length. Studies have demonstrated that miRNAs contribute to tumorigenesis, cell growth and differentiation. Multiple miRNAs are found with prominent roles in orthopedic diseases, especially in OP. For example, miR-19a-3p facilitates the osteogenic differentiation of human mesenchymal stem cells (hMSCs) by repressing the HDAC4, thereby ameliorating OP (5). In addition, Li QQ. et al. found that miR-7b-5p abates the adipose differentiation of hMSCs by dampening the expression of IRS2, thus relieving OP progression (6). On the other hand, miR-365a-3p strengthens OP evolvement by targeting RUNX2 and inhibiting osteogenic differentiation (7). Also, Li Z. et al. discovered that in ovariectomized (OVX) rats, miR-133a increased the level of osteoclast formation-related factors in the serum, changed the morphology of bone tissue and promoted osteoclastogenesis, thus inducing OP (8). The above studies indicate that miRNAs protect against or deteriorate OP. MiR-195-5p, one member of miRNAs, is 87 bp long and located at 17p13.1. A previous study indicates that miR-195-5p, as one member of the miR-15 family, reduces the differentiation and mineralization of primary osteoblasts via interfering with the BMP/Smad-pathway (9). Additionally, Li P. et al. showed that miR-195-5p was the most downregulated miRNA in the collapse of steroid-induced osteonecrosis of the femoral head (10). It is believed that miR-195-5p has a potential role in OP via affecting osteogenic differentiation.

SMADspecific E3 ubiquitin-protein ligase 1 (SMURF1) functions as an ubiquitin ligase that is specific for receptor-regulated SMAD proteins in the bone morphogenetic protein (BMP) pathway. SMURF1 has potent effects on cell motility, cell signaling, and cell polarity (11). Moreover, SMURF1 has been found involved in the progression of multiple diseases, such as acute kidney injury (12)

and spinal cord injury (13). Recent studies have revealed that SMURF1 plays an essential role in bone formation and embryonic development. It negatively regulates osteoblastic function and responses to bone morphogenetic proteins in a dose-dependent manner (14-15). For instance, Ahmad et al. found that miR-672-5p elevates osteoblastogenesis and osteogenesis mineralization by repressing SMURF1 *in vivo* and *in vitro*, thus reversing ovariectomy-mediated bone loss (16). Furthermore, miR-19b-3p facilitates osteogenic differentiation of BMSCs via restraining the profile of SMURF1 (17), which reveals that multiple miRNAs can take part in the progression of osteogenic differentiation through modulating SMURF1. Nevertheless, whether miR-195-5p affects osteogenic differentiation by modulating SMURF1 is still unknown.

Previous works have denoted that SMAD is a transcriptional activator of specific genes, and the SMAD-dependent BMP signaling pathway is essential for osteoblastogenesis (18). Diversified signals, such as mitogen-activated protein kinase (MAPK), serine/threonine kinase Akt, transforming growth factor β (TGF- β), fibroblast growth factor (FGF), and transcription factor RUNX2, all contribute to osteoblast differentiation (19-20). Targeting BMP-2/SMAD/RUNX2 pathway facilitates hBMSC proliferation and osteogenic differentiation (21-22). More importantly, Li L. et al. found that MacLactin F (MF) enhances osteoblast differentiation to repress OP by activating BMP-2/SMAD/Akt/RUNX2 on MC3T3-E1 cells (23). Kim KM. et al. also uncovered that CRT2 elevates SMURF1 mRNA expression to decrease BMP2-induced osteoblastic differentiation (24). However, whether miR-195-5p modulates the progress of OP by influencing SMURF1-mediated BMP-2/SMAD/Akt/RUNX2 needs to be further investigated.

In this study, both *in-vitro* and *in vivo* experiments were performed to explore the role of miR-195-5p in OP. It was found that miR-195-5p activated BMP-2/SMAD/Akt/RUNX2 by targeting SMURF1, thus promoting osteoblast differentiation and alleviating OP. The results of this study provide novel reference for the treatment of OP.

MATERIALS AND METHODS

Construction of OVX mouse model

Twenty female mice (six weeks old, 10-20g) (Animal Experiment Center of Zhejiang Province, China) were reared under specific pathogen-free (SPF) conditions in the laboratory with a light/dark cycle of 12 h and the humidity of 50±15% at 25±2°C. The mice were randomized into a sham group and an OVX group. The sham group was anesthetized by intraperitoneally injecting 5% chloral hydrate (at 400 mg/kg), and the OVX group was treated with the same method, but both ovaries were removed. After three months, the mice were anesthetized again using the above-mentioned method, and the distal femur scan was performed along the femoral axis with μ CT (SkyScan; Bruker Corporation, Billerica, MA, USA) to check the symptoms of OP after menopause. Adeno-associated virus (AAV) (Hanheng Biotechnology Co., Ltd Shanghai, China) was intravenously injected into the mice 4 h prior to the construction of the OVX mouse model to upregulate miR-195-5p (25). The normal mice received AAV-miR-195-5p injection or only solvent treatment (for sham group). All animal tests were handled according to the guidelines approved by the Ethics Committee of Taizhou Hospital of Zhejiang Province. The experiment was carried out observing the regulations of the National Institutes of Health on the care and use of laboratory animals.

Cell culture

The osteoblast precursor cell MC3T3-E1 (Cell Center of the Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C with 5% CO₂. MC3T3-E1 cells were cultured in an osteogenic medium supplemented with 100 nM dexamethasone, 10 mM β -glycerophosphate (Sigma, St. Louis, MO, USA) and 50 mM ascorbic acid to perform functional experiments of osteogenic differentiation.

Microgravity simulation

Microgravity (MG) microenvironment was used for dealing with MC3T3-E1 cells via a 2-D clinorotation (China Astronaut Research and Training Center, Beijing, China) (26). MC3T3-E1 cells (1×10^5 cells) were put on coverslips in 6-well plates. After the cells were adherent

to the wall, the coverslips were inserted into a chamber containing culture medium and a distance of 12.5 mm was kept from the rotational axis of the chamber. After the bubbles were cleared, the lids of the containers were screwed tightly. Then, these chambers were placed into a clinostat and rotated around a horizontal axis at 24 rpm. The vertical rotation groups were used as control.

Cell transfection

During the logarithmic growth phase, MC3T3-E1 cells were seeded in 6-well plates at 5×10^6 /well after trypsinization and sub-culture. The transfection was performed after cell stabilization. MiR-195-5p mimics and the negative control (miR-NC), small interfering RNA targeting SMURF1 (Si-SMURF1#1, #2, #3) and the negative control (Si-NC) were provided by GenePharma (Shanghai, China). The cells were incubated with 5% CO₂ at 37°C and were co-cultured with Lipofectamine® 3000 (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's protocol when the cells reached 30-50% confluence. After 24 h, the total cellular RNA was extracted for real-time fluorescent quantitative PCR to monitor the profile changes of miR-195-5p and SMURF1. The sequences of si-SMURF1 #1: 5'-AAUAUUGUCCGUAGAAUACUG-3'; #2 5'-ACAUAUAUAGUUAUUUCCA-3'; #3 5'-AAUUAACAGUGACAAAAGAA -3'; si-NC: 5'-UUCUCCGAACGUGUCACGUTT-3'.

Quantitative Real-time PCR

The head of femur of mice was isolated, and approximately 100 mg of the tissue was condensed and triturated in liquid nitrogen in a pre-cooled mortar. The TRIzol reagent (Invitrogen, Shanghai, China) was employed to isolate total cellular RNA from the head of femur of mice or MC3T3-E1 cells, which was then reversely transcribed into cDNA using the PrimeScript™ RT Reagent kit (Invitrogen, Shanghai, China) referring to the manufacturer's guidelines. Quantitative Real-time PCR (qRT-PCR) was implemented with the Bio-Rad CFX96 quantitative PCR system and SYBR following the supplier's procedures. The conditions for qRT-PCR: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, and annealing at 60°C for 30 s. GAPDH was the internal reference for ALP, RUNX2, Col1a1, and OCN, while U6 was for the internal control of miR-195-

5p. The $2^{-\Delta\Delta C_t}$ method was used for analyzing the relative expression of the detected genes. The experiment was repeated for three times. The primers were designed and synthesized by Guangzhou Ribo Biotechnology Co., Ltd (Table I).

Western blot

MC3T3-E1 osteoblast precursor cells and the ground head of femur tissues were harvested and rinsed with cold PBS three times. Then, 100~200 μ L RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) was used for lysis in ice water ultrasonically, and the protein purity was examined with the Bradford method using a BSA kit (Beyotime, Shanghai, China). The same amount of protein in each group was subjected to 10% SDS-PAGE

electrophoresis, and the protein on the gels was transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Afterward, the membranes were blocked at 4°C for 1 h, and then incubated with the primary antibodies (concentration 1:1000) of Anti-Bax antibody (ab32503), Anti-Caspase3 antibody (ab13847), Anti-Bcl2 antibody (ab182858), Anti-BMP-2 antibody (ab14933), Anti-RUNX2 antibody (ab76252), Anti-p-SMAD1 antibody (ab18834), Anti-SMAD1 antibody (ab40855), Anti-p-SMAD5 antibody (ab52903), Anti-SMAD5 antibody (ab40854), Anti-p-AKT Antibody (ab38449) and Anti-AKT antibody (ab18785) overnight at 4°C. After being rinsed twice with TBST, the membranes were incubated with the HRP-labeled goat anti-rabbit IgG (ab205718, 1:2500) at room temperature for 1 h. The

Table I. *Primer sequences of each gene*

Gene name	Primer sequences (5' → 3')
miR-195-5p	forward:AACCGGTAGCAGCACAGAAATG reverse:CAGTGCAGGGTCCGAGGT
SMURF1	forward:CCGCTTCAGTTCTCGAATGG reverse:TGAAAAGTTCTGCCTTGCCC
ALP	forward:TGAACCGCAGGATGTGAACT reverse:GAAGAAGGGGTGCTACGTCC
RUNX2	forward:TGATTTAGGGCGCATTCCTCA reverse:AGGATTGTGTCTGCCTGGGA
Colla1	forward:GGAGAGAGCATGACCGATGG reverse:GCTACGCTGTTCTTGCACTG
OCN	forward:ATTGTGACGAGCTAGCGGAC reverse:TCGAGTCCTGGAGAGTAGCC
U6	forward:CTCGCTTCGGCAGCACA reverse:AACGCTTCACGAATTTGCGT
GAPDH	forward:TGATCTTCATGGTCGACGGT reverse:CCACGAGACCACCACCTACAAC

above antibodies were all from Abcam (Cambridge, UK). Finally, the membranes were cleared three times, exposed with ECL chromogenic agent (Millipore, Bedford, MA, USA), and imaged with a membrane scanner.

BrdU assay

During the logarithmic growth phase, MC3T3-E1 cells were made into single-cell suspension and seeded in 24-well plates at 1×10^5 /well. After they were adherent to the wall, BrdU-labeled reagent (AmyJet Scientific Inc., China) was added as per operation instructions with 10 mol/L or without RA. Then, the plates were incubated with 5% CO₂ at 37°C. After culturing for 48 h, the cells were fixed by 4% polyformaldehyde and incubated with anti-BrdU antibody (Abcam). DAPI (Beyotime, Shanghai, China) was used for staining the nucleus. The immunofluorescent signal was observed under a immunofluorescent microscope. The positive cell number and the total DAPI positive cell number in three randomly chosen fields of view were chosen and counted under the microscope. Cell proliferation rate=BrdU-positive cell number/DAPI positive cell number, the cell proliferation rate in three fields was averaged.

Flow cytometry

The Annexin V-FITC/PI double staining method was adopted to monitor apoptosis using a commercially purchased Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, Shanghai, China). After MC3T3-E1 cells were trypsinized, collected, and seeded in 6-well plates. Then, the cell density was adjusted to 2×10^6 /well and further cultured for 24 h. Meanwhile, the supernatant was abandoned, and the cells were cleared twice with pre-cooled PBS and resuspended with $1 \times$ Binding buffer. Subsequently, 5 μ L AnnexinV-FITC and 5 μ L PI were added to the cell suspension, mixed well and incubated at room temperature for 15 min. Flow cytometry (BD Biosciences, SanJose, CA, USA) was employed to examine the apoptosis rate within 1 h. The apoptosis rate=the apoptotic cell number/(the apoptotic cell number + the normal cell number) $\times 100\%$. The procedures were carried out following the apoptosis kit instruction.

Dual-luciferase reporter assay

The dual-luciferase reporter gene assay was performed with the dual-luciferase reporter assay system (Promega,

Madison, WI, USA). The target fragments of wild-type and mutant SMURF1 were built and integrated into pGL3 vectors (Promega, Madison, WI, USA) to obtain pGL3-SMURF1-wild type (SMURF1-WT) and pGL3-SMURF1-mutant (SMURF1-MUT) reporter vector. SMURF1-WT or SMURF1-MUT were co-transfected with miR-195-5p mimics or negative control into MC3T3-E1 cells. Forty-eight hours later, the luciferase activity was determined following the supplier's guidelines. All tests were performed for three times.

RNA immunoprecipitation assay

RNA immunoprecipitation (RIP) assay was conducted with the EZMagna RIP RNA binding protein immunoprecipitation kit (Milligram) as per the kit instructions. The transfected MC3T3-E1 cells administered to MG microenvironment or not were lysed and immunoprecipitated with the anti-human argonaute 2 (Ago2) antibody (Abcam, Cambridge, UK) and the control IgG (the input group). After 48 h of incubation, the co-precipitated RNA was isolated and the SMURF1 enrichment was measured using qRT-PCR.

ALP activity assay

The alkaline Phosphatase activity detection kit (Yeasen Biotechnology Co., Ltd., Shanghai, China) was used to measure the ALP activity according to the manufacturer's instructions. MC3T3-E1 cells were cultured in an osteogenic medium supplemented with 100 nM dexamethasone, 10 mM β -glycerophosphate (Sigma, St. Louis, MO, USA) and 50 mM ascorbic acid to perform functional experiments of osteogenic differentiation. Next, MC3T3-D1 cells were transfected with miR-195-5p mimics or miR-NC or si-SMURF1 or si-NC under an MG environment. Cells were lysed with Triton X-100 lysis buffer (50 mM Tris, 150 mM NaCl, and 1% Triton X-100, pH10). The aliquots with a comparable amount of protein content were incubated with ALP substrate using ALP assay kit (Elabscience). Optical density (OD) of the p-nitrophenol was determined by a spectrophotometer at 520 nm. ALP activity was calculated relative to standard phenol solution and expressed as μ g/ml. Three independent experiments were performed.

Microcomputed tomography analysis

After the head of the femur of mice was fixed with 4%

paraformaldehyde for 24 h, a μ CT scan was performed by microcomputer tomography (μ CT) (Siemens, Germany). The microstructure of the femur was presented by the region of interest (ROI). A $2.5 \times 2.5 \times 3$ mm³ cube 15 μ m above the proximal epiphyseal growth plate was taken. COBRA 2.0 software was used for μ CT to assess relative bone volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), trabecular bone separation (Tb.Sp) and trabecular bone pattern factor (Tb.PF).

Hematoxylin-Eosin staining

Bone tissue samples from the mice were secured with 10% neutral buffer formalin for 24 h, decalcified for 4 weeks with 5% ethylenediaminetetraacetic acid. The solution was replaced every 3 days. The tissues were dehydrated with gradient ethanol (70%, 80%, 90%, 95% and 100%), permeated with xylene twice (5 in/time) paraffin-embedded and sliced with a thickness of 4 μ m. Finally, the sections were sealed with neutral resin. Hematoxylin-eosin (HE staining) was performed using an HE kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. The morphological differences were observed the using an optical microscope (Olympus, Japan).

Statistical analysis

Data were analyzed by SPSS20.0 (SPSS Inc., Chicago, IL, USA) and are presented as mean $\pm$ standard deviation ($\bar{x} \pm s$). The multivariate comparison was made by one-way ANOVA, and the pairwise comparison was implemented by the *t*-test. $P < 0.05$ indicated statistical significance.

RESULTS

MiR-195-5p was down-regulated in OP tissues

Firstly, an OVX mouse model was constructed and the pathological changes of femur were examined by HE staining. It was found that the trabeculae of the bone tissue sections in the sham group were clearly visible and neatly arranged, bone cells were visible in the cortex and trabeculae, and the nucleus was large. While in the OVX group, the trabeculae of the bone tissue were scattered and thinned, fibrosis and granulation tissue hyperplasia were seen around the trabeculae, and there were necrotic bone cells in the bone lacuna (Fig. 1A). μ CT analysis revealed that the

contents of BV/TV, Tb.N, and Tb.Th were notably abated, while the Tb.Sp and TbPF were higher in the OVX group compared with that in the sham group ($P < 0.01$, Fig. 1 B-F). Furthermore, qRT-PCR was applied to monitor the miR-195-5p expression in OVX mouse tissues. Interestingly, the miR-195-5p level in the OVX group was distinctly lower than that of the sham group ($P < 0.01$, Fig. 1G). The above results indicated that there was obvious osteoporosis in the bone tissue of OVX mice, and miR-195-5p was significantly downregulated in OVX mice.

Overexpressing miR-195-5p decreased the MG induced damage in MC3T3-E1 cells

Previous researches have reported that a simulated MG environment can inhibit osteoblast differentiation (27). To clarify whether miR-195-5p influences osteoblast differentiation under simulated MG conditions, the miR-195-5p overexpression model in MC3T3-E1 cells was constructed. The miR-195-5p level was examined. It was illustrated that miR-195-5p was downregulated in the MG group, while enhanced after miR-195-5p mimics transfection ($P < 0.01$ vs MG group, Fig. 2A). Additionally, qRT-PCR confirmed that the mRNA expression of osteogenic markers RUNX2, ALP, Col1a1, and OCN was attenuated in a simulated MG environment, and overexpression of miR-195-5p attenuated these effects ($P < 0.01$ vs MG group, Fig. 2B). Similarly, ALP activity was reduced in the MG group but enhanced with miR-195-5p upregulation ($P < 0.01$, Fig. 2C). In addition, BrdU and flow cytometry proved that compared with the control group, cell proliferation was dampened and apoptosis was increased under the simulated MG environment, and the miR-195-5p mimic transfection weakened the influence of the MG environment, enhanced cell proliferation and decreased apoptosis ($P < 0.01$, Fig. 2 D-E). Furthermore, western blot (WB) was implemented to verify the levels of apoptosis-related proteins, including Bax, Bcl2 and Caspase3. Interestingly, compared with the control group, the pro-apoptotic proteins Bax and Caspase3 were upregulated and the anti-apoptotic protein Bcl2 was down-regulated in the MG group, and miR-195-5p partially offset these effects ($P < 0.01$

vs MG group, Fig. 2F). The above findings suggest that the simulated MG environment inhibited the osteogenic differentiation and cell proliferation of MC3T3-E1 cells and strengthened cell apoptosis, while overexpressing miR-195-5p weakened MG-mediated effects.

MiR-195-5p targeted SMURF1 and activated BMP-2/SMAD/Akt/RUNX2

Through the Starbase database (<http://starbase.sysu.edu.cn/>), it was found SMURF1 contained a binding site with miR-195-5p (Fig. 3A). Then, a dual-luciferase reporter assay was implemented to further clarify the targeted association between miR-195-5p and SMURF1. As a result, miR-195-5p mimics dampened the luciferase activity of cells transfected with SMURF1-WT vector, while had no

influence on that of SMURF1-MUT ($P<0.05$, Fig. 3B). Additionally, RIP assay confirmed that after the miR-195-5p mimic transfection, the amount of SMURF1 precipitated in the Ago2 antibody group was more than that in the IgG group (both in the MG environment and normal environment), suggesting that SMURF1 bound to Ago2 protein through miR-195-5p ($P<0.05$, Fig. 3C). Moreover, WB confirmed that SMURF1 was down-regulated in MC3T3-E1 cell lines with MG treatment after the miR-195-5p mimics transfection ($P<0.05$ vs miR-NC group, Fig. 3D). Additionally, WB was implemented to examine the BMP-2/SMAD/Akt/RUNX2 expression in MC3T3-E1 cell lines with MG treatment, and it revealed that compared with the miR-NC group, BMP-2/SMAD/Akt/RUNX2 was upregulated after transfection of miR-195-5p mimics ($P<0.05$, Fig.

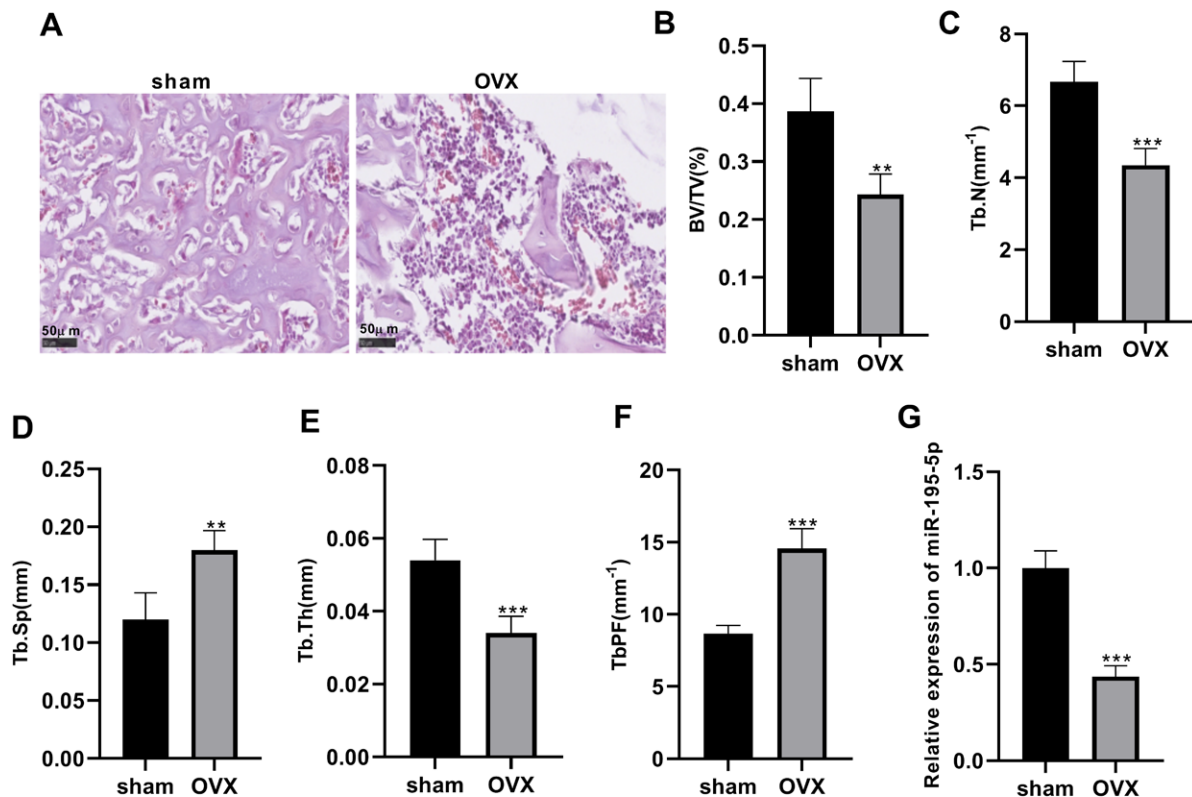


Fig. 1. miR-195-5p was down-regulated in OP tissues. The OVX mouse model was constructed. **A)** HE staining was implemented to check the pathological changes of femur. **B-F)** μ CT was employed to analyze the contents of BV/TV, Tb.N, Tb.Th, Tb.Sp and Tb.Pf. **G)** qRT-PCR was carried out to verify the miR-195-5p profile in OVX or sham mouse femur. ** $P<0.01$, *** $P<0.001$ (vs sham group). $N=5$. Scale bar=50 μ m.

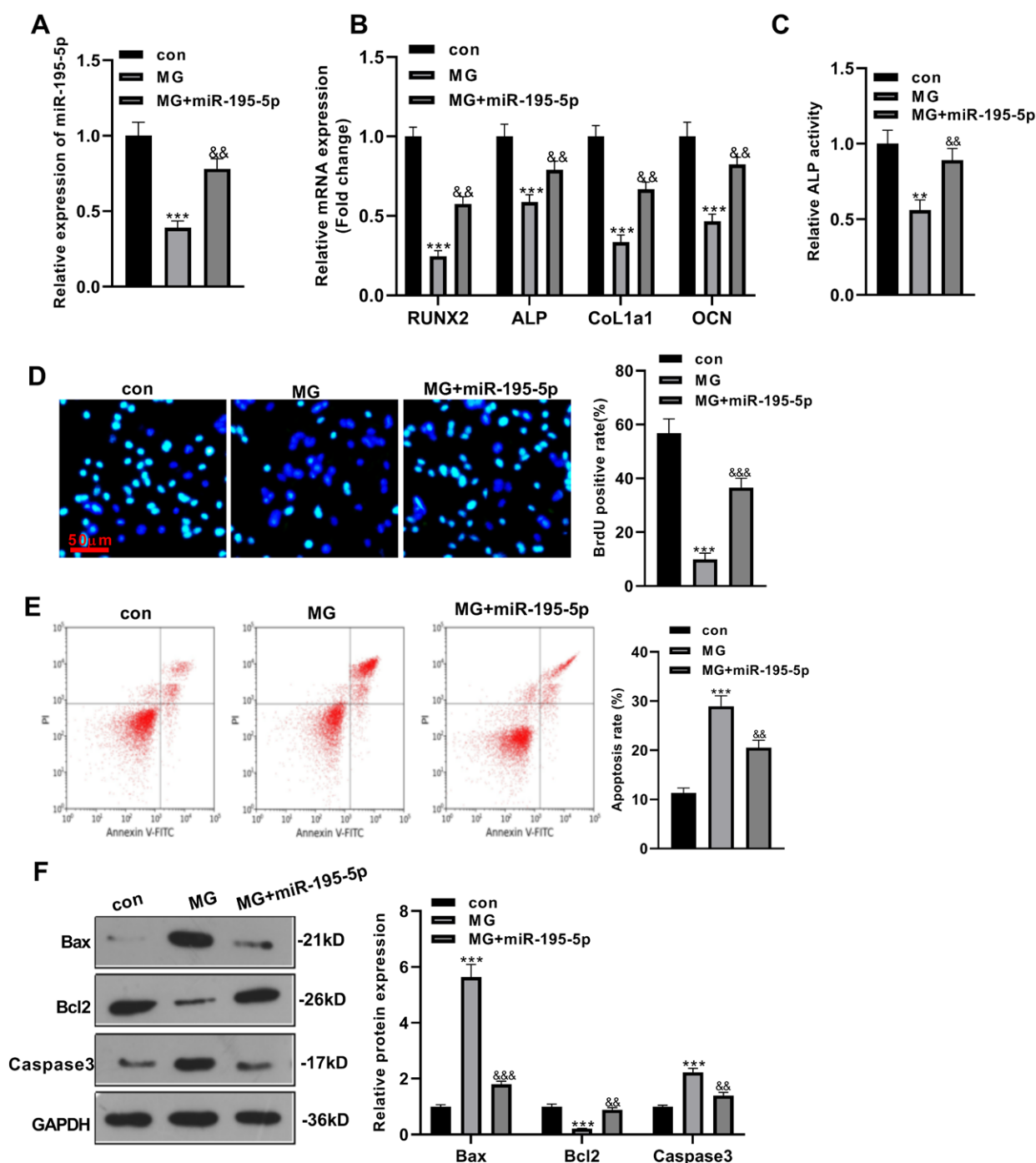


Fig. 2. Overexpressing miR-195-5p promoted osteogenic differentiation and reduced apoptosis of MC3T3-E1 cells in MG environment. The MG environment was simulated in MC3T3-E1 cells and miR-195-5p mimics were transfected into MC3T3-E1 cells. **A)** The miR-195-5p level was monitored by qRT-PCR. **B)** The mRNA expression of osteogenic markers RUNX2, ALP, Col1a1, and OCN was compared by qRT-PCR. **C)** ALP activity was detected by ALP detection kit. **D-E)** BrdU assay (**D**) and flow cytometry (**E**) were carried out to examine cell proliferation and apoptosis, respectively. **F)** WB was applied to determine the expression of apoptosis-related proteins Bax, Bcl2, and Caspase3 in MC3T3-E1 cells. *** $P < 0.001$ (vs con group). & $P < 0.05$, && $P < 0.01$ (vs MG group). $N = 3$. Scale bar = 50 μm .

3E), suggesting that miR-195-5p targeted SMURF1 and activated BMP-2/SMAD/Akt/RUNX2 pathway.

Knockdown of SMURF1 facilitated MC3T3-E1 osteogenic differentiation and inhibited apoptosis

The SMURF1 downregulation cell model was first established in MC3T3-E1 cells. It was found that the protein level of SMURF1 was considerably down-regulated after the transfection of si-SMURF1 ($P < 0.05$, Fig. 4A). Furthermore, MC3T3-E1 cells were subjected to MG environment. The protein profile of SMURF1 was further detected by WB. The results showed that the expression of SMURF1 was up-regulated in the simulated MG environment, while knockdown of SMURF1 substantially reduced the expression of SMURF1 ($P < 0.05$, Fig. 4B). Then, the mRNA levels of osteogenic markers RUNX2, ALP, Col1a1, and OCN were compared by qRT-

PCR, and ALP activity was also detected. It was proved that the above osteogenic markers, as well as the ALP activity, were down-regulated in a simulated MG environment, while the opposite results were observed after knocking down SMURF1 ($P < 0.05$, Fig. 4 C-D). Besides, BrdU and flow cytometry illustrated that compared with the control group, cell proliferation was abated and apoptosis was elevated under the simulated MG environment, while knocking down SMURF1 had the reverse effect ($P < 0.01$ vs MG group, Fig. 4 E-F). Moreover, WB was conducted to verify the levels of apoptosis-related proteins, including Bax, Bcl2 and Caspase3, and it was found that compared with the control group, the pro-apoptotic proteins Bax and Caspase3 were upregulated, and the anti-apoptotic protein Bcl2 was down-regulated in the simulated MG environment, while knocking down SMURF1 partially offset

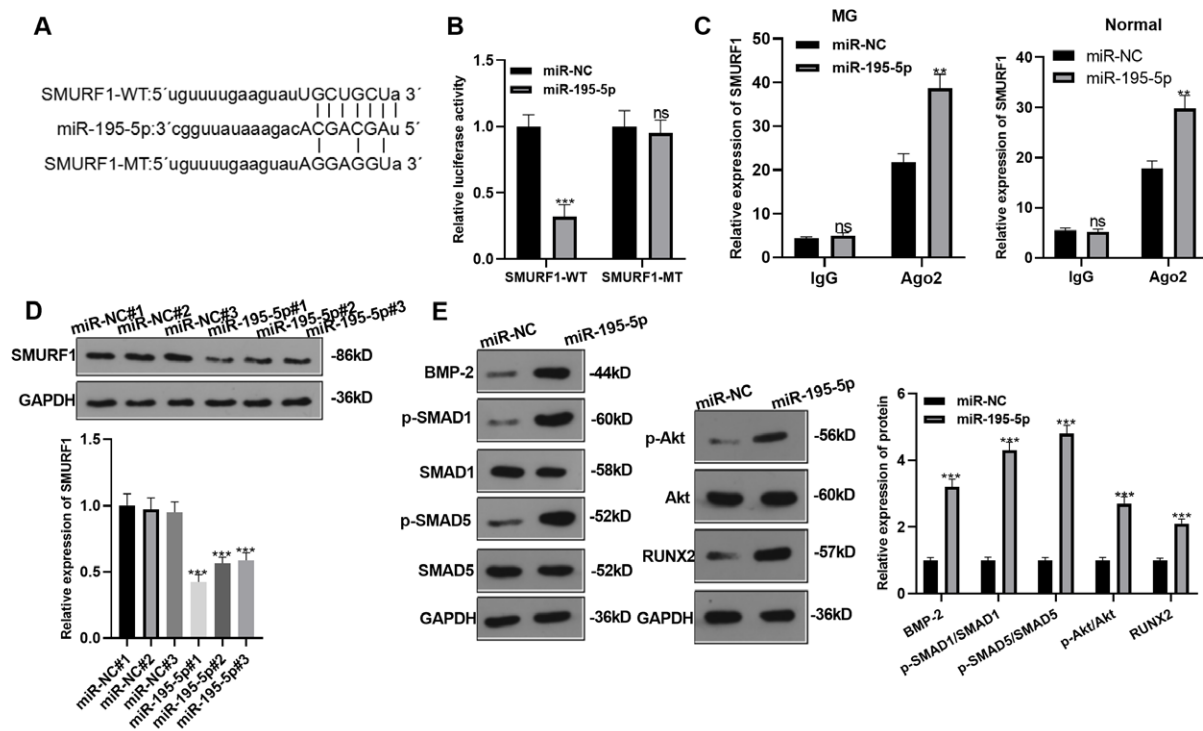


Fig. 3. MiR-195-5p targeted SMURF1 and activated BMP-2/SMAD/Akt/RUNX2 pathway in MC3T3-E1 cells. **A)** The Starbase database was employed to predict the binding sites of miR-195-5p and SMURF1. **B, C)** The binding association between miR-195-5p and SMURF1 was determined by dual-luciferase reporter assay and RIP. **D)** MC3T3-E1 cells with MG treatment were transfected with miR-195-5p mimics or miR-NC. WB was adopted to test the SMURF1 level in the cells. **E)** WB was applied to monitor the protein expression of BMP-2/SMAD/Akt/RUNX2 in the cells. $NsP > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (vs miR-NC group). $N = 3$.

these effects ($P < 0.01$, Fig. 4G). These findings indicated that knocking down SMURF1 attenuated the simulated MG environment-induced decrease in osteogenic differentiation and the increase in apoptosis.

MiR-195-5p activated the BMP-2/SMAD/Akt/RUNX2 signal by targeting SMURF1, thus repressing OP

To further clarify whether the miR-195-5p/SMURF1 axis affected the progress of OP, the mouse OVX model was constructed and AAV-miR-195-5p was injected into mice to induce the up-regulation of miR-195-5p ($P < 0.01$, Fig. 5A). Meanwhile, the bone tissue structure was observed by HE staining ($P < 0.01$, Fig. 5B). Additionally, μ CT analysis illustrated that in the OVX group, the BV/TV, Tb.N,

and Tb.Th in the OVX group were repressed, while the Tb.Sp and Tb.PF were elevated compared with the sham group. On the contrary, compared with the OVX group, BV/TV, Tb.N and Tb.Th levels were upregulated, while Tb.Sp and Tb.PF were down-regulated after AAV-miR-195-5p was injected *in vivo* ($P < 0.01$, Fig. 5 C-G). WB results disclosed that, in contrast with the sham group, the protein expression of SMURF1 was down-regulated in the AAV-miR-195-5p group, and up-regulated in the OVX group. Compared with the OVX group, the expression of SMURF1 in the OVX+AAV-miR-195-5p group was remarkably down-regulated ($P < 0.01$, Fig. 5H). Moreover, qRT-PCR illustrated that compared with the OVX group, the levels of osteogenic differentiation markers (RUNX2, ALP, Col1a1 and

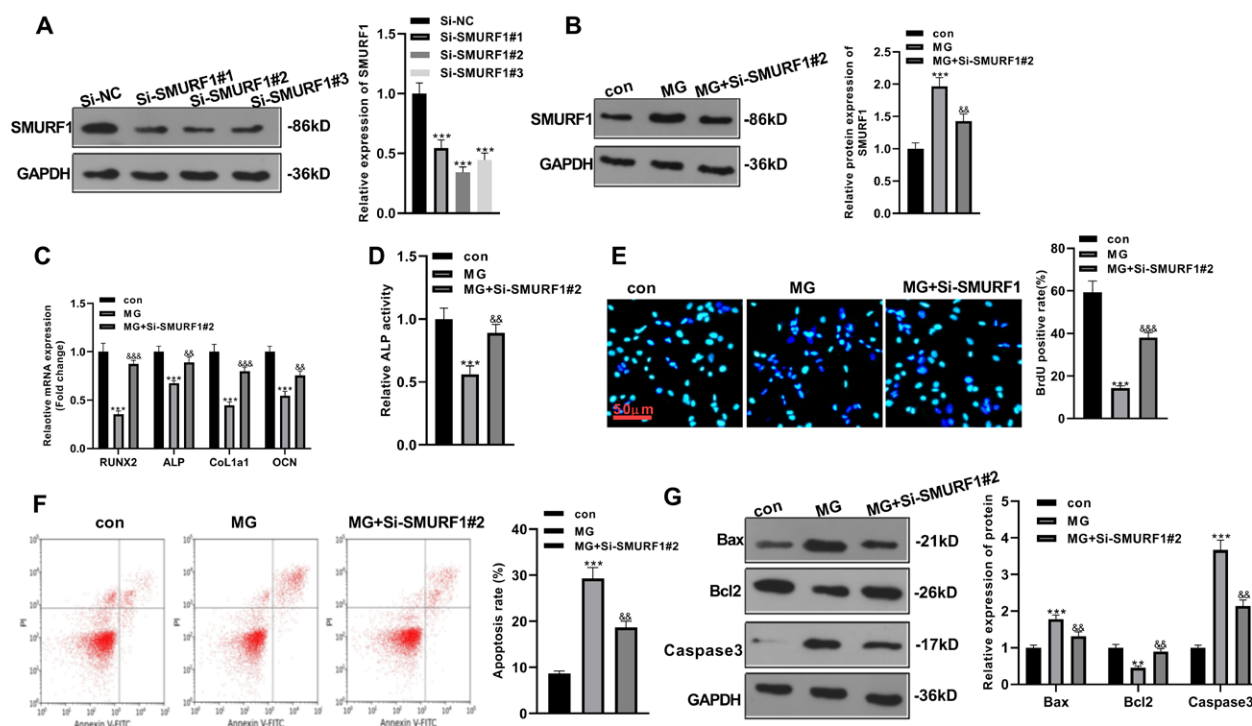


Fig. 4. Knockdown of SMURF1 enhanced osteogenic differentiation and dampened apoptosis in MC3T3-E1 cells. MC3T3-E1 cells were transfected with si-SMURF1 and the negative control (si-NC). **A**) WB was implemented to monitor the SMURF1 level. **B**) The MG environment was dealt with MC3T3-E1 cells with transfecting si-SMURF1#2. **B**) WB was implemented to monitor the SMURF1 level. **C**) The mRNA expression of osteogenic markers RUNX2, ALP, Col1a1, and OCN in MC3T3-E1 cells treated with MG environment were compared by qRT-PCR. **D**) ALP activity in MC3T3-E1 cells was determined. **E-F**) BrdU and flow cytometry were implemented to examine cell proliferation and apoptosis of MC3T3-E1 cells treated with MG environment. **G**) WB was carried out to determine the levels of apoptosis-related proteins Bax, Bcl2, and Caspase3 in MC3T3-E1 cells treated with MG environment. ** $P < 0.01$, *** $P < 0.001$ (vs con group). & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$ (vs MG group). $N = 3$.

OCN) were elevated after injection of AAV-miR-195-5p into mice (vs OVX group, $P < 0.01$, Fig. 5I). Furthermore, WB was implemented to verify the protein expression of apoptosis-related proteins Bax, Bcl2, Caspase3 and the BMP-2/SMAD/Akt/RUNX2 signal. It was found that compared with the OVX group, the pro-apoptotic proteins Bax and Caspase3 were down-regulated, and the anti-apoptotic protein Bcl2 and the BMP-2/SMAD/Akt/RUNX2 signal were upregulated in the OVX+AAV-

miR-195-5p group ($P < 0.01$, Fig. 5 J-K). The above results manifested that miR-195-5p prevented OP progression through SMURF1-BMP-2/SMAD/Akt/RUNX2 pathway (Fig. 6).

DISCUSSION

The imbalance of bone remodeling caused by increased osteoclastogenesis and decreased osteoblast formation can lead to multiple osteolytic

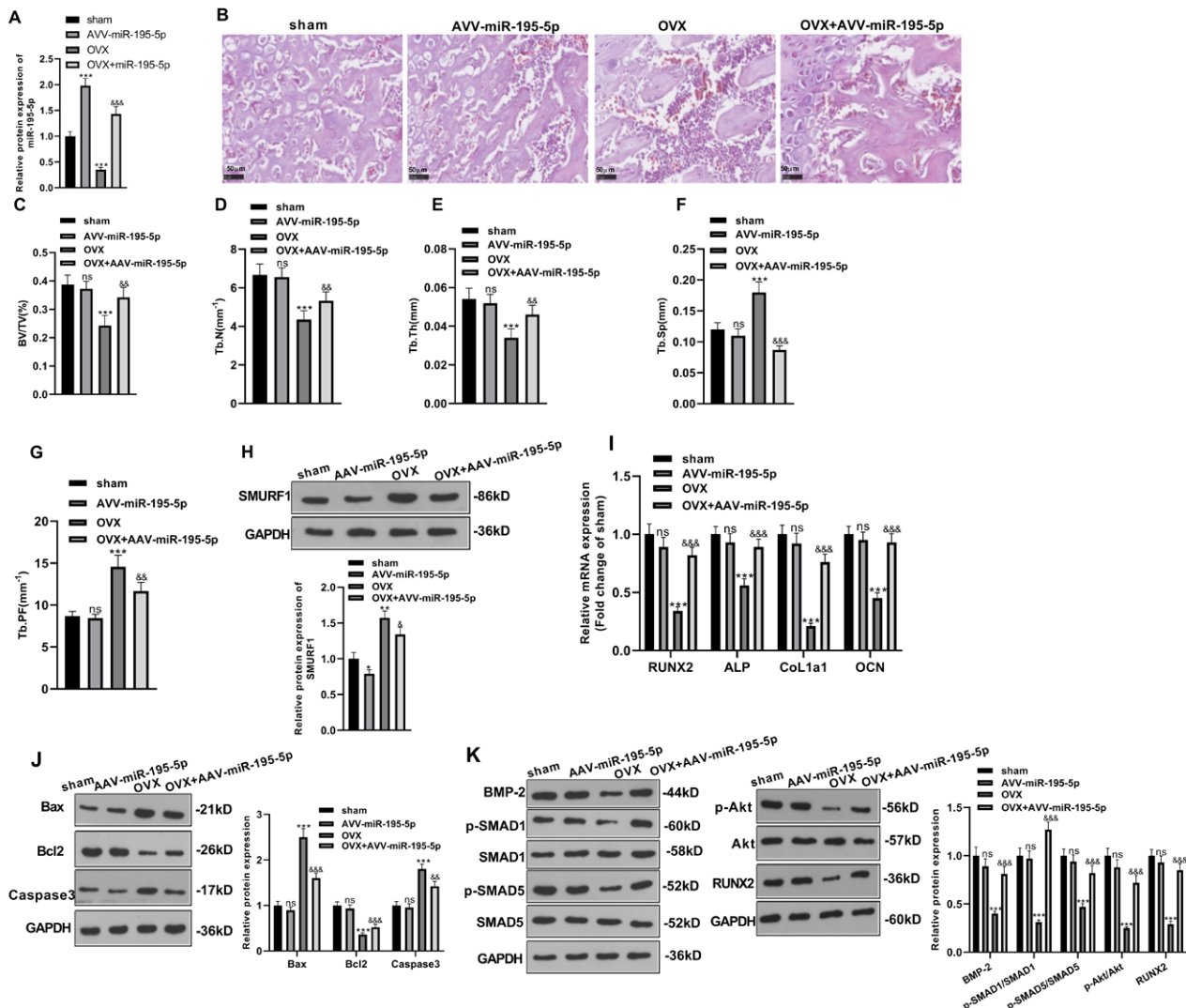


Fig. 5. MiR-195-5p activated BMP-2/SMAD/Akt/RUNX2 by targeting SMURF1, thus inhibiting OP. A mouse OVX model was constructed and AAV-miR-195-5p was injected into mice to induce the up-regulation of miR-195-5p. **A)** HE staining was conducted to observe the bone tissue structure. **B-F)** μ CT was employed to analyze the contents of BV/TV, Tb.N, Tb.Th, Tb.Sp, TbPF in the OVX mouse model. **G)** SMURF1 level in the bone tissues was evaluated by WB. **H)** The expression of osteogenic differentiation markers (RUNX2, ALP, Col1a1, OCN) was determined by qRT-PCR. **I, J)** WB was adopted to verify the protein expression of apoptosis-related proteins Bax, Bcl2, Caspase3 and BMP-2/SMAD/Akt/RUNX2. *** $P < 0.001$ (vs sham group), &#&# $P < 0.001$ (vs OVX group), &#&# $P < 0.001$ (vs OVX+AAV-miR-195-5p). $N = 5$. Scale bar = 50 μ m.

bone diseases such as erosive arthritis, bone metastases, freeze-dried osteolysis and OP (28). In recent years, OP has received increasing attention because of its dramatic increase in the risk of fractures and its significant negative impact on the health status and quality of life of the elderly (29). Therefore, this study constructed an OP model in mice and probed into the specific role and mechanism of miR-195-5p in OP progression. Our data suggested that miR-195-5p improves osteoporosis through activating BMP-2/SMAD/Akt/RUNX2 pathway via targeting SMURF1, which provides some reference for the treatment of OP at the molecular level.

As reported, various miRNAs contribute to OP. For instance, miR-1-3p stimulated osteogenesis and inhibited adipogenesis of bone marrow mesenchymal stem cells (BMSCs) via targeting frizzled-related

protein 1 (SFRP1), thus easing OP development (30). Wang XJ. et al. reported that miR-655-3p targets LSD1 and activates BMP-2/SMAD, thus slowing the progression of OP (31). In addition, miR-143 represses osteoclast formation and elevates osteogenic differentiation by targeting the RANK, NF- κ B and MAPK signaling pathways (32). More importantly, miR-195-5p, a member of miRNAs, can effectively prevent chondrocyte hypertrophy and is considered a candidate molecule for the therapeutic regimen of bone diseases such as OP (33). In osteosarcoma (OS), miR-195-5p overexpression led to inhibitive effects in cell proliferation, migration and invasion of OS cells (34). The above studies suggest that miR-195-5p plays a crucial role in orthopedic diseases. The present study manifested that miR-195-5p is down-regulated in OP tissues

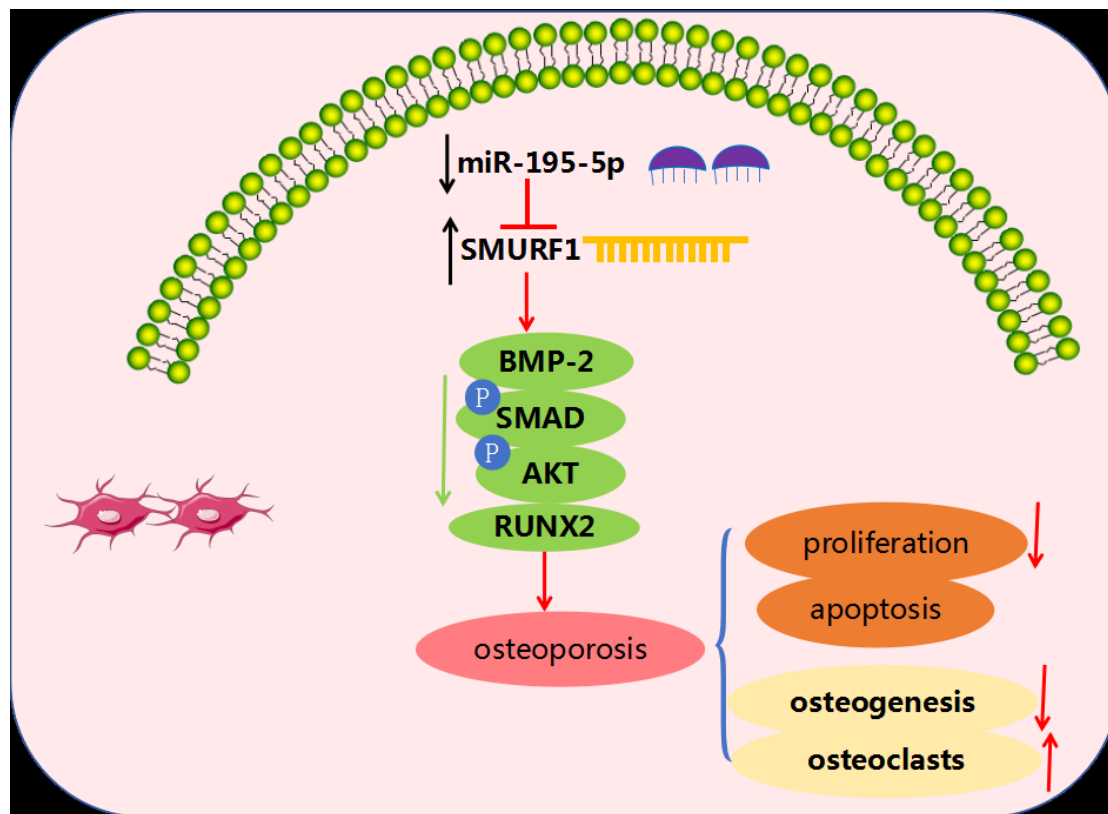


Fig. 6. Diagram of the mechanism. MiR-195-5p targeted SMURF1 and activated BMP-2/SMAD/Akt/RUNX2, thus promoting osteogenic differentiation and bone formation in MC3T3-E1 cells.

and MC3T3-E1 cells subjected to MG environment. Meanwhile, overexpressing miR-195-5p facilitates cell proliferation, inhibits apoptosis and elevates osteogenic differentiation both *in vitro* and *in vivo*. It is believed that miR-195-5p is a promising target in treating OP. However, Chang M et al. reported in 2017 that miR-195-5p is a mechanically sensitive gene that inhibits the expression of WNT3A, FGF2 and BMPR1A in the periodontal tissues of mice under orthodontic mechanical load during osteogenic differentiation induced by the mechanical load of human periodontal ligament cells, thus attenuating the osteogenic differentiation of cells (35). This may be because of the different roles of miR-195-5p in bone cells of different structures.

SMURF1 has been found to play a significant role in anti-virus (36), promoting the growth of glioma (37), and regulating myocardial differentiation (38). In the past few years, the function of SMURF1 in disrupting bone formation has gradually emerged. Studies have revealed that knocking down Cdh1 reduces the activity of SMURF1 and activates the MEKK2 signaling pathway to facilitate osteoblast differentiation (39). Also, Zhang Y. et al. discovered that small molecule compounds targeting the Ub binding region of the HECT domain enhance the activity of osteoblasts by inactivating SMURF1 ligase and stabilizing the protein level of the BMP signaling component SMAD1/5, providing therapeutic strategies for OP and other bone diseases (40). SMURF1, as a ubiquitin regulator, also plays its ubiquitylation-concerned role in some diseases. For instance, SHP-1 ubiquitination mediated by SMURF1 facilitates the proliferation and invasion of endometrial stromal cells via activating the profiles of STAT3 as well as its downstream targets MMP2 and MMP9 (41). More importantly, in bone-associated diseases, TNF induces systemic bone loss by inducing the expression of SMURF1 and promoting the ubiquitination and proteasome degradation of SMAD1 and RUNX2 (42). Jiang Y. et al. revealed that miR-25, secreted by BMSC-derived exosomes, modulates SMURF1-induced ubiquitin degradation of Runx2, thus speeding up the osteogenic differentiation, proliferation, and migration of osteoblasts (43). Similarly, our study illustrated that

SMURF1 was a functional target of miR-195-5p. Inhibiting SMURF1 strengthens the proliferation of MC3T3-E1 cells, relieves apoptosis, and enhances osteogenic differentiation. These results indicated that knocking down SMURF1 promotes osteoblast differentiation, which is consistent with a previous study conducted by Ahmad N. et al (16).

The BMP-2/SMAD/Akt/RUNX2 pathway in promoting osteogenic differentiation has been confirmed by multiple studies (21-23). The miRNA-mediated BMP-2/SMAD/Akt/RUNX2 pathway has also been revealed by several studies. For example, miR-128 upregulates BMP-2/RUNX2 by targeting DKK2 and enhancing the osteogenic differentiation of BMSCs (44). On the other hand, SMAD1/5 is the key molecule of BMP signal transduction. Wang Z. et al. found that Ethinyl lactone prevents bone loss in OVX rat models by activating estrogen- α /BMP-2/SMAD1/5 (45). In addition, the Akt pathway contributes to the biological function of osteoblast proliferation and osteogenic differentiation of MC3T3-E1 cells, and the Akt-mediated signaling pathway can be modulated by the RUNX2 activity (46-47). Furthermore, research by Sun M. et al. showed that miR-19b strengthens the formation of osteoblasts and reduces the osteoporotic bone phenotype in elderly OVX mice by regulating the PTEN/Akt/RUNX2 pathway (48). Similarly, the present research has also confirmed that overexpressing miR-195-5p activates BMP-2/SMAD/Akt/RUNX2, and the increased expression of transcription factors and biomarker genes, as well as proteins in osteoblasts may also be due to the induction of BMP-2/SMAD/Akt/RUNX2 signal transduction.

Overall, this study illustrates that miR-195-5p is down-regulated in OP tissues and cells, and promotes the activation of the BMP-2/SMAD/Akt/RUNX2 pathways by targeting SMURF1 to promote osteoblast differentiation and alleviate OP. It provides an essential molecular reference for the treatment and prognosis of OP. However, the *in-vitro* model of OP was established via employing osteoblasts to MG, which is rather different from the OVX mouse model as OP is triggered by estrogen dampening. This may reflect differences in the two

models and in the molecular mechanism that leads to OP. In addition, the direct role of miR-195-5p on the BMP/SMAD/Akt/RUNX2 pathway and how SMURF1 inactivates this pathway in OP remains elusive. In the future, further studies are needed to confirm the effects of miR-195-5p-SMURF1 axis on BMP/SMAD/Akt/RUNX2 pathway dysregulation in a hormone-induced osteoporosis model.

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Identification of miRNAs, mRNAs, lncRNAs, and circRNAs associated with hepatocellular carcinoma recurrence after interferon treatment

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To study the molecular mechanism of interferon-alpha (IFN- α) in the treatment of hepatocellular carcinoma (HCC) and the molecular markers that can predict the therapeutic effect, differentially expressed (DE)-miRNAs, -mRNAs, -lncRNAs, and -circRNAs were screened between 12 samples collected from 4 patients who had not received treatment (control), 4 patients who had received recombinant human interferon a-2b treatment (case1), and 4 patients who had relapsed after receiving recombinant human interferon a-2b treatment (case2). Enrichment analyses were performed to determine the principal functions of the DE-RNAs. We also constructed protein-protein interactions (PPI) and competing endogenous RNA (ceRNA) networks. In addition, a series-cluster analysis was performed to analyze changes in gene expression across different groups of HCC. Furthermore, the expression of the genes were verified in the Cancer Genome Atlas (TCGA) database. A total of 36 union DE-miRNAs, 175 union DE-mRNAs, 65 union DE-lncRNAs, and 52 union DE-circRNAs were obtained between the control vs case1, and case2 vs case1 groups. DE-mRNAs were mainly involved in the mitochondrial inner membrane. DE-circRNAs were mainly enriched in the Golgi apparatus. ceRNA network contained 68 DE-mRNAs, 26 DE-miRNAs, 45 DE-lncRNAs, and 23 DE-circRNAs. A total of 24 DE-miRNAs, 175 DE-mRNAs, 65 DE-lncRNAs, and 52 DE-circRNAs were classified into eight profiles, respectively. A total of 26 genes showed a significant correlation with prognosis of HCC ($p < 0.05$). Some genes may be used to predict the efficacy of IFN- α in the treatment of HCC. The results may lay a foundation for investigating the different sensitivities of IFN- α in the treatment of HCC.

Hepatocellular carcinoma (HCC) is a common epithelial malignant tumor originating in the liver. HCC has the fifth highest incidence of cancer globally and has the third highest mortality in tumor-related deaths. Thus, it is a disease that seriously affects human health and quality of life (1). The incidence of HCC varies with etiology, race, sex, age, and region.

The main causes of HCC are viral liver disease, alcoholic liver disease, aflatoxin exposure, and other chronic liver diseases, such as hepatitis B virus infection (the most common liver disease in China) (2). At present, the methods of radical treatment of HCC include resection, radiofrequency ablation, and liver transplantation (3). Although approximately

Key words: hepatocellular carcinoma; differentially expressed genes; protein to protein interaction network; competing endogenous RNA; series-cluster analysis

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30% of patients can receive radical treatment, up to 80% of patients experience recurrence of the disease within five years of treatment, with half of these patients having recurrence within two years after treatment (4). Therefore, it is necessary to explore the pathogenesis of HCC and provide new ideas for the treatment and diagnosis of HCC.

Interferon is a cytokine with a wide range of biological activities; it not only has an antiviral effect but can also enhance anti-tumor immune response, inhibit tumor angiogenesis and cell proliferation, and induce cell differentiation and apoptosis (5, 6). It has been reported that interferon-alpha (IFN- α) is one of the drugs that can effectively inhibit the recurrence of HCC metastasis (7). Moreover, IFN- α has also been shown to inhibit the recurrence and metastasis of HCC in animal models (LCI-D20) (8). Further randomized controlled clinical trials have demonstrated that IFN- α can effectively delay early postoperative recurrence and improve overall survival time in patients with HCC. However, the overall efficacy has not been satisfactory, due to the differences in IFN- α sensitivity in the treatment of HCC (9, 10).

The development of tumors is the result of the correlation of multiple genes (11-13). Accumulating evidence has suggested that non-coding RNAs may be involved in various biological processes, including proliferation, apoptosis, invasion, and metastasis. Loss of mir-23b promotes bladder cancer cell migration and invasion (14). A comparison of the transcriptional analysis of breast cancer tissues and normal breast tissues revealed that mir-125b, mir-145, mir-21, and mir-155 were significantly abnormally expressed (15). The increased expression of long non-coding RNA HOX (lncRNA HOX) is related to metastasis and poor prognosis of lung cancer (16). The overexpression of lncRNA PVT1 accelerates the occurrence and development of cancer and reduces the chemical sensitivity of cancer patients. Compared with normal liver tissues, PVT1 is highly expressed in HCC, which accelerates cell proliferation (17, 18). The expression of hsa_circ_0005075 is related to tumor size in HCC (19). Other researchers have found that the expression of hsa_circ_0001649 is different in HCC tissues

and adjacent normal liver tissues; specifically, hsa_circ_0001649 expression is down-regulated in HCC (20). Although HCC metastasis-related genes have been extensively studied and some molecules related to the recurrence of HCC metastasis have been found, there is still no successful molecular target of metastasis for clinical application; therefore, intervention in HCC recurrence and metastasis is still a challenging problem and one that must be solved. In the present study, using transcriptome analysis, we aimed to investigate the different sensitivities of IFN- α in the treatment of HCC and explore the potential therapeutic targets and biomarkers of this interferon, so as to improve its clinical efficacy.

MATERIALS AND METHODS

Patients and sample preparation

A total of 12 HCC patients who had received curative resection at Fudan University Shanghai Cancer Center (Shanghai, China) were enrolled in this study. Among them, four patients had received no postoperative recombinant human interferon a-2b treatment were set as control group. The other eight patients had been injected with recombinant human interferon a-2b after surgery (30 μ g twice a week). Four months after treatment, four of the eight patients who had received postoperative treatment developed tumor recurrence and metastasis (case2 group), while the other four patients displayed no such symptoms (case1 group). The Child-Pugh scoring system was used to evaluate preoperative liver function (21). Tumor differentiation was determined using the method of Edmondson and Steiner (22). The 2010 International Union Against Cancer TNM classification system was used to assess the tumor stage. The clinicopathological characteristics of these patients are shown in Table I. The differences of clinical characteristics of HCC patients in different groups were analyzed using χ^2 tests. Values of $p < 0.05$ were considered statistically significant. This study was approved by the Research Ethics Committee of Fudan University Shanghai Cancer Center (050432-4-1212B). Informed consent was obtained from all the enrolled patients.

RNA extraction and sequencing

Outpatient imaging data was collected from all 12

patients in the study. Once recurrence was evident in any of the patients, peripheral blood mononuclear cell samples were immediately collected from all 12 HCC patients. The diagnosis of recurrence referred to the guidelines of the diagnosis of HCC. Total RNA was extracted with TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the quality was evaluated with reference to NanoDrop ND-1000 (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The integrity of extracted RNA was detected using standard denaturing agarose gel electrophoresis. RNA sequencing was performed using the Illumina HiSeq 2500 Sequencing System.

Sequencing data preprocessing and mapping

Fast-QC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) (23) was used to assess the quality of the sequenced data obtained. Hisat2 software (GRCh38) (24) was used to map the RNA-seq data.

Differential expressed gene screening

EB-Seq algorithm was used to screen counts for differentially expressed (DE)-miRNAs, -mRNAs, -lncRNAs, and -circRNAs in the control vs case1, and case2 vs case1 groups (25). The screening criteria were set as $|\log_2 \text{ fold change (FC)}| > 0.585$ and false discovery rate (FDR) < 0.05 . Ggplot2 (version 3.2.1) and gplots (version 3.0.1.2) of R software were used to draw the volcano and heat maps (26; 27).

Venn analysis

The DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs obtained from the comparison between the control vs case1, and case2 vs case1 groups were subjected to Venn analysis according to the up- and down-regulation, respectively. The union genes were used in the next analysis.

Enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) (28) and Gene Ontology (GO) (29) analysis of union genes were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8, <https://david-d.ncicrf.gov/>) (30). GO analysis includes biological process (BP), molecular function (MF), and cell component (CC). Enrichment results were considered significant when the number of

enriched genes in the parameter count was ≥ 2 , with a $p\text{-value} < 0.05$.

Protein-protein interaction network construction

The Protein-protein interaction (PPI) edges were predicted using String database v11 (<https://string-db.org/>) (31). The input gene set consisted of the two groups of DE-mRNA union genes, and the species was *Homo sapiens*. The threshold was set at a PPI score = 0.4. The PPI network was constructed using Cytoscape (version 3.4.0 <http://chianti.ucsd.edu/cytoscape-3.4.0/>) (32).

Target gene prediction and competing endogenous RNA (ceRNA) analysis

Miranda (score ≥ 150 , energy ≤ -20) (33) and RNAhybrid (Energy ≤ -25) (34) were used to predict the target DE-mRNAs, -lncRNAs, -circRNAs, and -miRNAs. The result of the union of the two algorithms was used as the final target gene prediction result. The target genes prediction result under Miranda and RNAhybrid in a previous step were used to screen ceRNAs. In the process of screening ceRNA, the same regulation of lncRNA/circRNA and mRNA was adopted.

Series-cluster analysis

STEM software (<http://www.cs.cmu.edu/~jernst/stem/>) (Short Time-series Expression Miner) (version 1.3.11) (35) was used to analyze the trend of the DE-mRNAs, -lncRNAs, -circRNAs, and -miRNAs in the union of control vs case1, and case2 vs case1 groups through the changes in the expression over time. The threshold was set at a correlation coefficient of gene expression > 0.7 and $p\text{-value} < 0.05$. The gene annotation source was *Homo sapiens*. Gene modules that clustered significantly in STEM were observed, and genes with consistent expression trends were individually pooled.

Screening of prognostic-related genes

The gene expression RNAseq data of patients with HCC were downloaded from The Cancer Genome Atlas (TCGA) database. We also downloaded the phenotype data through the Genomic Data Commons (GDC) tool from TCGA. In series-cluster analysis, the median expression value of genes whose expression trends were significantly different between the three groups was used to distinguish tumor samples into high-expression group

and low-expression group using R package (version 2.42-6). Log-rank test and Kaplan-Meier (K-M) survival curve were employed to show the different survival probability between gene high expression group and low expression group. $P < 0.05$ was used as criteria.

RESULTS

Clinical data analysis

In this study, we collected the clinical information of all 12 patients including gender,

age, HBsAg, Edmondson grade, tumor size, TNM stage, alpha fetoprotein (AFP), tumor number, tumor encapsulation, and microvascular invasion. The results showed that there were no significant differences in clinical characteristics between the three groups ($p > 0.05$, Table I).

Screening of DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs

The DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs of control vs case1, and case2 vs case1

Table I. The clinical characteristics of 12 hepatocellular carcinoma (HCC) patients.

	Control (N=4)	Case 1 (N=4)	Case 2 (N=4)	P-value
Gender				
Male	4.00 (100%)	4.00 (100%)	4.00 (100%)	1
Age (years)				
<=50	1.00 (25.0%)	0 (0%)	1.00 (25.0%)	0.549
>50	3.00 (75.0%)	4.00 (100%)	3.00 (75.0%)	
HBsAg				
Positive	4.00 (100%)	4.00 (100%)	4.00 (100%)	1
Edmondson grade				
I-II	3.00 (75.0%)	2.00 (50.0%)	2.00 (50.0%)	0.71
III-IV	1.00 (25.0%)	2.00 (50.0%)	2.00 (50.0%)	
Tumor size (cm)				
<=5	4.00 (100%)	3.00 (75.0%)	2.00 (50.0%)	0.264
>5	0 (0%)	1.00 (25.0%)	2.00 (50.0%)	
TNM stage				
I	4.00 (100%)	3.00 (75.0%)	3.00 (75.0%)	0.549
IV	0 (0%)	1.00 (25.0%)	1.00 (25.0%)	
AFP (ng/ml)				
<=20	0 (0%)	0 (0%)	1.00 (25.0%)	0.336
> 20	4.00 (100%)	4.00 (100%)	3.00 (75.0%)	
Tumor number				
Single	4.00 (100%)	4.00 (100%)	4.00 (100%)	1
Tumor encapsulation				
Complete	2.00 (50.0%)	2.00 (50.0%)	1.00 (25.0%)	0.71
Incomplete	2.00 (50.0%)	2.00 (50.0%)	3.00 (75.0%)	
Microvascular invasion				
Absent	4.00 (100%)	4.00 (100%)	4.00 (100%)	1

Note: AFP represents alpha fetoprotein.

groups were screened (Fig. 1). In the control vs case1 group, 23 DE-circRNAs (14 up-regulated and 9 down-regulated), 60 DE-mRNAs (21 up-regulated and 39 down-regulated), 14 DE-lncRNAs (3 up-regulated and 11 down-regulated), and 23 DE-miRNAs (5 up-regulated and 18 down-regulated) were obtained (Fig. 1A). In the case2 vs case1 group, 31 DE-circRNAs

(17 up-regulated and 14 down-regulated), 122 DE-mRNAs (80 up-regulated and 42 down-regulated), 54 DE-lncRNAs (6 up-regulated and 48 down-regulated), and 15 DE-miRNAs (6 up-regulated and 9 down-regulated) were obtained (Fig. 1B).

Union DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs were identified

Venn plots were constructed for the integration analysis of the screened DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs (Fig. 2). For up-regulated DE-miRNAs, 11 union miRNAs were obtained, and there was no intersection between the control vs case1, and case2 vs case1 groups (Fig. 2A). For down-regulated DE-miRNAs, 25 union miRNAs were obtained, and there were two intersections between the control vs case1 and case2 vs case1 groups (Fig. 2B). A total of 94 union up-regulated and 81 union down-regulated DE-mRNAs were obtained according to the Venn analysis (Fig. 2 C, D). For DE-lncRNAs, there were nine union up-regulated and 56 union down-regulated DE-lncRNAs, which included one down-regulated intersection gene (Fig. 2 E, F). For DE-circRNAs, there were 30 union up-regulated genes and 22 union down-regulated genes (Fig. 2 G, H). These union genes were used for further analyses.

GO and KEGG analysis

Up-regulated DE-mRNAs were mainly enriched in mitochondrial inner membrane, endocytic vesicle membrane, and extracellular exosome. Up-regulated DE-mRNAs were mainly enriched in some KEGG pathways, including biosynthesis of antibiotics and influenza A (Fig. 3A). For down-regulated DE-mRNAs, they were mainly enriched in the regulation of transcription and DNA-templated, GO:0003700-transcription factor activity, and sequence-specific DNA binding. Down-regulated DE-mRNAs mainly participated in the hematopoietic cell lineage pathway (Fig. 3B).

For down-regulated DE-circRNAs, these genes were mainly enriched in the Golgi apparatus, unfolded protein binding, and membrane. Mannosidase alpha class 1A member 2 (MAN1A2), UDP-glucose glycoprotein glucosyltransferase 2 (UGGT2), and calnexin (CANX) mainly participated

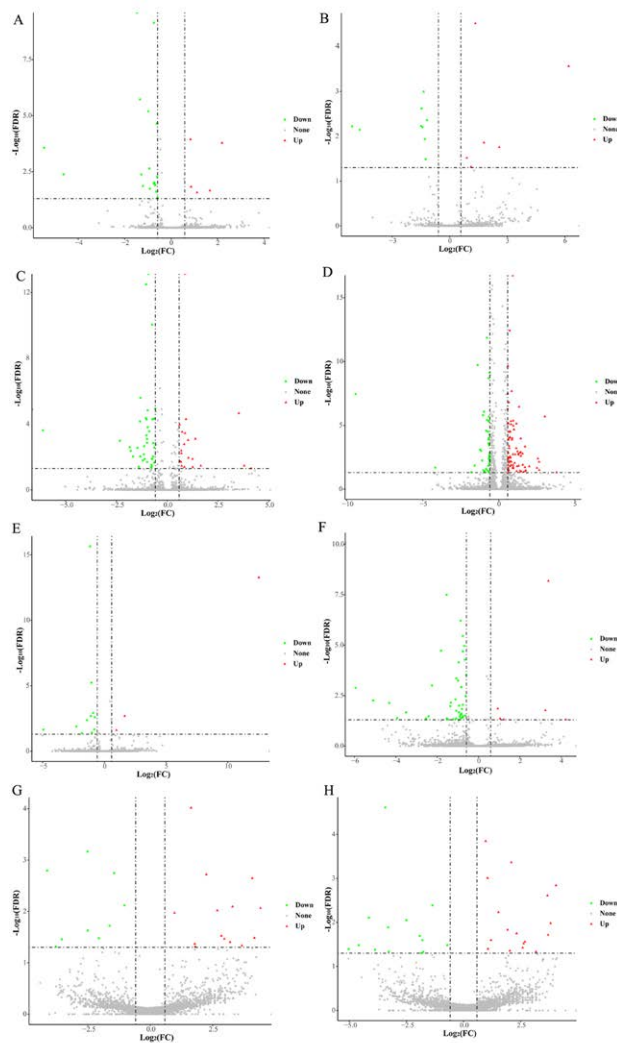


Fig. 1. Volcano plot of miRNA (A, B), mRNA (C, D), lncRNA (E, F), and circRNA (G, H). A, C, E and G represent miRNA, mRNA, lncRNA and circRNA screened between control group and case1 group, respectively. B, D, F, and H represent miRNA, mRNA, lncRNA and circRNA screened between case1 group and case2 group, respectively. Green square represents the down-regulated differentially expressed gene. Red triangle represents the up-regulated differentially expressed gene. Grey dot represents genes with insignificant differences in expression.

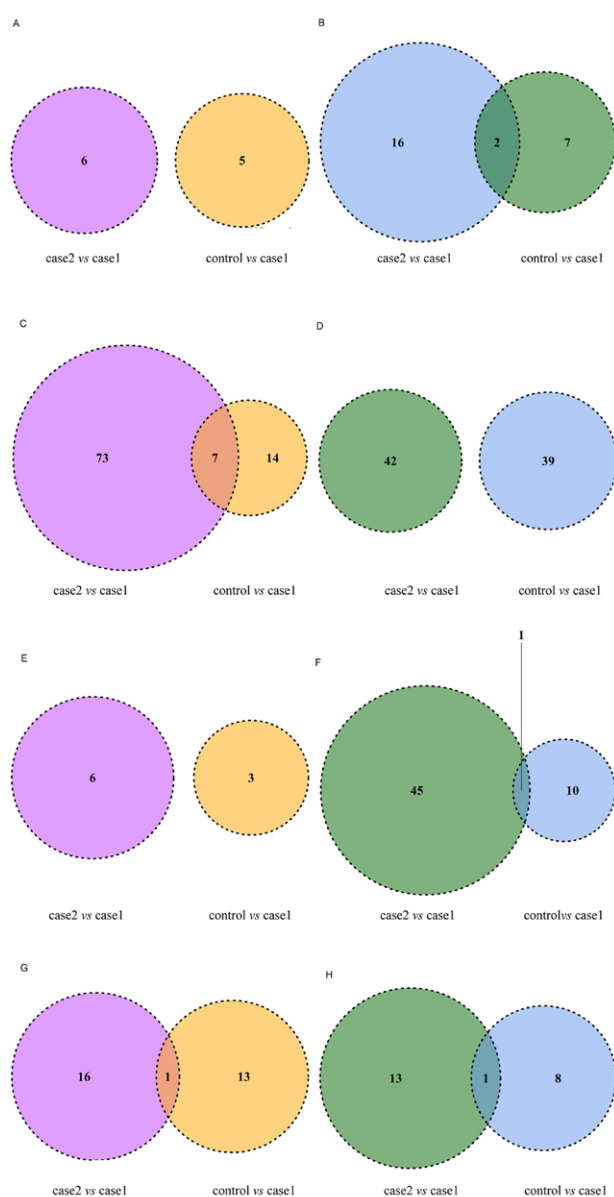


Fig. 2. Venn diagram of differentially expressed-miRNA (A), -mRNA (B), -lncRNA (C), and -circRNA (D). Solid purple circle represents the up-regulated differentially expressed-miRNA (A), -mRNA (B), -lncRNA (C), and -circRNA (D) in the control vs case1 group. Solid yellow circle represents the up-regulated differentially expressed-miRNA (A), -mRNA (B), -lncRNA (C), and -circRNA (D) in the control vs case1 group. Solid blue circle represents the down-regulated differentially expressed-miRNA (A), -mRNA (B), -lncRNA (C), and -circRNA (D) in the case2 vs case1 group. Solid green circle represents the down-regulated differentially expressed-miRNA (A), -mRNA (B), -lncRNA (C), and -circRNA (D) in the case2 vs case1 group.

in protein processing in the endoplasmic reticulum pathway (Fig. 3C). The enrichment results of 22 down-regulated DE-circRNAs showed that they were mainly enriched in clathrin-coated vesicle and endocytic recycling (Fig. 3D).

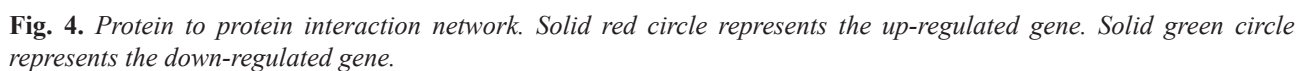
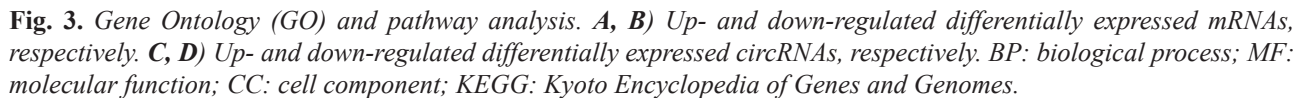
PPI analysis

To explore the relationships between proteins, a PPI network was constructed. There were 100 proteins (66 up-regulated and 34 down-regulated) and 166 edges in the PPI network (Fig. 4). The proteins with the 11 highest values of degree were CD19 molecule (CD19), SRC proto-oncogene, non-receptor tyrosine kinase (SRC), CD22 molecule (CD22), CD68 molecule (CD68), C-X-C motif chemokine ligand 10 (CXCL10), paired box 5 (PAX5), BLK proto-oncogene, src family tyrosine kinase (BLK), CD1d molecule (CD1D), C - X - C motif chemokine receptor 5 (CXCR5), membrane spanning 4-domains a1 (MS4A1), and thymidylate synthetase (TYMS).

Target prediction and ceRNA analysis

Due to the presence of multiple miRNA metal response elements (MREs), miRNAs can bind to mRNA through MRE, leading to the degradation of mRNA or inhibition of its translation. Therefore, miRNA mainly regulates mRNA expression level in a negative regulatory manner. In contrast, lncRNA and circRNA can also adsorb miRNAs, thus affecting the regulation of miRNA on mRNA. Thus, lncRNA and circRNA can be used as ceRNAs. circRNA-miRNA-mRNA, and lncRNA-miRNA-mRNA interaction networks were constructed.

The integrated ceRNA network contained 162 nodes, which included 29 up-regulated DE-mRNAs, 39 down-regulated DE-mRNAs, 11 up-regulated DE-miRNAs, 15 down-regulated DE-miRNAs, 4 up-regulated DE-lncRNAs, 41 down-regulated DE-lncRNAs, 13 up-regulated DE-circRNAs, and 10 down-regulated DE-circRNAs. There were 291 edges in the ceRNA network (Fig. 5). As shown in Fig. 5, hsa-miR-1268a, hsa-miR-3195, hsa-miR-4786-3p, and hsa-miR-5096 were the potential targets of CD22, and these may be regulated by several circRNAs (RAB11FIP1, KIAA1586, FNTA,



and PICALM). Meanwhile, several lncRNAs (BTBD6P1, BMS1P13, BTBD6P1, CCL3P1, LINC00926, LOC100507616, LOC107984113, LOC401312, MCM3AP-AS1, RMRP, and RPS27P3) may also regulate the miRNAs mentioned above.

Series-cluster analysis

A series-cluster analysis was performed to identify potential profiles representing the overall expression patterns of DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs (Fig. 6). Of these, 24 DE-miRNAs, 175 DE-mRNAs, 65 DE-lncRNAs, and 52 DE-circRNAs were classified into eight profiles, respectively. For DE-mRNAs (Fig. 6B), the gene expression trends

included in profile 4 (51 DE-mRNAs) and profile 6 (64 DE-mRNAs) showed significant differences between the three groups (correlation coefficient of gene expression > 0.7 and p -value < 0.05). For DE-lncRNAs (Fig. 6C), 46 DE-lncRNAs were included in the profile 6, and the expression trends of the genes contained in this profile were significantly different between the three groups (correlation coefficient of gene expression > 0.7 and p -value < 0.05).

Prognostic analysis

A total of 386 HCC tumor samples were collected from TCGA. The 161 genes (115 DE-mRNAs and 46 DE-lncRNAs) whose expression trends were

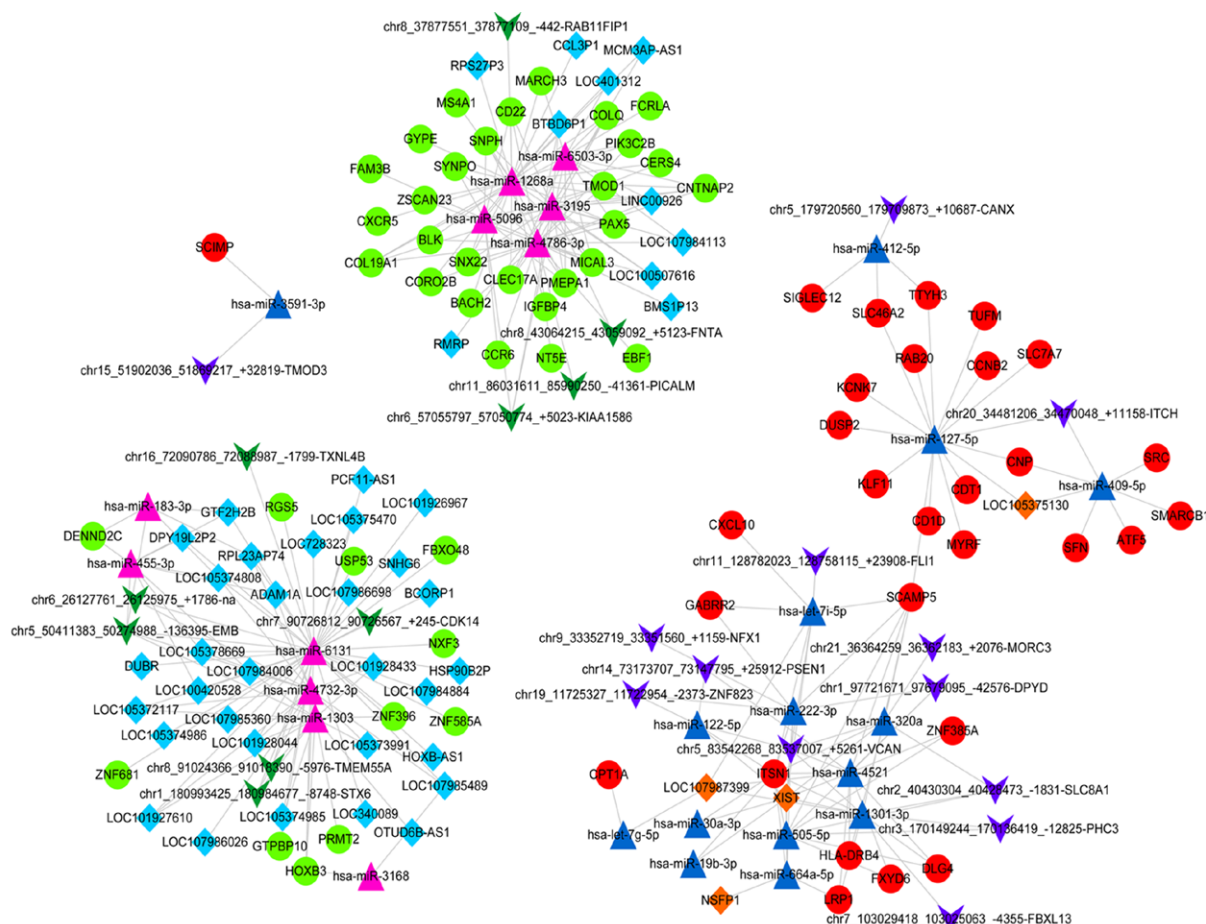


Fig. 5. Competing endogenous RNA network in hepatocellular carcinoma. Red circular node represents up-regulated mRNA. Green circular node represents down-regulated mRNA. Purple inverted triangle node represents up-regulated circRNA. Dark green inverted triangle node represents down-regulated circRNA. Orange diamond node represents up-regulated lncRNA. Blue diamond node represents down-regulated lncRNA. Pink triangle node represents up-regulated miRNA. Dark blue triangle node represents down-regulated miRNA.

significantly different between the three groups were matched to 125 genes in the TCGA database. Based on the prognostic analysis, 26 genes out of 125 genes showed a significant correlation with prognosis of HCC ($p < 0.05$, Fig. 7).

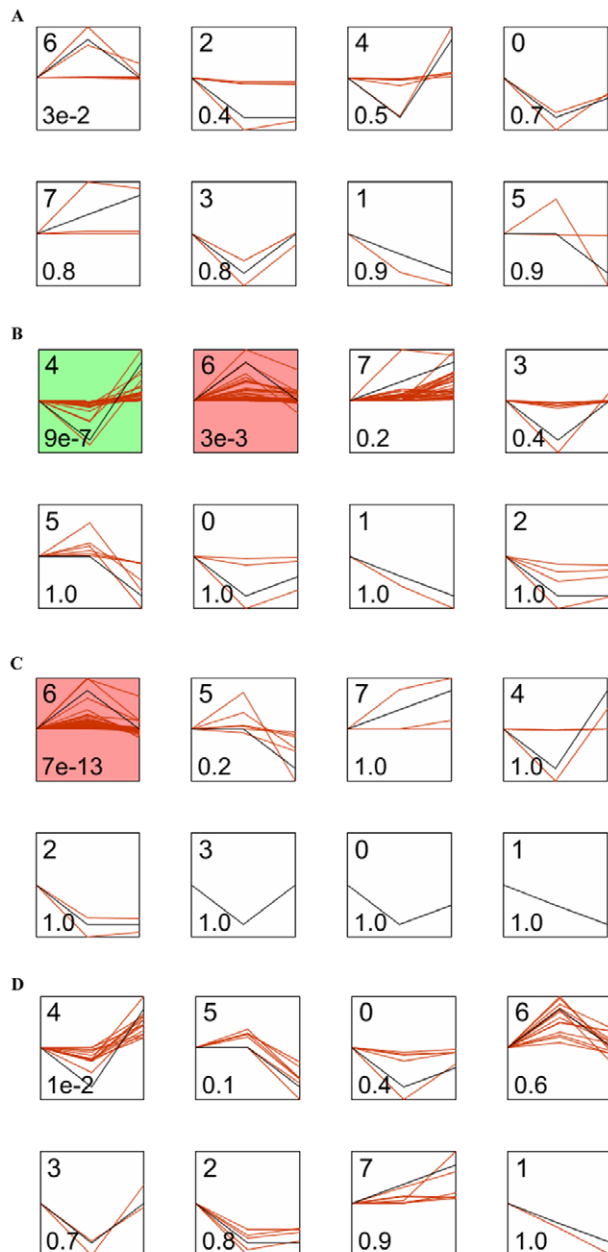


Fig. 6. Series-cluster analysis of differentially expressed-miRNAs (A), -mRNAs (B), -lncRNAs (C), and -circRNAs (D). Profile number is shown at the top left of each square. Square with colored background represents the genes in this profile had a significant variation tendency.

DISCUSSION

HCC is a common malignant tumor that is often clearly diagnosed only in the late stage, at which point the opportunity of surgical resection is lost, resulting in a high fatality rate (36). The recurrence and metastasis of HCC have been the main obstacles to the long-term survival of HCC patients. IFN- α has proven to be effective in the treatment of liver cancer, but its sensitivity varies with different patients (10). It has been reported that the expression of microRNA-26 can predict the effect of IFN- α . Patients with low microRNA-26 expression had shorter overall survival, but better response to interferon treatment than those with high microRNA-26 expression (37). In this study, more biomarkers were screened which could be used as predictors of the efficacy of IFN- α in the treatment of HCC.

We screened several union DE-miRNAs, -mRNAs, -lncRNAs, and -circRNAs between the control vs case1, and case2 vs case1 groups. In the series-cluster analysis, *PAX5* was defined in the profile 6, the expression level of which showed a peak state in the case1 group. The expression level of *PAX5* in patients treated with IFN- α was significantly higher than that in patients not treated with IFN- α , and the expression of *PAX5* in patients with recurrence of liver cancer showed a downward trend compared with patients without recurrence, which indicated that the expression of *PAX5* may be used as a marker to indicate the effect of IFN- α in the treatment of HCC. Enrichment analysis showed that *PAX5* was enriched in humoral immune response term. Several studies have also shown that methylation of *PAX5* is associated with HCC (38-40). Currently, *PAX5* has been reported to be related with leukemia and acute lymphoblastic leukemia (41, 42). We also found that *PAX5* was regulated by hsa-mir-3195, and hsa-mir-3195 was significantly up-regulated in the HepG2 cell line with Bcl-2 over-expression, which was demonstrated to be related to the malignant progression of the tumor (43). MS4A1 was classified in the 6 profile in the series-cluster analysis, which was consistent with the expression trend of *PAX5*. One study reported that MS4A1 is down-regulated in the low immune score of HCC, while the high immune score is significantly associated with

better prognosis in HCC patients (44). Our validation results also showed that high expression of MS4A1 was associated with a better prognosis of HCC. CD22 is mainly expressed in mature B cells, and it is a cell

surface adhesion molecule that performs the function of regulating the activation of B cells, which is helpful for the control of the sensitivity of B cells against the original reaction (45). In the present study, we found

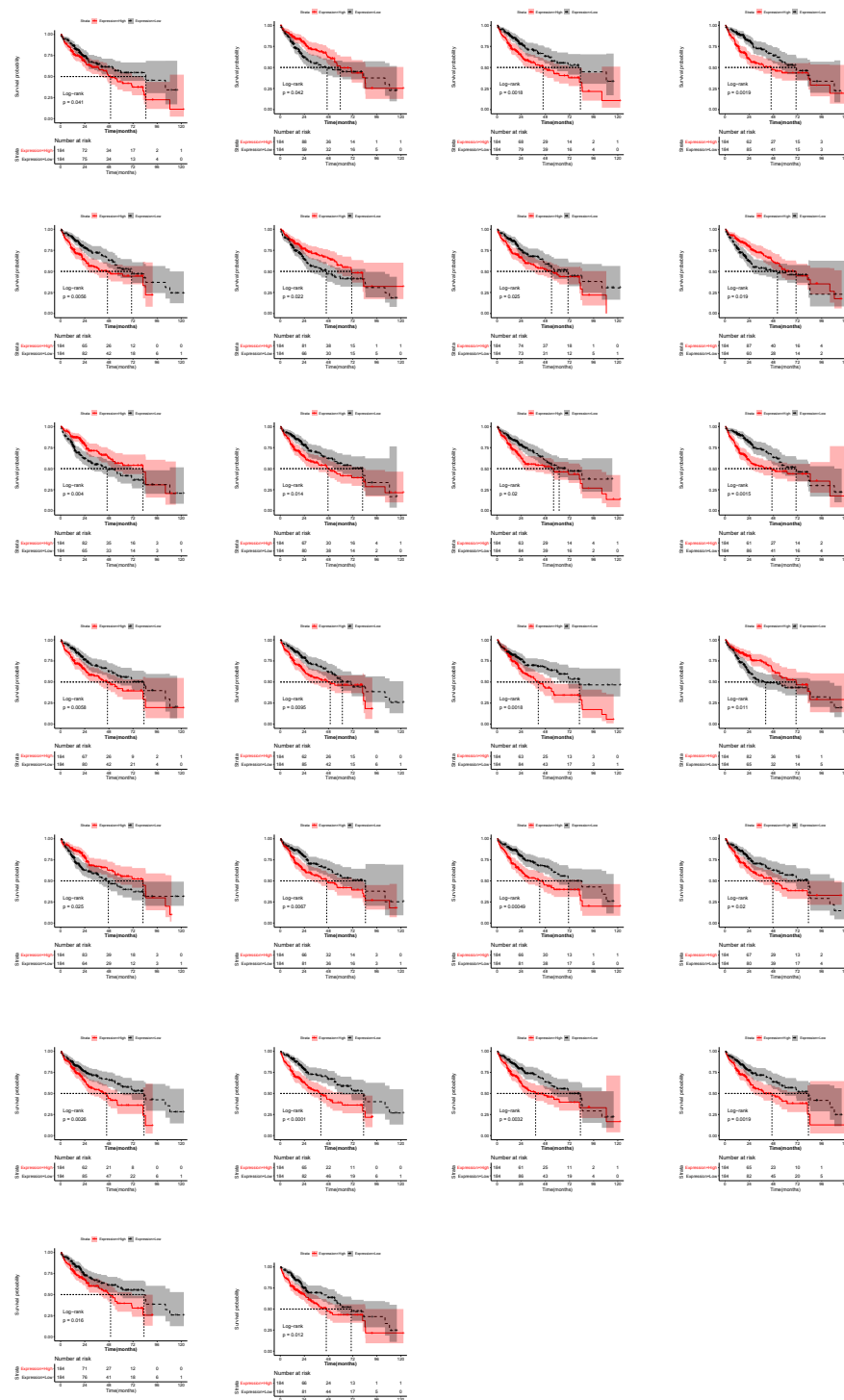


Fig. 7. K-M plot showed the survival probability results of high and low gene expression level in TCGA cohort.

that CD22 was mainly enriched in several pathways, including cell adhesion molecules, hematopoietic cell lineage, and B cell receptor signaling pathway. Another study has shown that *CD22* and sialoadhesin ligands are strongly expressed on the surface of HCC cells (46). In this study, *CD22* was down-regulated in the control compared with the case1 group; that is, the expression of *CD22* in HCC patients was down-regulated after treatment with drugs. However, *CD22* was up-regulated in the blood samples of post-intervention recurrent patients (case2), which was higher than that of the case1 patients (Fig. 6B). We found that *CD22* was regulated by four miRNAs (hsa-miR-4786-3p, hsa-miR-5096, hsa-miR-3195, and hsa-miR-1268a). lncRNAs bind to miRNAs, buffering like a sponge and reducing the function of interfering target mRNAs encoding proteins. In this study, BTBD6P1, LINC00926, LOC100507616, LOC107984113, MCM3AP-AS1, and RMRP could competitively bind to hsa-miR-3195 and regulate the expression of *CD22*. The expression trends of lncRNAs mentioned above and *CD22* were consistent. circRNA RAB11FIP1 could also competitively bind to hsa-miR-3195 and regulate the expression of *CD22*.

To date, many studies have reported the screening of tumor biomarkers related to HCC. Monoacylglycerol lipase (*MAGL*), an important lipolytic enzyme, has been found to be up-regulated in various tumor tissues, such as melanoma and breast cancer (47). It was also found that *MAGL* could promote the development of HCC by inducing the epithelial-mesenchymal transition (48). Heat-shock protein 27 can be detected in the serum of 90% of HCC patients, but not in normal individuals (49).

There are still some limitations to this study. First of all, in general, the tumor size, the number of nodules and other factors are closely related to the risk stratification of cancer, which is related to the risk of tumor recurrence. Therefore, the risk stratification of patients is very important, but there is no consensus on risk stratification at present, which seriously affects the treatment of patients, or whether it is suitable to use IFN- α (50). Secondly, a larger sample size experiment with multi center cooperation is also necessary (51). Thirdly, the

dosage of drugs used by the patients included in this study was the same, and the most appropriate dosage for different patients may be different, which may affect the effect of treatment (52).

In conclusion, bioinformatics methods were used to screen out some markers that may be used to predict the efficacy of IFN- α in the treatment of HCC, which laid the foundation for exploring the molecular mechanism of IFN- α in the treatment of HCC. Further study may also help improve the clinical therapeutic efficiency of this therapy in the future.

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Analysis of long non-coding RNA expression profiles in disuse osteoporosis using microarray and bioinformatics

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Disuse osteoporosis (DOP) is one of the major consequences of long space flights. DOP also occurs in patients with spinal cord injuries and prolonged bedridden states that can have a severe impact on human health. Bone marrow mesenchymal stem cells (BMSCs) are multipotent stromal cells that play an important role in bone homeostasis. Long non-coding RNAs (lncRNAs) are involved in regulating osteogenic differentiation of BMSCs, and their abnormal expression might lead to the formation of orthopedic diseases. However, the specific mechanism of DOP has not yet been elucidated. All sequencing data were obtained from Gene Expression Omnibus (GEO) datasets. The limma package of R was applied to identify DEmRNAs and DElncRNAs. Pearson correlation coefficients (PCC) between DElncRNA-DEmRNA expression levels were calculated. Functional annotation was performed for DEmRNAs co-expressed with DElncRNAs. In addition, the Cytohubba plug-in in Cytoscape was applied to determine the top 10 hub genes. Finally, connectivity map (CMap) analysis was used to identify potential therapeutic drugs for DOP. The gene expression data, GSE100930 and GSE17696, were retrieved from the GEO database. A total of 2,212 differentially expressed mRNAs (DEmRNAs) and 22 differentially expressed lncRNAs (DElncRNAs) were obtained. Gene ontology (GO) functional terms, Kyoto Encyclopedia of Genes, and Genomes (KEGG) pathway enrichment analysis reveal 30 significant GO terms and 13 significant pathways. A coding–non-coding gene co-expression (CNC) network was constructed to study the potential role of hub-DElncRNAs and their co-expressed DEmRNAs in DOP. The lncRNAs, GSN-AS1, SNHG12, and EPB41LA4A-AS1, were significant in the CNC network and potential regulators of DOP development. Three bioactive compounds (scoulerine, kinetin riboside, dexamabiolol) with potential therapeutic significance for DOP were obtained through the Connectivity Map (CMap) analysis. Our study revealed a new mechanism for a lineage shift of bone marrow mesenchymal stem cells under microgravity, and linked the function of protein-coding mRNAs with ncRNAs, which may contribute to the development of new therapies for DOP.

The lack of mechanical loading is related to a range of physiological changes in bones at the whole organism and cellular levels (1). The balance of osteoblasts and

osteoclasts is disrupted at the cellular level under conditions of microgravity and simulated microgravity, resulting in significant bone loss and osteoporosis (2, 3).

Key words: long non-coding RNAs; disuse osteoporosis; bioinformatics; CMAP; BMSCs

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In space flight, the bone loss experienced by astronauts can reach 2% of the total bone mass in one month, which is equivalent to more than one year of bone loss in postmenopausal women (4). Therefore, research under microgravity conditions is useful to reveal the pathogenesis of disuse osteoporosis.

Bone mesenchymal stem cells (BMSCs) are multipotent stromal cells with the ability to self-renew and differentiate into osteoblasts, adipocytes, myoblasts, and chondroblasts (5). Their osteogenic and fat-forming processes are maintained under strict control that stabilizes bone health (6). Therefore, it is critical to study the molecular mechanisms involved in DOP that regulate proliferation, osteoblast differentiation, and adipocyte differentiation of bone marrow mesenchymal stem cells.

Long non-coding RNAs (lncRNAs) are a type of non-coding RNAs with lengths exceeding 200 nucleotides (7). They have been proven to play essential roles in cell differentiation, apoptosis, gene regulation, and carcinogenesis. Recent studies have shown that lncRNAs are involved in regulating osteogenic differentiation of bone marrow mesenchymal stem cells, and their abnormal expression could lead to the onset of orthopedic diseases (8, 9). Li et al. (10) demonstrated that decreased expression of lncRNA H19, which was induced by mechanical unloading, led to the development of DOP through inhibition of Wnt signaling by promoting Dkk4 expression. However, few studies have explored the regulatory mechanism of lncRNA expression in BMSCs under microgravity, and the specific underlying mechanisms remain unknown.

In the present study, we constructed for the first

time an lncRNA regulatory network for BMSCs under microgravity to study the role of lncRNA expression in the molecular mechanisms that regulate BMSC differentiation. Our study revealed a new mechanism that involved a lineage shift of BMSCs under microgravity. This new information could contribute to the development of new therapeutic strategies for bone loss associated with mechanical unloading.

MATERIALS AND METHODS

Affymetrix microarray data

The gene expression dataset, GSE100930, was mined from the Gene Expression Omnibus (GEO) database (Table I). This dataset was created using the platform GPL6244 [HuGene-1_0-st], which was first published by Bradamante S. et al. Six specimens were retrieved, including three flight samples and three ground samples that were used for subsequent analysis. The medium used for the samples contained 1 α , 25-dihydroxy Vitamin D3 (Vit D3) to simulate the *in vivo* environment (11).

Identification of DEmRNAs and DElncRNAs

The R software (version 3.5.2; <https://www.r-project.org>) and Bioconductor software package 3.8 (<https://www.bioconductor.org/>) were used to preprocess the gene expression datasets. R software was used through the Agilent platform to perform preprocessing and normalization of the Series Matrix Files (TXT files).

The limma package of R was applied to identify DEmRNAs and DElncRNAs by performing differential expression analysis between FOM (flight, osteogenic medium) and GOM (ground, osteogenic medium)

Table I. Basic information on the two microarray datasets from GEO

Data source	Platform	Author	Year	Region	Sample size (flight/ground)	Detected RNA type
GSE100930	GPL6244	Bradamante	2019	Italy	3/3	mRNA and lncRNA
GSE17696	GPL6246	Monticone	2010	Italy	3/4	mRNA

conditions. A p-value threshold of 0.05 and log₂ (fold change) greater than 1 were used as the cut-off criteria for this analysis.

Enrichment analysis

Since lncRNAs do not encode proteins, but some encode peptides, the prevalent method for studying their function is “guilt-by-association,” using functional annotation of their targeted coding RNAs (mRNAs). Pearson correlation coefficients (PCC) between

delncRNA-demRNA expression levels were calculated to explore the potential role of DElncRNA and DEmRNA in BMSCs under microgravity conditions. Then, DElncRNA-DEmRNA paired with PCC absolute values ≥ 0.99 and p-values < 0.05 were identified as co-expressed DElncRNA-DEmRNA pairs. The clusterProfiler package of R was used to functionally annotate the DEmRNAs, which were co-expressed with DElncRNAs. Functional and pathway terms from gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used,

Table II. Top 30 significant DEGs in flight samples compared to normal controls.

mRNA	Locus	Strand	log ₂ (fold change)	Regulation	P.Value
BRINP1	chr9:121928907-122131745	-	2.781786667	UP	1.57E-12
CCL20	chr2:228678558-228682280	+	-6.144293333	DOWN	6.92E-14
CDH6	chr5:31193796-31329253	+	4.08031	UP	3.02E-14
CH25H	chr10:90965386-90967071	-	-4.071296667	DOWN	2.00E-14
CLDN1	chr3:190023493-190040222	-	-3.814733333	DOWN	5.27E-13
CXCL1	chr4:74735109-74749385	+	-3.270856667	DOWN	3.33E-13
CXCL2	chr4:74962752-74965010	-	-4.706886667	DOWN	1.56E-12
CXCL8	chr4:74606275-74609433	+	-7.643156667	DOWN	1.62E-13
DDIT3	chr12:57910371-57914300	-	-2.588316667	DOWN	2.22E-12
ERO1LB	chr1:236378855-236445285	-	-3.798856667	DOWN	4.91E-14
FGF7	chr9:44344297-44345687	-	2.967583333	UP	3.30E-12
GALNT1	chr18:33234524-33291794	+	2.55008	UP	1.36E-12
GCH1	chr14:55308723-55369542	-	-4.028006667	DOWN	1.96E-14
GSTM2	chr1:110210667-110252890	+	3.32016	UP	3.64E-12
IL1A	chr2:113531492-113542971	-	-5.558756667	DOWN	3.41E-16
IL1B	chr2:113587328-113594360	-	-4.973856667	DOWN	8.38E-14
ITGA8	chr10:15555948-15762124	-	4.642286667	UP	1.65E-13
LIF	chr22:30636441-30642759	-	-5.675853333	DOWN	9.04E-16
MMP3	chr11:102706528-102714342	-	-4.32811	DOWN	1.10E-14
NAMPT	chr10:36811744-36813162	-	-3.490296667	DOWN	3.98E-12
NREP	chr5:111064509-111093429	-	2.664496667	UP	7.69E-13
PARM1	chr4:75858298-75975610	+	-5.06695	DOWN	3.43E-15
PIEZO2	chr18:10670850-10697814	-	2.87898	UP	7.30E-13
PROS1	chr3:93591957-93692910	-	2.909886667	UP	1.86E-12
PRSS23	chr11:86511562-86522275	+	3.13503	UP	1.36E-12
SERPINB2	chr18:61554939-61571122	+	-5.988626667	DOWN	1.32E-13
SLC19A2	chr1:169432465-169455241	-	-3.065753333	DOWN	1.90E-12
SLC3A2	chr11:62623518-62656353	+	-3.82254	DOWN	2.99E-13
TNFAIP3	chr6:138188458-138204449	+	-4.003913333	DOWN	1.17E-12
TRIB3	chr20:361273-378205	+	-3.385446667	DOWN	4.06E-14

respectively, for the functional and pathway enrichment analysis. A p-value threshold of 0.05 was used as the cut-off value for reporting significant GO terms and KEGG pathways.

Re-construction of the DElncRNA-DEmRNA co-expression network

First, the DElncRNA-DEmRNA co-expression pairs annotated in GO and KEGG were selected. Among these co-expression pairs, the positive and negative pairs were identified based on their PCC values. The DElncRNA-DEmRNA network was visualized using Cytoscape software (version 3.7.2; <http://cytoscape.org/>).

Establishment of PPI network and identification of hub genes

The genes obtained by the above analysis were screened and mapped to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (<https://string-db.org/>), and then visualized using Cytoscape 3.7.2 software. In addition, the Cytohubba plug-in in Cytoscape was applied to determine the top 10 hub genes.

Connectivity Map (CMap) Query

The CMap dataset of cellular signatures catalogs transcriptional responses of human cells to chemical and genetic perturbation. A total of 27,927 perturbagens have been profiled to produce 476,251 expression signatures. After submitting up-regulated and down-regulated genes as up-regulated and down-regulated signatures (<https://clue.io>), so-called connectivity scores were estimated based on pattern-matching algorithms to find functional associations between drugs, genes and diseases through transient characteristics of common gene expression changes. The connectivity score represented the degree of similarity with the expression profile, and bioactive compounds with the lowest connectivity score were selected as potential therapeutic drugs to inhibit the occurrence of disease.

Validation of the Gene Expression Omnibus (GEO) dataset

The GSE17696 dataset was obtained from the GEO (<https://www.ncbi.nlm.nih.gov/geo/>). This dataset consisted of four microgravity samples and four ground samples (Table I). All samples were murine bone marrow stromal cells (BMSC). The dataset was used to validate the observed expression trends of the DEmRNAs.

RESULTS

Differential expression analysis of lncRNAs and mRNAs in BMSCs from flight and ground samples

A total of 2,212 significant DEmRNAs (975 upregulated DEmRNAs and 1,237 down-regulated DEmRNAs) were obtained using a p-value threshold of 0.05 and log2 fold change greater than 1 (Fig. 1 A-B). The top 30 significant DEmRNAs are shown in Table II. Twenty-two DElncRNAs were identified in BMSCs using the aforementioned cut-off criteria, including 12 downregulated and 10 upregulated DElncRNAs (Fig. 1C). The DElncRNAs are reported in Table III.

Enrichment analysis

PCC values were calculated based on the expression levels of 2,212 DEmRNAs and 22 DElncRNAs; 4,251 co-expression pairs were identified using the absolute value of $PCC \geq 0.99$ and a p-value threshold of 0.05. The 1,318 DEmRNAs related to DElncRNAs were used for enrichment analysis. The main functions of the DEmRNAs were analyzed using Gene Ontology Biological Processes (BP), Cellular Compartments (CC), and Molecular functions (MF) terms (Fig. 2). The reticulum stress response, oligosaccharyl transferase activity, and endoplasmic reticulum lumen were the most significantly enriched terms identified based on the enrichment analysis. These processes might be involved in BMSC differentiation.

KEGG pathways have been widely used for the systematic analysis of gene characteristics by combining higher-level pathway information with gene information. Focal adhesion was the most highly enriched pathway among the upregulated DEmRNAs, while protein processing in the endoplasmic reticulum was the most highly enriched pathway among the downregulated DEmRNAs (Fig.3).

lncRNA-mRNA co-expression network

406 DEmRNAs and 18 DElncRNAs underwent additional screening, and the pathway and functional enrichment results were used to construct the coding–non-coding gene co-expression (CNC) network. 1,379 co-expression pairs were obtained, including

595 negative co-expression pairs and 784 positive co-expression pairs (Fig. 4). The network consists of 424 nodes and 1,379 edges. Further analysis of this network revealed that the following lncRNAs were

hubs: GSN-AS1 (degree=197), EPB41L4A-AS1 (degree=195), TP53TG1 (degree=190), MIR155HG (degree=168), RNF185-AS1 (degree=150), and SNHG12 (degree=122).

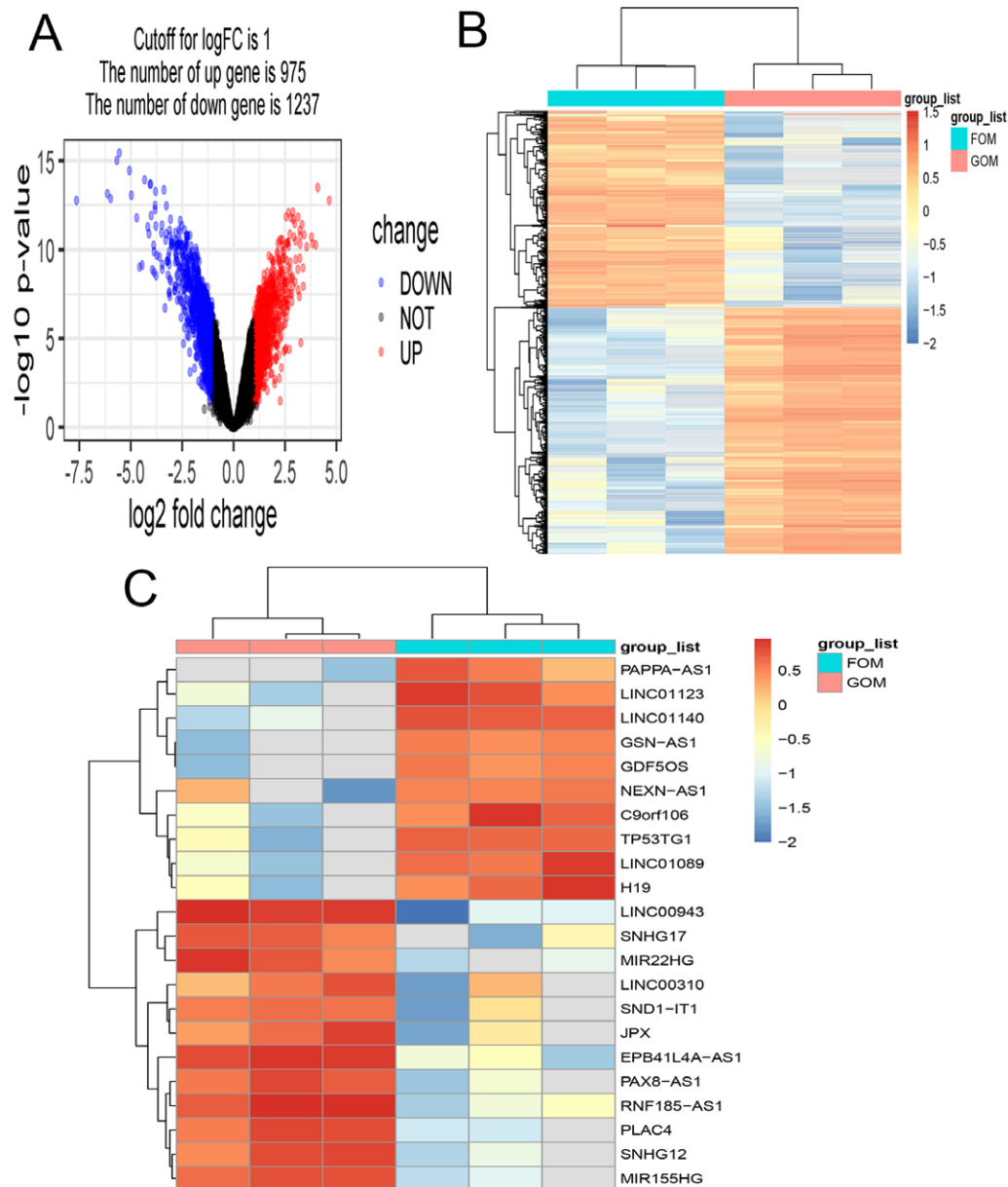


Fig. 1. Overview of aberrantly expressed lncRNAs and mRNAs in flight samples. **A)** The Volcano Plot of mRNA differential expression analysis. Each point represents one mRNA, with red ones representing up-regulation and blue ones representing down-regulation. **B, C)** Hierarchical clustering showing a distinguishable lncRNA or mRNA expression profile between the two groups and homogeneity within groups. Each row represents the gene and each column represents the sample. The red color denotes the degree of upregulation and the blue color denotes the degree of downregulation. FOM represents the flight sample, while GOM represents the ground sample.

Establishment of PPI network and identification of hub genes

Ten hub genes were selected based on Cytohubba plug-in in Cytoscape and ordered by Maximal clique centrality(MCC) values. Further, three key co-expression pairs were screened through hub genes and hub lncRNAs: GSN-AS1/TMED2, EPB41L4A-AS1/BET1, and MIR155HG/BET1.

Identification of three bioactive compounds by CMap analysis

After submitting the top 30 DEmRNAs including 10 upregulated signatures and 20 downregulated signatures into the CMap database, three chemicals

(scoulerine, kinetin riboside, dexamabiol) with the three highest negative enrichment scores were found to provide potential therapeutic targets for DOP (Table IV).

Validation using the GEO dataset

The GSE17696 dataset was firstly preprocessed. One sample (GSM42476) in the microgravity group was determined to be an outlier using Principal Component Analysis (PCA) and removed. Statistical analysis of this dataset validated the directionality of the changes of the hub DEmRNAs, including TMED2, CD59, BET1, COPB2, USO1, COPG1. Differential expression results of the mRNAs under

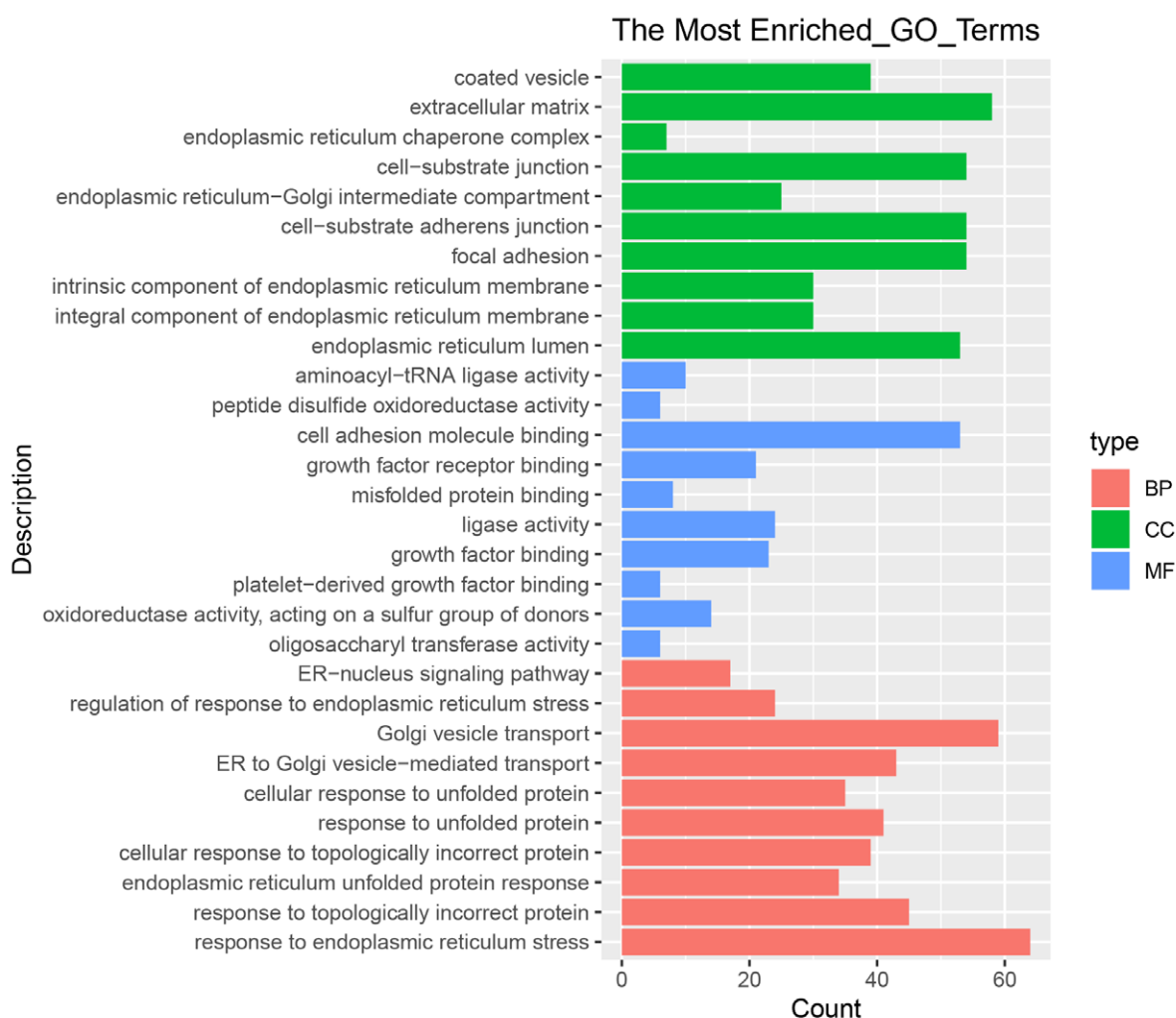


Fig. 2. Gene Ontology (GO) analysis of DEmRNAs co-expressed with DElncRNAs. GO analysis was conducted on three different functional terms types: biological processes (BP), cell compartments (CC), and molecular function (MF). The ordinate represents the enriched GO term, and the abscissa represents the number of enriched DEmRNAs.

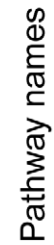


Fig. 3. *Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEmRNAs co-expressed with DElncRNAs. The enriched pathway, represented by red, is composed of upregulated DEmRNAs, while the enriched pathway, represented by blue, is composed of downregulated DEmRNAs.*

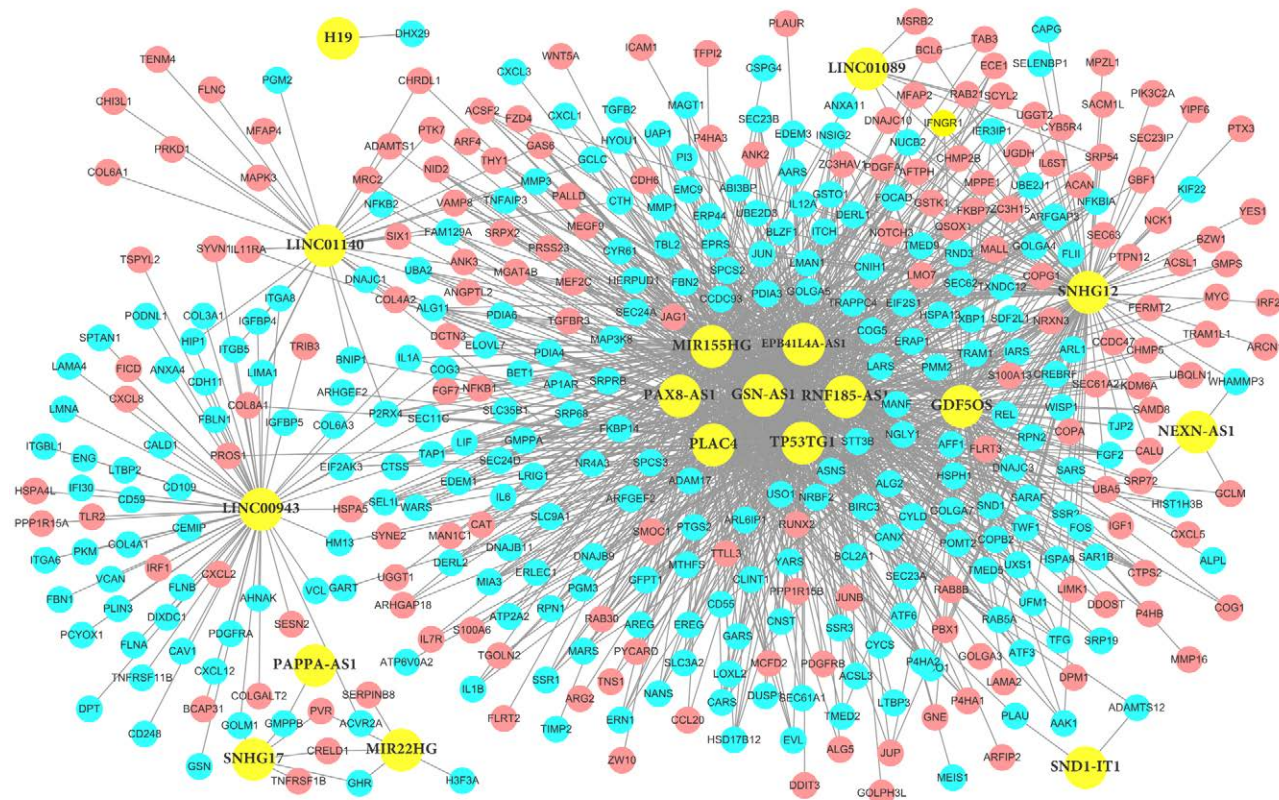


Fig. 4. The CNC network. The co-expression pairs of DElncRNA-DEmRNA annotated in GO and KEGG were selected. Nodes in yellow are DElncRNAs. The red nodes represent the DEmRNAs positively correlated with the DElncRNAs. The blue nodes represent DEmRNAs negatively correlated with DElncRNAs. The lines represent relationships between genes.

Table III. Significant differentially expressed lncRNAs in flight samples compared to normal controls.

LncRNA	Locus	Strand	log2 (fold change)	Regulation	P Value
LINC01140	chr1:87633819-87634887	+	2.335206667	UP	1.66E-09
SNHG12	chr1:28905050-28909495	-	-1.660443333	DOWN	1.66E-06
NEXN-AS1	chr1:78347033-78355224	-	1.068373333	UP	3.15E-05
H19	chr11:2016408-2019072	-	1.048703333	UP	0.000664515
LINC00943	chr12:127210816-127230782	+	-1.770926667	DOWN	1.49E-10
LINC01089	chr12:122233723-122234448	-	1.630683333	UP	1.63E-05
MIR22HG	chr17:1614803-1619505	-	-1.64985	DOWN	1.28E-06
PAX8-AS1	chr2:113993113-114024579	+	-1.499643333	DOWN	9.25E-07
LINC01123	chr2:111140398-111140801	-	1.00176	UP	0.000125041
GDF5OS	chr20:34020827-34023278	+	1.06136	UP	7.19E-06
SNHG17	chr20:37050294-37050422	-	-1.122396667	DOWN	4.73E-07
MIR155HG	chr21:26946292-26946356	+	-1.588626667	DOWN	1.76E-06
LINC00310	chr21:35552978-35555546	+	-1.15529	DOWN	0.000940444
PLAC4	chr21:42548791-42558269	-	-1.170203333	DOWN	2.22E-07
RNF185-AS1	chr22:31601299-31601515	-	-1.551576667	DOWN	7.65E-06
EPB41L4A-AS1	chr5:111496223-111498004	+	-1.172893333	DOWN	1.07E-05
SND1-IT1	chr7:127637562-127640130	+	-1.893763333	DOWN	3.52E-08
TP53TG1	chr7:86954541-86974842	-	1.108993333	UP	9.91E-07
C9orf106	chr9:132083295-132087184	+	1.0361	UP	5.50E-06
PAPPA-AS1	chr9:119160436-119162885	-	1.915926667	UP	1.24E-07
GSN-AS1	chr9:124043046-124047808	-	2.371993333	UP	4.67E-11
JPX	chrX:73219444-73220142	+	-1.50431	DOWN	2.28E-05

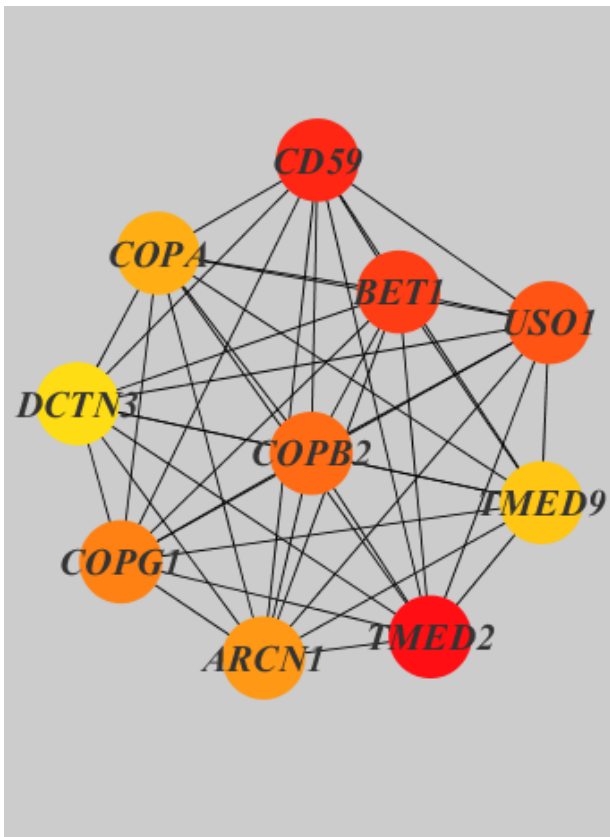


Fig. 5. Identification of hub genes in the PPI network with the Cytoscape plugin cytoHubba.

microgravity revealed no significant mRNA CD59 and downregulation of the remaining mRNAs, which supported our sequencing data (Fig. 6).

DISCUSSION

Disuse osteoporosis is a common disease experienced by spaceflight astronauts and patients with spinal cord injury, and it has been associated with severe damage to human health. LncRNAs in bone diseases, including osteonecrosis of the femoral head (12), knee osteoarthritis (13), and postmenopausal osteoporosis (14), have been studied previously. However, the association of lncRNA expression profiles with mechanical unloading has not been reported. The lncRNA expression profile for BMSCs in DOP was examined for the first time in the present study.

The calculation of correlation coefficients based on the expression levels of lncRNAs and mRNAs is essential to identify potential target genes for lncRNAs (15). Twenty-two significant differentiated lncRNAs were identified based on the differential expression analysis. LncRNAs have greater biological significance when they are associated

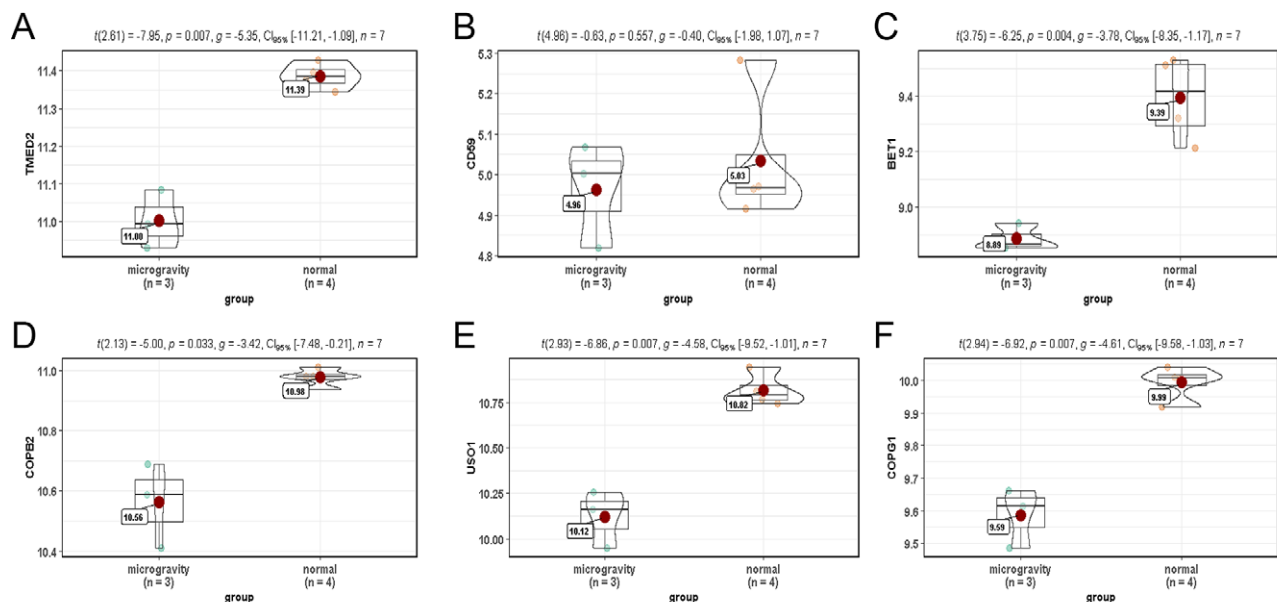


Fig. 6. Validation of selected hub DEMRNAs in GSE17696. The x-axis shows microgravity (case) and normal control groups, and the y-axis shows the level of gene expression. Student's t-tests were used to calculate the significant differences between microgravity (case) and normal control groups. A) TMED2; B) CD59; C) BET1; D) COPB2; E) USO1; F) COPG1.

with more DEmRNAs. Based on the functional and pathway enrichment analysis of the DEmRNAs using GO and KEGG terms, endoplasmic reticulum stress was the most significant GO term and endoplasmic reticulum protein processing was the most significant KEGG pathway term. Previous studies have shown that endoplasmic reticulum stress and its related biological processes play a critical role in the onset of DOP (16-18). Stress on the endoplasmic reticulum (ER) is thought to be related to cellular damage to the protein-folding mechanism, which is responsible for the biosynthesis, folding, assembly, and modification of many soluble and membrane proteins (19). Li et al. reported that endoplasmic reticulum stress could reduce the formation of osteoblasts, increase the number of osteoclasts, and promote their migration and adhesion (16). This finding is consistent with the results of the enrichment analysis in this study. It provides validation of our results and strengthens the reliability of our dataset.

Focal adhesion was significant in both GO and KEGG analyses. Focal adhesion involves a large number of macromolecules through which mechanical forces and regulatory signals are transmitted between the extracellular matrix (ECM) and the interacting cells. More precisely, focal adhesion is a subcellular process that mediates the regulatory role of cells in ECM adhesion, including signaling events (20). It has been shown *in vivo* that

focal adhesion complexes assemble in response to mechanical overload (21, 22). The study by Samarel et al. showed that the focal adhesion pathway plays an important role in heart failure (23). It has been suggested that focal adhesion deserves more attention during the development of DOP, but this observation needs to be verified by future studies. Our findings also revealed the significance of lncRNAs and co-expressed DEmRNAs (hubs) in the constructed co-expression network for DOP.

In the present study, we focused on examining the differentially expressed hub lncRNAs in microgravity. The Gelsolin Antisense RNA 1(GSN-AS1) is a lncRNA located in the q33.2 region of chromosome 9 and containing 4,763 bases. At present, there are few studies on GSN-AS1, but its co-expressed TMED2 has included a large number of studies. Transmembrane emp24 domain-containing protein 2(TMED2), the first hub gene in our study, can promote the proliferation of preosteoblasts and was down-regulated in our DOP model (24). This co-expression pair of GSN-AS1/TMED2 deserves further study. The small nucleoli host gene 12 (SNHG12), also known as LNC04080, is a lncRNA located in the p35.3 region of chromosome 1 (25). It consists of 1,867 bases and encodes four small-sized nucleolar RNAs through its splicing introns (SNORA66, SNORA61, SNORA16A, and SNORD99) (26). SNHG12 has

Table IV. Three compounds with the highest negative connectivity scores in the CMap analysis

Name	ID	Target	Score	MOA
scoulerine	BRD-K62609077	ADRA1D, ADRA2A, GABRA1	-99.37	Adrenergic receptor antagonist, GABA receptor antagonist, Serotonin receptor antagonist
kinetin-riboside	BRD-K94325918	None	-99.19	Apoptosis stimulant
dexanabinol	BRD-A15010982	CNR1,CNR2,GLRA1,GLRA2, GLRA3,GPR55, GRIN1, NFKB1	-98.7	Glutamate receptor antagonist

been previously associated with various cancers, and its mechanisms of action have been related to the regulation of proliferation and metastasis of osteosarcoma through the NOTCH pathway (27). NOTCH3, an important member of the NOTCH family, inhibits the osteogenic differentiation of BMSCs (28). It is noteworthy that the DElncRNA, SNHG12, is highly negatively correlated with the DEmRNA, NOTCH3, in our study (Pearson correlation coefficient ≥ 0.99 , $p\text{-value} < 0.05$). These findings suggest that the lncRNA SNHG12 regulates the proliferation and differentiation of BMSCs in microgravity via the NOTCH signaling pathway. Another DElncRNA, EPB41LA4A-AS1, was highly positively correlated with hub DEmRNA BET1. Previous studies have shown that overexpression of EPB41LA4A-AS1 promotes BMSC proliferation (29), and BET1 can participate in vesicular transport from the endoplasmic reticulum (ER) to the Golgi complex (30). The function of BET1 matches the most significant GO term and KEGG pathway term. Further study of their specific mechanisms of interaction and their possible regulatory roles in BMSCs in microgravity could be an interesting future research direction.

Based on CMap analysis, three chemicals scoulerine, kinetin riboside(KR) and dexamethasone were identified as potential therapeutics for treating DOP. Scoulerine, an isoquinoline alkaloid isolated from *Corydalis* plants, has been reported to possess potent anti-proliferative and pro-apoptotic function in cancer cells (31, 32). In addition, scoulerine also promotes cell viability reduction and apoptosis by activating ROS-dependent ER stress in colorectal cancer cells. The above discussion has shown that ER stress is a key pathway in the development of disuse osteoporosis. Hence, scoulerine could be an important drug for treating DOP. Kinetin riboside, an N6-substituted derivative of adenosine, was initially shown to selectively inhibit the proliferation of cancer cells and induce their apoptosis (33). Dexamethasone (HU-211) is an artificially synthesized cannabinoid derivative that exerts neuroprotective effects through anti-inflammatory and antioxidant effects (34). All of these drugs deserve further exploration and study.

However, there were some limitations noted in

our study. Firstly, the sample size for the BMSCs of the microarray dataset was relatively small, although the sample numbers met the statistical significance of biological duplication. The low sample number could influence the results of the differential expression analysis of the lncRNAs and mRNAs. Secondly, only a few differentially expressed mRNAs were validated. In addition, due to the small number of open data sets that met the research criteria, we chose the sequencing data from *Mus musculus* for verification. Finally, although the CNC networks of the lncRNAs and mRNAs were constructed in this study, the mechanisms of these lncRNAs and mRNAs still need to be confirmed by additional specific studies.

In conclusion, after bioinformatics analysis of gene expression data of BMSCs under microgravity, we constructed the first CNC network of BMSCs under microgravity and revealed the potential role of co-expressed pairs (GSN-AS1/TMED2, SNHG12/NOTCH3, and EPB41LA4A-AS1/BET1) in the regulation of BMSCs under microgravity. Based on the functional enrichment analysis, we hypothesize that after the loss of stress, the dysregulated lncRNAs interfere with signaling pathways that affect the endoplasmic reticulum(ER) stress and focal adhesion, which in turn promote the development of DOP. In addition, based on the above key genes, we identified three bioactive compounds (scoulerine, kinetin-riboside, dexamethasone) with potential therapeutic significance. Our findings provided a new perspective for understanding the pathogenesis of DOP and establishing new roads for finding molecular targets for its treatment.

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Pogostone attenuates traumatic brain injury-induced inflammatory response and neurological damage via regulating NF- κ B and PPAR- γ pathways

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Neuroinflammation occurs after traumatic brain injury (TBI) and induces secondary brain injury. This study was aimed at probing the neuroprotective effects of pogostone on TBI-induced neurological damage and inflammatory response. The TBI model of mice was established with Feeney DM method. The neuroprotective effects of pogostone were determined using modified neurological severity scores (mNSS), rotarod test, brain water content detection, and HE staining. Microglial activation and expression of pro-inflammatory cytokines were also examined to evaluate the anti-inflammatory effects of pogostone. The expression levels of PPAR- γ and NF- κ B were quantified by Western blot and immunofluorescence. We demonstrated that the motor, learning, and memory functions of the mice in the TBI group, compared with those in the Sham group, were significantly decreased, accompanied by increased brain edema, neuronal apoptosis, and microglial activation. With pogostone treatment, the mice had lower mNSS scores and better motor scores compared with the mice in the TBI group. Furthermore, brain edema, neuronal apoptosis, microglial activation, expressions of inflammatory factors [including tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and iNOS] in brain tissue of the mice were inhibited by pogostone treatment, and NF- κ B signaling was significantly suppressed. Moreover, PPAR- γ expression in brain tissues of the mice was up-regulated after pogostone treatment. However, these effects of pogostone were significantly suppressed by PPAR- γ inhibitor T0070907. In conclusion, pogostone can probably ameliorate traumatic brain injury-induced inflammatory response and neurological damage via regulating NF- κ B and PPAR- γ pathways, and it is a promising neuroprotective drug.

Traumatic brain injury (TBI) is identified as a severe disease, which may cause disability and death (1). Following the primary damage occurring at the time of the injury, secondary injury mediated by pathological processes such as post-traumatic ischemia

and hypoxia, abnormal status of calcium channels, and lipid peroxidation, usually lasts for days, months, or even years, thus leading to a variety of neurological dysfunctions (2). The excessive inflammatory responses figure prominently in secondary injury

Key words: traumatic brain injury (TBI); pogostone; PPAR- γ ; NF- κ B; inflammatory response

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of TBI (3). For instance, the expression level of pro-inflammatory factors, tumor necrosis factor- α (TNF- α), interleukin(IL)-1 β , and IL-6 included, are usually up-regulated in the lesions, which can further cause astrogliosis, neuron death, blood-brain barrier destruction, brain edema, and other pathological changes. TNF- α can activate resting microglia or astrocytes, and contribute to the inflammatory response of the central nervous system (CNS) (4). IL-1 β partakes in a variety of pathological processes such as tissue destruction and edema formation (5). Moreover, IL-6 is a pleiotropic cytokine which can aggravate the inflammation (6). Inhibiting the expression of pro-inflammatory factors following TBI can significantly reduce TBI-mediated neurological impairment.

NF- κ B signaling features prominently in regulating expression of inflammatory genes, cell survival, and neuronal differentiation in the CNS (7). RelA (p65), RelB, c-Rel, p105/p50, and p100/p52, five members of this family, form homodimer or heterodimers (8). Under the inactive state, NF- κ B is retained in the cytoplasm by inhibitory protein κ B (I κ B). Upon activation, NF- κ B is phosphorylated and translocated into the nuclei, promoting transcriptional regulation of the target genes (7, 8). TNF- α and IL-1 β can not only cause inflammatory damage, but also further activate NF- κ B, leading to the formation of inflammatory cascade (9).

Peroxisome proliferator-activated receptors (PPARs), a class of ligand-dependent transcription factors, belong to the nuclear hormone receptor superfamily (10). PPARs have many biological effects, such as promoting adipocyte differentiation and lipogenesis, enhancing the body's sensitivity to insulin, inhibiting inflammatory factor production (11). PPARs have three subtypes, PPAR- α , PPAR- β , and PPAR- γ , among which PPAR- γ is a ligand-activated nuclear receptor that affects the pathogenesis of many diseases, including diabetes, atherosclerosis, and airway inflammation. PPAR- γ activation reduces neuroinflammation and exerts direct neuroprotection after CNS injury (12). During the development of brain injury, PPAR- γ acts as the primary gatekeeper controlling cytoprotective stress responses, increasing cell survival and recovery of homeostasis. Reportedly, PPAR- γ reduces neuroinflammation by suppressing NF- κ B pathway and activating Nrf2/ARE axis to

counteract oxidative stress and ameliorate tissue injury (9). Furthermore, the activation of PPAR- γ promotes the repair of gray matter and white matter, reconstruction of neurovascular units, regression of inflammation, and long-term functional recovery (13).

Pogostemon cablin (Blanco) Benth. (Labiatae), as a traditional Chinese medicine, has been used for the treatment of fatigue, thermoplegia, etc., in China (14). Its main bio-active component is pogostone, which has anti-inflammatory effects (15). Reportedly, pogostone can attenuate carrageenan or protein-induced mouse foot swelling and has a strong analgesic effect on pain caused by physical and chemical stimulation and a certain antipyretic effect on rat fever induced by 2,4-dinitrophenol (16). However, the therapeutic effect of pogostone on CNS disease warrants further investigation. In this study, we investigated the therapeutic effects and mechanisms of pogostone on neurological impairment in TBI mice. Our study found that pogostone could significantly promote neurological recovery in TBI mice by inhibiting brain edema, neuronal apoptosis, microglial activation, and excessive inflammatory response following TBI, which was mediated by its regulatory functions on PPAR- γ and NF- κ B pathways.

MATERIALS AND METHODS

Animal grouping, drug administration and establishment of TBI model

All experiments were by approved by the Ethics Review Board of Hubei University of Science and Technology, and the Animal Ethics Committee of Chongqing Red Cross Hospital. (Approval Number: 2018952). Eighty male C57BL/6 mice (8-12 weeks old, 18-22 g) were randomly divided into four groups: Sham group (Sham), TBI group (TBI), TBI + pogostone group (TBI + Po), and TBI + pogostone + PPAR- γ inhibitor group (TBI + Po + T007). T0070907 (T007) [purity of 99.34%; concentration of 20 μ M; 2 mg/kg body weight (17)] was employed as the inhibitor of PPAR- γ . Pogostone (20 mg/kg body weight) was administrated via intraperitoneal injection within 0.5 h after TBI.

The animals were raised in a temperature-controlled environment (22 $\pm$ 3°C) with 12 h light/dark cycle and free access to chow and water. A mouse model of controlled cerebral injury was established by modified Feeney DM

free fall epidural impact method: after pentobarbital sodium anesthesia (50 mg/kg), the skin was cut 2 mm behind the lateral coronal suture and 2 mm beside the midline, and a bone hole with a diameter of 5 mm was drilled, and the dura mater remained intact, and the 30 g weight hammer fell freely from the impact rod (height: 20 cm) to induce the mouse craniocerebral injury (the injury depth was about 3 mm). After that, bone wax was utilized to close the bone hole, and the scalp was sutured. The mice in the Sham group only had the drilled bone hole without hammering, and the TBI + pogostone group was administered 0.5 h after TBI. The mice in the TBI + pogostone + PPAR- γ inhibitor group was simultaneously injected with pogostone and PPAR- γ inhibitors (T0070907). The TBI group was injected with an equal dose of normal saline.

Measuring neurological injury

Modified Neurological Severity Score (mNSS) was employed to examine neurological function of the mice. The higher score indicated the severe neurological impairment. The neurological function was measured at 1, 3, 7, 14, and 28 days, respectively, after TBI. The mNSS is a neurological function scoring system, including motor, sensory, reflex, and balance tests. The mNSS score is graded from 0 to 18, and the scores represented neurological defects: 13-18 for severe injury, 7-12 for moderate injury, 1-6 for mild injury, and 0 for normal status.

Rotarod test

Mice received 2-day training before TBI, with a rotating rod which was gradually accelerated from 5 rpm to 45 rpm in 5 min. During this process, the latency of the fall was recorded when the mouse fell off the rod. The latency at 3, 7, 14, and 28 days, respectively, was examined after TBI.

Brain water content measurement

Wet-dry method was employed to detect brain water content of the mice. The mice in different groups were euthanized and then the brain was immediately removed. Subsequently, 200 $\pm$ 20 mg of cerebral cortex that was 2 mm from the edge of the bone window was chosen. The wet weight of the tissue was firstly weighed on the electronic analytical balance, and after that, the tissues were dried at in an oven for 24 h before the dry mass was measured. Brain water content (%) = [(wet mass - dry mass)/wet mass] $\times$ 100%.

Hematoxylin-eosin staining (HE) staining

Brain tissues were taken and fixed with 4% paraformaldehyde, immediately frozen and then sectioned using a freezing microtome (Leica, Solms, Germany). Coronal sections (20 μ m) were prepared and then placed on a glass slide in an oven at 37°C for 5-6 h to allow the sections to adhere closely. The sections were then treated with xylene and gradient ethanol, and washed with PBS. Subsequently, the sections were soaked with hematoxylin staining solution for 3 min and rinsed with water for 1 min. Next, they were soaked in hydrochloric acid differentiation solution for 2 s, rinsed with water for 5 min, soaked with eosin staining solution for 2 min, and washed with tap water for 30 s. After being soaked in 95% alcohol and absolute ethanol, neutral resin was added, and the sections were observed and evaluated under a microscope by two independent pathologists.

Immunohistochemistry (IHC)

Deparaffinized coronal sections of brain tissues (20 μ m) were placed in 3% H₂O₂ for 5 min to inactivate the endogenous enzyme and then washed 3 times with distilled water. Subsequently, the sections were immersed in 0.01 M citrate buffer, heated to boiling in a microwave oven, and the heating lasted 5 min. The above steps were repeated 2 times. Next, the sections were incubated with 5% BSA blocking solution at 37°C for 30 min, after which diluted anti-Iba1 antibody (1:100, Abcam, Cambridge, UK) was added and the sections were incubated overnight at 4°C. After being rinsed with PBS for 5 min 3 times, the sections were incubated with goat anti-rabbit H&L (HRP) (1:500; Abcam, Cambridge, UK) secondary antibody at 37°C for 30 min. With DAB employed as a chromogenic agent, counterstaining was performed with hematoxylin. Subsequently, the number of positively immunostained cells in each section was counted in 5 visual fields under a microscope, and the staining intensity was examined by two independent pathologists.

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) staining

Neuronal apoptosis was examined by the TUNEL kit (Maiyol Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. With permeabilization solution (0.1% Triton X-100, 1% sodium citrate), brain sections were permeabilized for 2

min on ice, followed by being incubated with 50 ml of TUNEL reaction mixture (5 ml of enzyme solution and 45 ml of label solution) at 37°C for 60 min. After being rinsed with PBS, the specimens were incubated with 1 µg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining solution (Beyotime Biotechnology, China) for 15 min at ambient temperature. Fluorescence signals were obtained through a fluorescence microscope. Five fields of view were randomly selected around the injury site, and the numbers of TUNEL-positive cells and total cells were counted.

Western blotting

The brain tissues were homogenized and lysed with RIPA buffer (Beyotime, Shanghai, China) and the total protein was extracted after centrifugation. The protein concentration was measured by BCA method. After denaturation, the protein samples were separated on 12% SDS-polyacrylamide gel electrophoresis for 60 min and then transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). After that, the membranes were blocked using 5% skim milk at ambient temperature for 2 h and then incubated with diluted anti-β-actin antibody (ab8226, 1:1000, Abcam, Cambridge, UK), anti-Caspase-3 antibody (ab32351, 1:1000, Abcam, Cambridge, UK), anti-NF-κB p65 antibody (ab207297, 1:1000, Abcam, Cambridge, UK), and anti-PPAR-γ antibody (ab41928, 1:1000; Abcam, Cambridge, UK), respectively, at 4°C overnight. After the membranes were washed with TBST for 10 min 3 times, horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (ab205718, 1:500; Abcam, UK) was added to incubate with the membranes at room temperature for 1 h. Next, the membranes were washed with TBST for 10 min 3 times. Enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Scientific, Rockford, IL, USA) was employed to visualize the bands. ImageJ software (National Institutes of Health, USA) was used to quantify the gray intensity of the protein bands.

Enzyme-linked immunosorbent assay (ELISA)

The expression levels of inflammatory factors IL-1β, IL-6, and TNF-α in brain tissue were quantified by ELISA kit (Boster Biotech, Wuhan, China). After the tissues were homogenized and centrifuged, the supernatants were transferred into 96-well plates coated with primary antibodies, and incubated for 2.5 h and then rinsed. After biotinylated antibodies were added into every well, and

the supernatants were incubated with HRP-conjugated streptavidin and chromogen reagent, the absorbance at 450 nm of each well was measured using a microplate reader (Synergy2, BioTek, USA). Then the levels of IL-1β, IL-6, and TNF-α were quantified according to the standard curve.

Statistical analysis

All measurement was performed three times. The data was processed employing the SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and t-test was utilized to compare differences between two groups. The difference was statistically significant at $P < 0.05$.

RESULTS

Pogostone promoted neurological recovery of TBI Mice

To pinpoint the neuroprotective effects of pogostone on TBI mice, a TBI mouse model was established, the findings of which showed that the mNSS score of the mice in the TBI group, compared with that in the Sham group, was significantly increased, and the rotation test time was markedly reduced. Moreover, HE staining showed an increase in the number of apoptotic neurons, and the water content in the brain tissue of the mice was observably increased. After pogostone treatment, mNSS scores, neuronal apoptosis and water content of the brain tissue were reduced, and the latency to fall was longer, compared with the TBI group. After the mice were treated with T0070907, the effects of pogostone were reversed (Figs. 1-2).

Pogostone attenuated TBI-induced neuronal apoptosis

To further clarify the effect of pogostone on TBI-induced neuronal apoptosis, IHC and Western blot were used to detect the apoptotic markers in the brain tissues of the mice. It was shown that the number of TUNEL positive cells and caspase-3 protein expression in TBI group were observably higher than those in Sham group, which were both inhibited by the treatment of pogostone; after the T0070907 treatment, compared with TBI + Po group, the number of TUNEL positive cells and caspase-3 protein expression were markedly increased (Fig. 3).

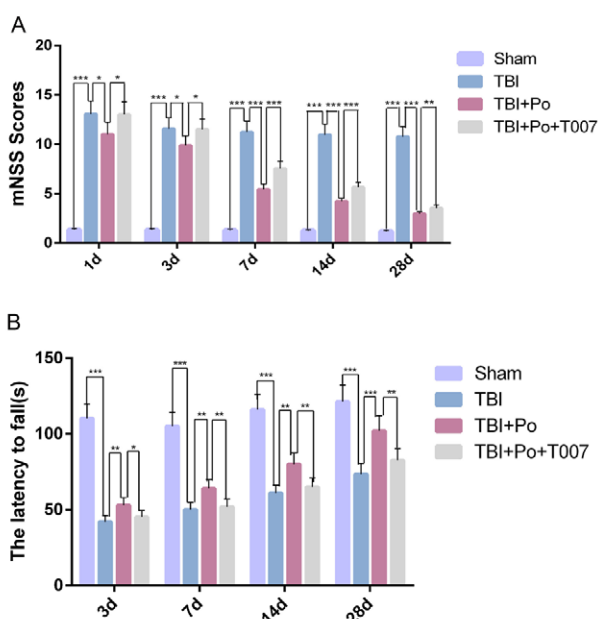


Fig. 1. Pogostone could significantly improve the neurological function of TBI mice. **A)** The neurologic function of the mice in each group was assessed by mNSS. **B)** The locomotion ability of the mice in each group was measured by Rotarod test. All experiments were performed in triplicate. The quantitative data were expressed as the mean $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NS: $P > 0.05$.

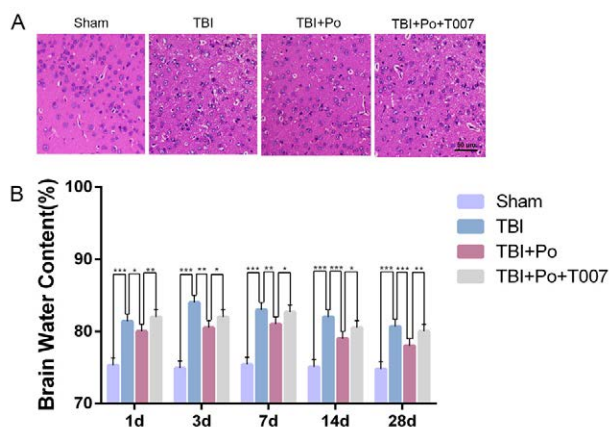


Fig. 2. Pogostone could attenuate brain edema after TBI in mice. **A)** HE staining was used to evaluate the morphological changes of the neurons in the brain of the mice. Bar=50 μ m (40x). **B)** Brain tissue water content of the mice in each group was measured by dry-wet method. All experiments were performed in triplicate. The quantitative data were expressed as the mean $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NS: $P > 0.05$.

Pogostone inhibited the proinflammatory response of microglia

To elaborate the effects of pogostone in regulating TBI-induced inflammation, IHC and ELISA were performed to detect microglial activation. It was elucidated that the number of activated microglia in the TBI group was dramatically higher than that in the Sham group, accompanied by up-regulation of the expression levels of pro-inflammatory factors including TNF α , IL-1 β and IL-6; with the treatment of pogostone, the number of activated microglia and the levels of pro-inflammatory factors were decreased compared with the TBI group, however, those effects were eliminated by T0070907 (Fig. 4).

Pogostone inhibited the expression of NF- κ B via regulating PPAR- γ

As PPAR- γ figures prominently in TBI-induced microglial activation and subsequent inflammatory responses, immunofluorescence and Western blot were used to investigate the expressions of PPAR- γ and NF- κ B in the lesions. The total protein expression levels of PPAR- γ and NF- κ B in the TBI group were found to be significantly higher than those in the Sham group, but the nuclear translocation of PPAR- γ was not obvious. With the treatment of pogostone, the expression of NF- κ B protein was inhibited while the total protein expression and nuclear translocation of PPAR- γ were significantly increased; however, PPAR- γ inhibitor not only increased the expression of NF- κ B but also decreased the expression of PPAR- γ protein and nuclear translocation compared with that of the TBI + Po group (Fig. 5).

DISCUSSION

TBI is regarded as a major cause of death and disability (18). The inflammatory response is a leading cause of secondary injury following the primary injury of TBI, which is seriously life-threatening. In view of this, using a mouse model, the present study explored the therapeutic effect of pogostone on neurological damage after TBI and the regulatory effect on inflammatory response. Our results indicated that pogostone could not only attenuate neurological dysfunction, cerebral edema,

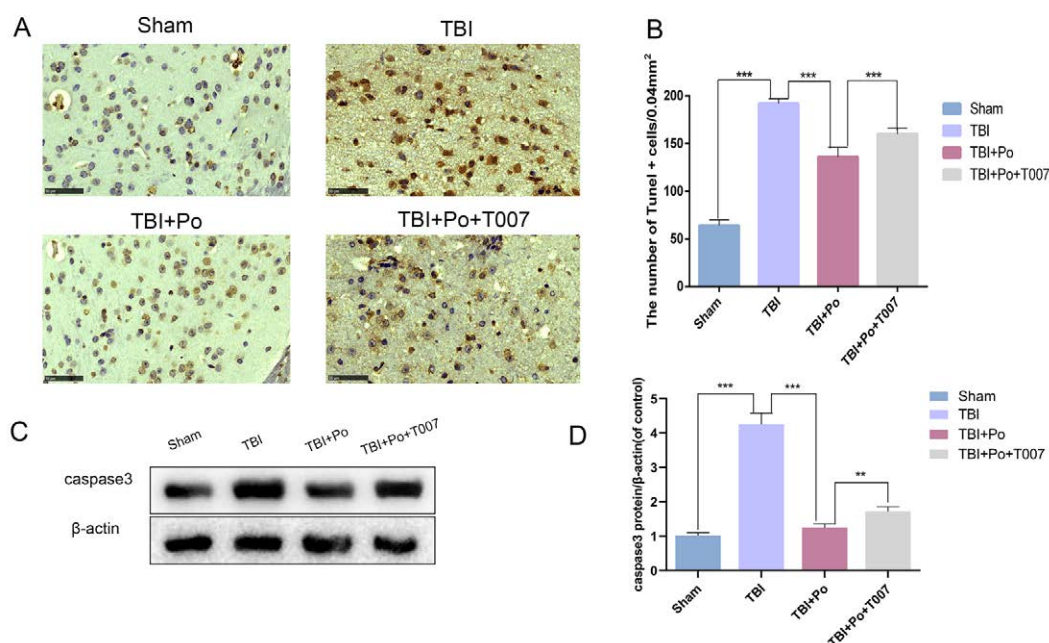


Fig. 3. Pogostone could effectively inhibit the apoptosis of neurons. **A, B)** Immunohistochemistry was used to detect the expression of caspase-3 in neurons to examine neuronal apoptosis, and statistical analysis were made. Bar = 50 μ m (40x) **C, D)** Western Blot method was used to detect the expression of caspase-3 in the brain tissues of the mice in each group. All experiments were performed in triplicate. The quantitative data were expressed as the mean $\pm$ S.D. *** $P < 0.001$, NS: $P > 0.05$.

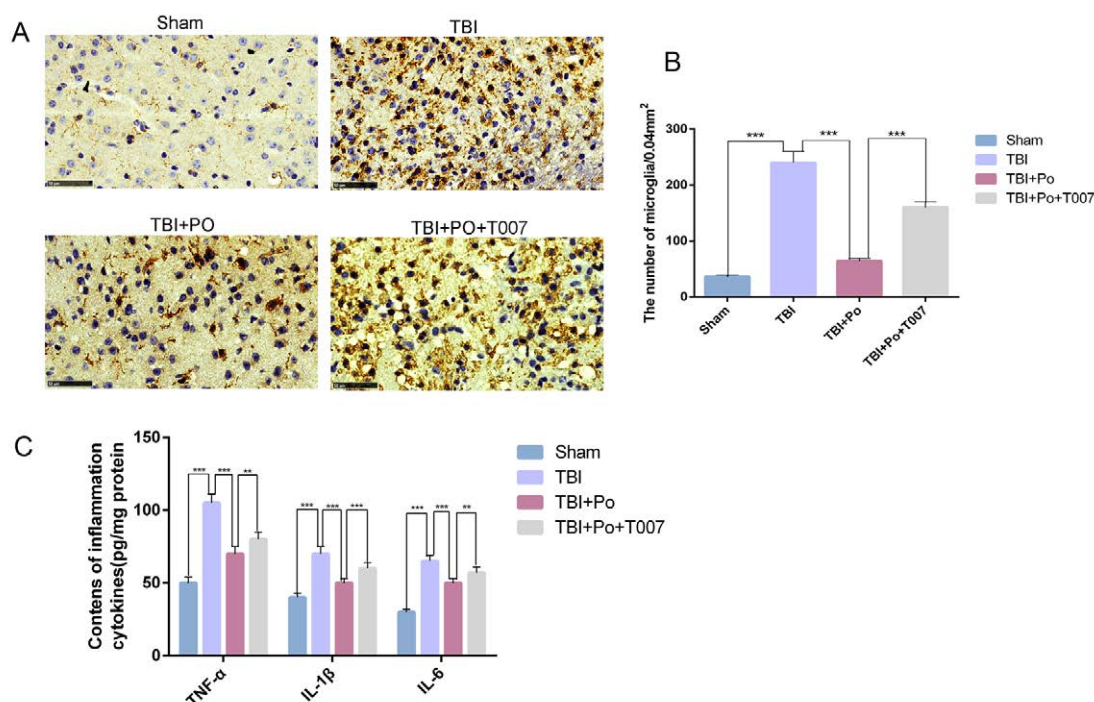


Fig. 4. Pogostone could effectively inhibit inflammatory reaction of microglia. **A, B)** The microglia (marked by Iba1) were detected by immunohistochemistry. Bar = 50 μ m (40x). **C)** The levels of inflammatory factor IL-1 β , IL-6, TNF- α were detected by ELISA. All experiments were performed in triplicate. The quantitative data were expressed as the mean $\pm$ S.D. ** $P < 0.01$; *** $P < 0.001$, NS: $P > 0.05$.

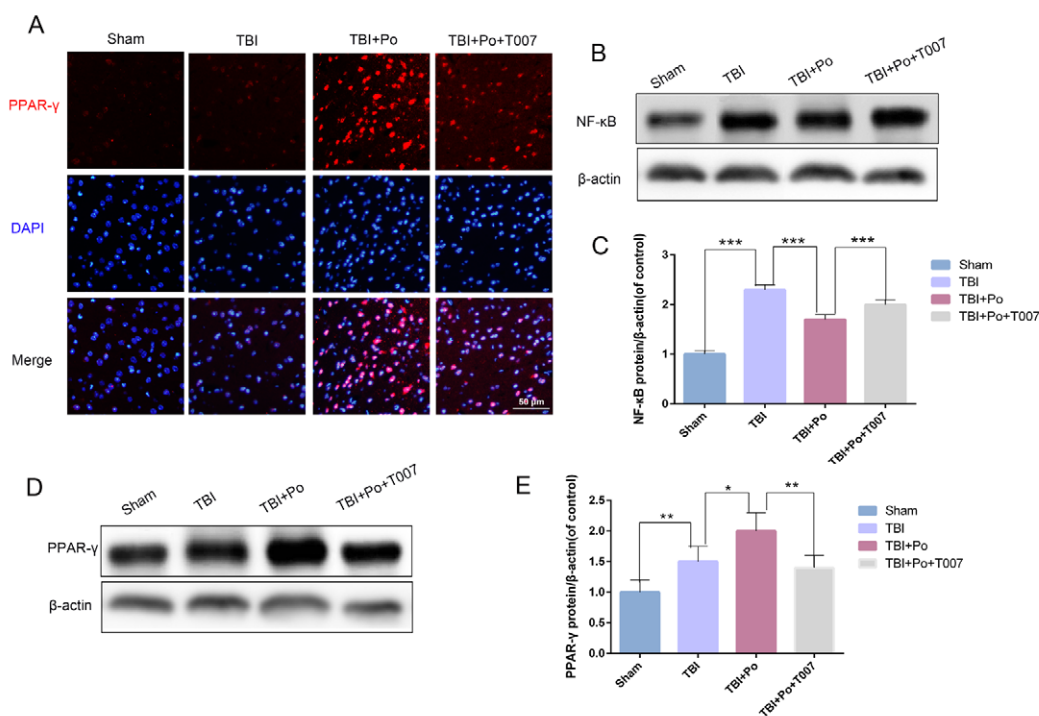


Fig. 5. Pogostone inhibited TBI-activated NF- κ B activation through PPAR- γ signaling pathway. **A)** Immunofluorescence was used to detect the expression of PPAR- γ . Bar= 50 μ m. **B-E)** The protein expression levels of NF- κ B and PPAR- γ were determined by Western blot. All experiments were performed in triplicate. The quantitative data were expressed as the mean $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, NS: $P > 0.05$.

and neuronal apoptosis induced by TBI, but also inhibit expression levels of pro-inflammatory factors via modulating NF- κ B and PPAR- γ .

TBI is accompanied by complicated pathophysiological changes in which noxious mechanical forces are responsible for tissue damage and dysregulation of CNS homeostasis (19). On the other hand, microglia, which act as the first line of defense of the central nervous system against injury or disease, is vital in neuroinflammation after TBI (20). After neuronal injury, the normal function of microglia is disrupted, and excessive inflammatory factors (IL-1 β , IL-6, TNF- α , and iNOS) are produced by activated microglia and then released to the brain lesions (21), causing neuronal dysfunction and cell death. Many studies indicate that effective regulation of inflammatory response, such as by drugs or genetic intervention, can potentially reduce brain edema, and cell apoptosis as well as neurological impairment after TBI. For example, hyperbaric oxygen therapy

can impede apoptosis by inhibiting the expression of inflammatory factors to protect the integrity of the blood-brain barrier and promote the functional recovery from TBI (22). In addition, endocannabinoids can inhibit the inflammatory response after TBI, and weaken TBI-induced neurological dysfunction (23). The results of this study showed that pogostone reduced the mNSS score in mice, increased the latency to fall, and decreased brain tissue edema and neuronal apoptosis, suggesting that pogostone could protect neurological function after TBI. On this basis, we also found that pogostone could significantly reduce the accumulation of microglia and the expressions of pro-inflammatory factors in the lesions, indicating that pogostone could suppress the inflammatory response after TBI.

Several studies report that PPAR- γ has a prominent role in promoting neurogenesis, enhancing neural plasticity, and improving repair and functional recovery (24). PPAR- γ is expressed in

microglia and astrocytes, both of which are involved in neuroinflammatory activities in neurological diseases and brain injury (25). PPAR- γ can compete with activator-1 (AP-1) to bind to the synergistic activators CBP and p300, thereby inhibiting the AP-1 signal pathway and repressing the inflammatory response (26). PPAR- γ activation can reprogram the immune response, attenuate neuronal and glial death after CNS injury, and regulate microglia activation and the phenotypic transformation, thus promoting phagocytosis of apoptotic cells and ameliorating inflammation after ischemia (26). Additionally, PPAR- γ can inhibit the activities of transcription factors, such as transcription factor activating protein-1 and NF- κ B. In the inflammatory response after TBI, PPAR- γ can directly bind to the subunit p65/p50 of NF- κ B, form a transcriptional repressor complex, and then reduce the activation of NF- κ B p65 (27). In the present study, we authenticated that pogostone could markedly suppress the expression of NF- κ B, whilst elevating the expression of PPAR- γ and its nuclear translocation. In addition, PPAR- γ inhibitor T0070907 could aggravate neurological damage, brain edema, and inflammatory response in mice, reversing the effects of pogostone. These data implied that pogostone could probably repress the secondary injury after TBI by repressing PPAR- γ /NF- κ B pathway.

In conclusion, this study verifies that pogostone can alleviate the degree of cerebral edema after TBI in mice, improve neurological function, and have neuroprotective effects. Moreover, pogostone inhibits TBI-induced microglia activation and increases the expressions of inflammatory factors. From the perspective of mechanisms, pogostone inhibits TBI-activated NF- κ B signaling through regulating PPAR- γ . Nonetheless, the neuroprotective functions and safety of pogostone in clinical application require further investigation in the future.

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Synergistic effects of miR-505-3p and uremic serum on the proliferation of vascular smooth muscle cells

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This study aimed to assess the synergistic effects of miR-505-3p and uremic serum on the proliferation of vascular smooth muscle cells (VSMCs) *in vitro*. Serum samples from uremic patients and healthy controls were collected and treated with 10% serum. Meanwhile, the samples were divided into eight groups: uremic serum, healthy serum, uremic serum + miR-505-3p inhibitor, uremic serum + miR-505-3p negative control (NC), uremic serum + miR-505-3p mimics, healthy serum + miR-505-3p inhibitor, healthy serum + miR-505-3p NC, and healthy serum + miR-505-3p mimics. The proliferation and apoptosis of cells and protein expression of miR-505-3p in these groups were detected. Compared with the healthy serum group, the expression level of miR-505-3p in the uremic serum group was elevated, and the difference was statistically significant ($P < 0.05$). Moreover, the uremic serum + miR-505-3p mimics group had the highest proliferation activity and the lowest rate of apoptosis, and the expression level of caspase-3 was significantly down-regulated ($P < 0.05$), while that of PLK1 was noticeably up-regulated. Inhibition of miR-505-3p significantly reduced uremic serum-mediated proliferation of VSMCs ($P < 0.05$), and the underlying mechanism was found to be associated with the miR-505-3p-caspase-3/PLK1 pathway.

Uremia refers to a clinical syndrome characterized by metabolic abnormalities and multiple organ failure in the whole body when chronic renal failure enters the end-stage. Compared with ordinary individuals, uremic toxin content in blood of uremic patients significantly increases, which may lead to disorders of water, electrolyte, acid-base balance or other clinical symptoms, and then may result in pathological changes in other organs and tissues in the whole body (1-3). The renal tissue of uremic patients is mainly fibrotic, causing the loss of almost all renal functions. In the early-stage of renal failure, the fibrosis of renal tissues is initiated (4, 5). With the progression of fibrosis, there may be an incidence

of metabolic acidosis, as well as the appearance of some serious symptoms, threatening other organs. To date, uremia has been extensively disseminated worldwide, and its main treatment approach is hemodialysis or kidney transplantation (6, 7). When entering the advanced stage, the concentration of uremic toxin (> 200 mL) in patients is significantly higher than that in healthy individuals. According to the molecular weight of the toxins, they can be divided into small-, medium-, and large-sized molecules. Although the symptoms of uremia often show the accumulation of various uremic toxins, its main cause is closely associated with the changes in genetic materials (8, 9). Therefore, the study

Key words: vascular smooth muscle cells; uremic serum; miR-505-3p; synergistic effects

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of uremia from the level of genetic materials may provide a new theoretical basis for slowing down renal damage and metabolic imbalance *in vivo*.

Vascular smooth muscle cells (VSMCs) are predominant constituents of the blood vessel wall and contribute to the regulation of blood pressure. Hypoxia (decreased oxygen delivery) and ischemia (reduced blood flow) are relatively common stressors of cells. After sensing the hypoxia signaling, VSMCs may increase the load and adjust the circulation of blood flow to improve the ventilation/perfusion ratio, while continuous exposure to hypoxia promotes the proliferation of VSMCs, as well as thickening of the vascular wall. Under exposure to hypoxia, several signaling pathways are involved in VSMCs (10). For instance, hypoxia leads to the imbalance of redox state in cells, which may cause down-regulation of Kv1.5 channel or changes in activity, decrease K⁺ outflow, and depolarize cell membrane potential, thereby activating voltage-dependent calcium channels to promote calcium influx, while intracellular increase of Ca²⁺ concentrations can activate the related kinase and gene expression regulated by calcium, and promote cell proliferation and survival (11-13). In addition, the activity of hypoxia-inducible factor (HIF) prolyl hydroxylase (PHD) could be inhibited, degradation of HIF-1 α could be reduced, and HIF-1 β could be transferred to the nucleus to activate the hypoxic response. The hypoxia response element (HRE) mediates the proliferation of VSMCs, phenotype transformation, metabolic changes, and also promotes vascular remodeling (14, 15). VSMCs not only possess the feature of self-regulation, but are also regulated by the secretion of other cells, which may influence their proliferation, secretion, and other phenotypes.

MicroRNA (miRNAs) are natural, small, non-coding RNA (ncRNA) molecules that post-transcriptionally regulate the expression levels of a large number of genes involved in various biological processes, including cell proliferation, differentiation, and apoptosis, either through mRNA degradation or translation inhibition. An ncRNA is transcribed from a DNA sequence that is also considered as an RNA gene; these types of RNA are transcribed directly by type III RNA polymerases

or are processed indirectly from a large transcript of type II RNA polymerases. The majority of miRNAs are transcribed from DNA sequences into primary miRNAs (pri-miRNAs) and are classified into precursor miRNAs (pre-miRNAs) and mature miRNAs. The most frequent processing model suggests that DGCR8 recognizes the pri-miRNA at the ssRNA-dsRNA junction and directs Drosha to a specific cleavage site ~11 base pairs (bp) from the junction on the stem where Drosha cuts to liberate a ~60-70 bp miRNA hairpin precursor (pre-miRNA). Dicer cleaves a double-stranded RNA (dsRNA) and a pre-microRNA (pre-miRNA) into short dsRNA fragments, namely small interfering RNA (siRNA) and miRNA, respectively. These fragments have a length of approximately 20-25 bp with a two-base overhang on the 3' ends. Dicer facilitates the activation of the RNA-induced silencing complex (RISC), which is essential for RNA interference (16, 17). In RISC, a single-stranded mature miRNA and a target miRNA form partial complementary pairing, which mediates the degradation or translation inhibition of miRNA in a classical manner. The mechanism of RNA interference depends on the incomplete complementary pairing of homologous miRNA sequences, which determines that the same miRNA can theoretically target several target miRNAs. In addition, miRNA possesses the ability of tissue-specific gene expression, indicating that miRNA only interacts with highly expressed target miRNAs in specific tissues. Therefore, miRNA plays multiple biological roles by inhibiting different target genes in different tissues and cells. Furthermore, miRNA plays a major role in the regulation of gene expression and controlling various cellular processes, such as cell proliferation, differentiation, and apoptosis.

MiR-505-3p has been reported to be involved in various diseases. For instance, Cheng et al. showed that miR-505-3p inhibits glioma growth by targeting high mobility group box 1 (HMGB1) (18). In familial hypercholesterolemia, miR-505-3p can regulate chemokine receptor up-regulation in macrophages (19). Moreover, miR-505 has been shown to suppress the growth of hepatocellular carcinoma cells via targeting insulin-like growth

factor type 1 receptor (IGF-1R) (20). In addition, miR-505 could enhance M2 polarization in a mouse model of choroidal neovascularization by targeting transmembrane protein 229B (21). However, the role of miR-505-3p in the proliferation of VSMCs has still remained elusive. The present study aimed to assess the synergistic effects of miR-505-3p and uremic serum on the proliferation of VSMCs *in vitro*.

MATERIALS AND METHODS

Sample collection

From January 2017 to December 2019, 40 uremic patients (age, 32-55 years; 24 males and 16 females) and 40 healthy controls (age, 29-56 years; 21 males and 19 females) were included in this study; there was no significant difference in age and sex ratio between the two groups. Serum samples were collected from all the participants. The study protocol was approved by the Ethics Committee of Shaanxi Provincial People's Hospital (Xi'an, China), and patients or their family members signed the written informed consent form prior to the start of the study.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from serum samples and VSMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription of RNA was performed via a PrimeScript-RT reagent kit. The qRT-PCR was carried out using qPCR SYBR Green/ROX Master Mix (Thermo Scientific Fisher, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered as an internal standard. All tests were carried out in triplicate. The sequences of primers used for qRT-PCR are presented in Table I.

Transfection and grouping

The samples were divided into eight groups: uremic serum group, healthy serum group, uremic serum + miR-505-3p inhibitor group, uremic serum + miR-505-3p negative control (NC) group, uremic serum + miR-505-3p mimics group, healthy serum + miR-505-3p inhibitor group, healthy serum + miR-505-3p NC group, and healthy serum + miR-505-3p mimics group. MiR-505-3p inhibitor (miR-505-3p expression was down-regulated), miR-505-3p mimics (miR-505-3p expression was up-regulated), and NC were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China) and transfected into cells with Lipofectamine 2000 (Thermo Fisher Scientific). VSMCs were collected 24 h after completing the transfection. All tests were carried out in triplicate.

Cell proliferation assay

The VSMCs were digested and a cell suspension containing 1.0×10^4 cells/mL was prepared. Subsequently, 100 μ L of cell suspension were inoculated into each well, and incubated overnight at 37°C in presence of 5% CO₂. When the cells reached a confluency of 60-70%, the Dulbecco's modified Eagle's medium (DMEM) with a low fetal bovine serum (FBS) concentration was changed. After that, 200 μ L of cell counting kit-8 (CCK-8) solution (Gibco, New York, NY, USA) was added into each well and incubated at 37 °C for 1 h. The optical density was measured at a wavelength of 450 nm with a microplate reader. All the tests were carried out in triplicate.

Cell migration and invasion assays

For the cell migration and invasion assays, the Transwell chambers were coated with 100 μ L BD Biosciences Matrigel overnight in a cell incubator. Then, 3×10^4 cells were trypsinized and re-suspended in a 200 μ L

Table I. Sequences of primers used for qRT-PCR

miR-505-3p	F: 5'- CGTCAACACTTGCTGGT -3'
	R: 5'- GGTCCAGTTTTTTTTTTTTTTTAGGA -3'
GAPDH	F: 5'-AGAAGGCTGGGGCTCATTTG-3'
	R: 5'-AGGGGCCATCCACAGTCTTC-3'

serum-free medium. The re-suspended cells were placed into the upper chambers of a 24-well Transwell plate (pore size, 8 mm; BD Biosciences, Franklin Lakes, NJ, USA). The lower chambers were filled with a 700 μ L complete medium with 10% FBS. Following incubation for 24 h at 37°C, non-invaded cells were removed from the upper chamber with a cotton swab. The cells were then stained with 4,6-diamino-2-phenylindole (DAPI) and counted under a fluorescence microscope. All tests were carried out in triplicate.

Flow cytometry

The transfected VSMCs were cultured in 6-well plates. To perform flow cytometry, the cells were digested with trypsin and fixed with 70% ethanol for 24 h at 4°C. The fixed cells were washed three times with phosphate-buffered saline (PBS) and centrifuged at 1,000 rpm for 5 min at room temperature. The supernatant was subsequently discarded. The cells were incubated with propidium iodide (PI) for 10 min and subsequently treated with 200 μ L of PBS, RNase A, and PI for 10 min at 4°C in the dark. Finally, the cells were screened by 400-mesh sieves and analyzed by a flow cytometer (FACSCanto II; BD Biosciences). The data were analyzed using the ModFit 4.1 software (Verity Software House, Inc., Topsham, ME, USA). All tests were carried out in triplicate.

Western blot analysis

After 24 h of transfection, cells were washed three times with PBS and incubated with radioimmunoprecipitation assay (RIPA) lysis buffer for 30 min on ice. After that, the protein concentration was quantified using a bicinchoninic

acid (BCA) protein assay kit. Samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes. Subsequently, the membranes were incubated with primary antibodies against PLK1, caspase-3, DHX15, and LDOC1 overnight at 4°C, and washed with TBST. Then, the membranes were treated with horseradish peroxidase (HRP)-labeled goat-anti-rabbit immunoglobulin G (IgG) secondary antibody and incubated at room temperature for 1 h. Following TBST washing for 6 times at room temperature (5 min/wash), the membranes were incubated, and the bands were then visualized by an enhanced chemiluminescence (ECL) reagent. All tests were carried out in triplicate.

Statistical analysis

SPSS 19.0 (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) software were used to perform statistical analysis. Two groups of experiments were compared using the Student's *t*-test. One-way analysis of variance (ANOVA), followed by the Tukey's post hoc test was applied to analyze differences among multiple groups. $P < 0.05$ indicated significant difference.

RESULTS

The expression level of miR-505-3p was up-regulated in uremic patients

The expression level of miR-505-3p in 40 uremic patients and 40 healthy controls was measured by qRT-PCR. The results showed that the expression of miR-505-3p was up-regulated in uremic patients (Fig. 1).

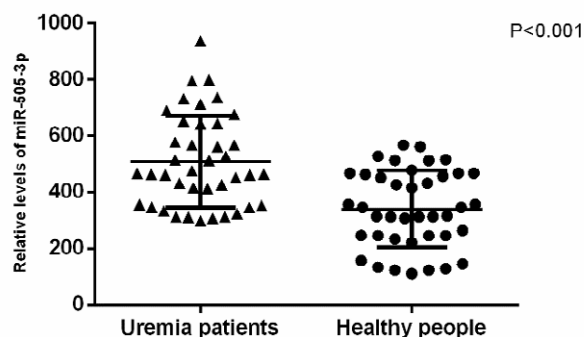


Fig. 1. The expression level of miR-505-3p in uremic patients and healthy controls

Expression levels of miR-505-3p in different groups

After transfection of miR-505-3p NC, miR-505-3p mimics, and miR-505-3p inhibitors into VSMCs, qRT-PCR revealed that the expression level of miR-505-3p was significantly elevated with miR-505-3p mimics, while that was decreased with miR-505-3p inhibitors (Fig. 2).

MiR-505-3p could promote the proliferation of VSMCs

The effects of miR-505-3p and uremic serum on viability and migration of VSMCs were assessed by

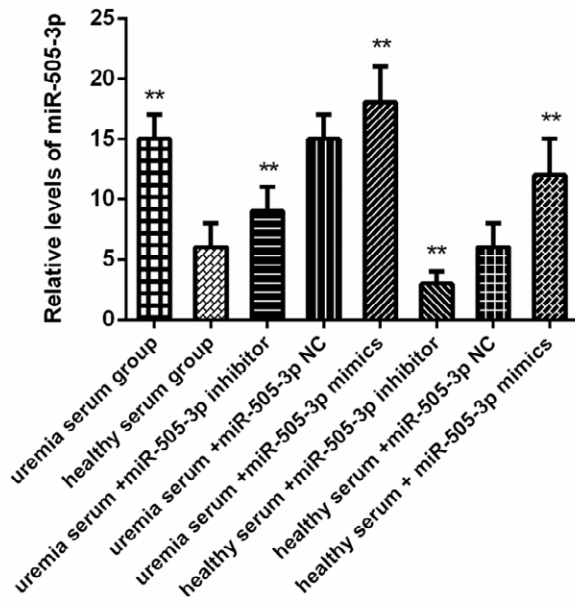


Fig. 2. The expression level of miR-505-3p in different groups

the CCK-8 and cell migration assays, respectively. As shown in Fig. 3A, the proliferation rate of VSMCs in the uremic serum group was higher than that in healthy serum group. It was found that the uremic serum + miR-505-3p mimics group had the highest proliferation rate, while the lowest proliferation rate was noted in the healthy serum + miR-505-3p inhibitor group. The cell invasion assay showed a trend similar to that illustrated in Fig. 3B, and the invasiveness of VSMCs in the uremic serum group was significantly higher than that in the healthy serum group. However, the invasiveness of VSMCs in the uremic serum + miR-505-3p mimics group was the highest, while it was the lowest in the healthy serum + miR-505-3p inhibitor group.

MiR-505-3p inhibited apoptosis of VSMCs

The effects of miR-505-3p and uremic serum on the apoptosis of VSMCs were detected by flow cytometry. As shown in Fig. 4, the apoptotic ability of VSMCs in the uremic serum group was lower than that in the healthy serum group. The apoptotic ability of the uremic serum + miR-505-3p mimics group was

the weakest, while that of the healthy serum + miR-505-3p inhibitor group was the strongest. Furthermore, it was found that miR-505-3p significantly inhibited apoptosis of VSMCs ($P < 0.05$).

MiR-505-3p could influence VSMCs via miR-505-3p-caspase-3/PLK1 pathway

miRanda software was used to analyze potential targets of miR-505-3p, and four high-score mRNAs (PLK1, caspase-3, DHX15, and LDOC1) were identified. The results of Western blotting indicated that PLK1 and caspase-3 were regulated by the miR-505-3p-caspase-3/PLK1 pathway (Fig. 5).

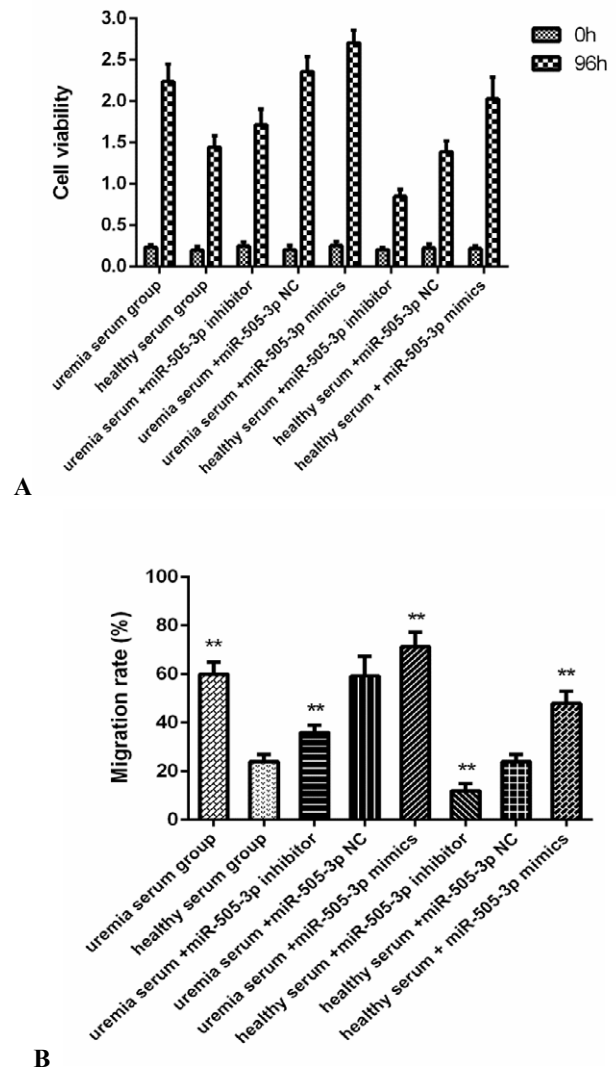


Fig. 3. MiR-505-3p suppressed migration and invasion of VSMCs

DISCUSSION

Chronic kidney disease (CKD) is characterized by gradual loss of kidney function, and is increasingly recognized as a global public health concern.. A previous study reported that the prevalence of CKD is about 11% in China (22). CKD develops into end-stage renal disease (ESRD). Although a successful kidney transplantation confers a notable increase in life expectancy over dialysis therapy for patients with ESRD, the survival of kidney transplant recipients is significantly shortened by atherosclerotic cardiovascular disease. For patients with CKD who need vascular access for acute hemodialysis, it is advised to use a non-cuffed or a cuffed, percutaneously inserted catheter (23, 24). Vascular access is truly the “lifeline” for hemodialysis

patients, and its dysfunction directly increases the burden of ESRD and reduces the survival rate of CKD patients. However, the one-year patency rate of vascular access after arteriovenous fistula is only 63%, which may be related to the fact that uremia can promote neointimal hyperplasia (NH), causing stenosis and dysfunction, while its specific mechanism remains to be further studied.

The miRNAs associated with atherosclerosis have provided new insights into the diagnosis and treatment of cardiovascular diseases. miRNAs bind to a specific sequence at the 3' UTR of target mRNAs to induce translational repression and mRNA deadenylation and decapping, and gene expression is suppressed at the post-transcription level. To our knowledge, miRNAs play an important role in a series of physiological and pathological

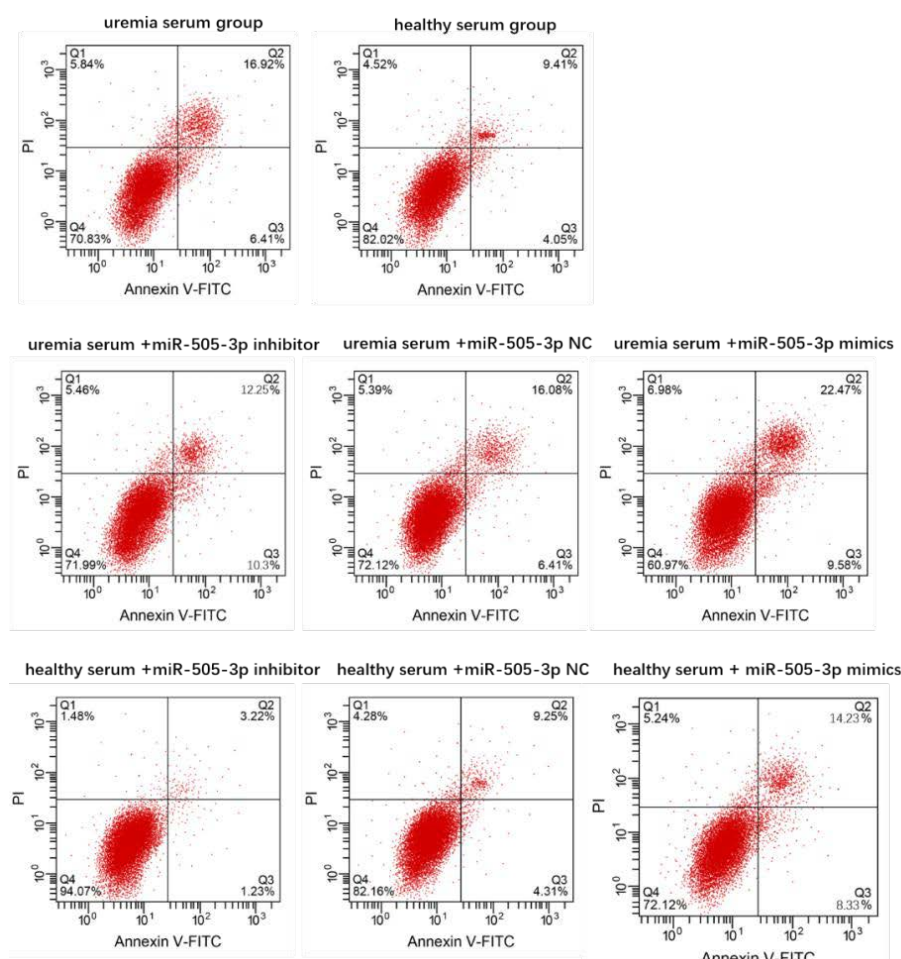


Fig. 4. Results of flow cytometry in different groups

processes, such as cell proliferation, differentiation, stem cell maintenance, and tumorigenesis (25-27). A previous study reported that miRNAs are closely correlated with angiogenesis, and they can regulate angiogenesis-associated biological functions. Angiogenic factors contribute to the angiogenesis process *in vivo* (28).

The expressions of miRNAs were found to be important in the regulation of proliferation, invasion, and migration of VSMCs. A previous study demonstrated that overexpression of miR-221/222 could promote proliferation of VSMCs by inhibiting P27KIP1/P57KIP1 (29). MiR-181b was found to activate the PI3K and mitogen-activated protein kinase (MAPK) pathways, enhancing the proliferation of VSMCs (30). Furthermore, proliferation of VSMCs plays a critical role in the progression of atherosclerosis (31). During the development of atherosclerosis, VSMCs migrate from the media into the intima and proliferate within the lesions in response to mediators secreted by monocytes and lymphocytes. Chronic renal failure may worsen atherosclerosis, the main driver of coronary artery disease, by basic glucose-lipid dysmetabolism (32).

It has been shown that the main pathological basis of NH is that multiple risk factors stimulate VSMCs

to over-proliferate and migrate to the intima. Under normal circumstances, VSMCs maintain a contractile phenotype to regulate elasticity and function of blood vessels. During disease development, VSMCs are stimulated by extrinsic factors (e.g., uremia) to form a synthetic phenotype. At this time, the cells begin to proliferate and an inflammatory reaction occurs. The internal environment of VSMCs could be stimulated by uremic serum and normal serum *in vitro* (33, 34).

However, despite the high expressions of miRNAs in VSMCs, our understanding of the role of miRNAs in the regulation of VSMC proliferation was not comprehensive. As the novelty of the present research, the results highlighted the important role of miR-505-3p in the regulation of VSMC proliferation. We herein detected miR-505-3p levels in the uremic serum group and healthy serum group. It was further confirmed that uremic serum, as a risk factor, could significantly promote the proliferation of VSMCs compared with healthy serum, and miR-505-3p could intensify this effect, eventually causing intimal hyperplasia.

We further investigated the regulatory role of mir-505-3p in proliferation of VSMCs, and it was found that under the stimulation of uremic serum + mir-505-3p, the expression level of caspase-3 decreased, while the expression level of PLK1 increased.

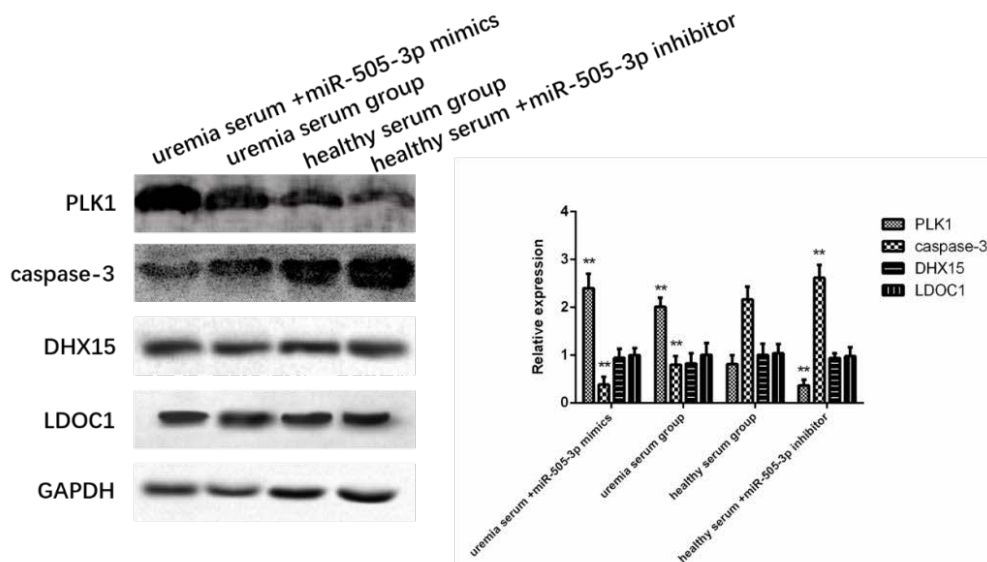


Fig. 5. MiR-505-3p could influence VSMCs through miR-505-3p-caspase-3/PLK1 pathway

PLK1 is a member of a highly conserved serine/threonine kinase family that plays pivotal roles in mitosis, cytokinesis, and DNA damage response in eukaryotic cells. PLK-1 also targets a variety of substrates that regulate cell cycle progression, and this is achieved either in a CDK-1-dependent or self-dependent manner (35-37). Additionally, a number of scholars pointed out that PLK1 plays an important regulatory role in DNA replication and DNA repair during mitosis. By detecting the phosphorylation level of H2A histone, Yim et al. found that after PLK1 deletion, DNA damage was noticeable (38). Emi1 is an anaphase inhibitor of the APC/C complex that prevents geminin from being destroyed by the pre-RC complex before DNA replication. Caspase family is a group of cysteine proteases that specifically cleave aspartic acid and play an important role in cell apoptosis. In mammals, apoptotic caspases are subdivided into two categories, initiator caspases and effector caspases. Initiator caspases have a long N-terminal prodomain, providing the molecular platform for caspase activation. Studies have confirmed the important role of epigenetic factors in the regulation of the expression of caspase-3 gene (39, 40). Yakovlev et al. found that developmental down-regulation of caspase-3 expression in brain of rats is associated with transcriptional silencing, in which age-dependent differential DNA methylation and histone acetylation play a substantial role (41). Recent studies have suggested that HIF-1 is capable of transcriptional activation of caspase-3. For instance, Wang et al. studied the role of HIF-1 in the apoptosis induced by hypoxia-reoxygenated rat cardiomyocytes. Their results showed that hypoxia-reoxygenation stimulation could up-regulate the expression levels of HIF-1 and caspase-3 in primary rat neonatal cardiomyocytes and induce cell apoptosis. They co-transfected the ESE-3 vectors and the luciferase reporter gene, containing the caspase-3 promoter region into the PC3 cell line and found a three-fold increase in the luciferase activity (42).

In summary, the results of the present study, for the first time, showed that mir-505-3p could interact with uremic serum to induce the proliferation of VSMCs and inhibit their apoptosis, and miR-505-3p could influence VSMCs via miR-505-3p-caspase-3/

PLK1 pathway. Therefore, mir-505-3p can be a new target for the treatment of uremia and its associated complications.

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Circ_0078619 facilitates oral squamous cell carcinoma progression via miR-142-5p/ROCK1 axisJY. Qiu^{1,2} and J. Yuan²¹*Department of Stomatology, Beijing Shijitan Hospital, Capital Medical University, Beijing, China;* ²*Institute and Department of Stomatology, Chinese People's Liberation Army General Hospital, Beijing, China**Received April 30, 2021 – Accepted August 24, 2021*

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The present study is aimed at investigating the effects of circular RNA circ_0078619 on oral squamous cell carcinoma (OSCC) cell growth, migration, invasion, and apoptosis, as well as its mechanism. The GSE131182 dataset was downloaded from Gene Expression Omnibus (GEO), and GEO2R was adopted to analyze the differentially expressed circular RNAs in OSCC tissues compared with normal tissue samples. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted for detecting the expressions of circ_0078619, microRNA (miRNA, miR)-142-5p, and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) mRNA. Western blot was adopted to quantify the expression of ROCK1 protein in OSCC cells. Cell counting kit-8 (CCK-8) assay, 5-bromo-2'-deoxyuridine (BrdU) assay, Transwell assay, and flow cytometry were employed to assess cell proliferation, migration, invasion, and apoptosis. Bioinformatics was adopted to predict the targeted relationship between miR-142-5p and circ_0078619 or ROCK1 mRNA 3'UTR, and RNA immunoprecipitation and dual-luciferase reporter gene assays were used for validation. Moreover, Pearson correlation analysis was utilized to analyze the correlation among their expressions. Circ_0078619 expression was elevated in OSCC cells and tissues, and its high expression was strongly associated with advanced TNM (tumor, node, metastases) stage and the poor differentiation of tumor tissue. Knockdown of circ_0078619 repressed OSCC cell growth, migration, and invasion, and promoted cell apoptosis. MiR-142-5p was directly targeted by circ_0078619, which negatively modulated its expression. ROCK1 was a target of miR-142-5p, and ROCK1 expression could be positively and indirectly modulated by circ_0078619. Circ_0078619 and miR-142-5p expressions were negatively correlated, while circ_0078619 and ROCK1 expressions were positively correlated in OSCC tissues. Circ_0078619 promoted OSCC cell growth, migration, and invasion, and suppressed apoptosis by modulating the miR-142-5p/ROCK1 axis.

Globally, head and neck cancer (HNC) is identified as the sixth most common carcinoma (1) of which oral squamous cell carcinoma (OSCC) has the second-worst prognosis (2). Surgical treatment is preferred for OSCC patients with early stage disease, while for those

with advanced stage disease or with recurrence and metastasis, surgery combined with radiotherapy and chemotherapy is currently the main treatment strategy (3). Over the past few decades, despite great advances in the diagnostic methods and treatment approaches

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for OSCC, the prognosis of OSCC patients is still unsatisfactory, and, specifically, the five-year overall survival rate remains only 40-60% (4). In this context, further exploration of the molecular mechanism of OSCC and seeking novel targets for the effective therapy of OSCC are necessary (5).

As a type of non-coding RNA, circular RNAs (circRNAs) do not possess a 3'-end poly (A) tail and a 5'-end cap, and they have a covalent closed-loop structure, so they are resistant to RNase (6-8). CircRNAs participate widely in physiological and pathological processes and feature prominently in the tumorigenesis and progression of various cancers (9-11). In OSCC, circ_0005379 can inhibit OSCC cell growth, migration, and invasion, both *in vitro* and *in vivo* (12); circ_0055538 can suppress OSCC cell migration and invasion, induce apoptosis, and restrain the growth of transplanted tumor in nude mouse (13). In the present work, we downloaded GSE131182 dataset from Gene Expression Omnibus (GEO) database, and analyzed the differentially expressed circRNAs in OSCC tissues compared with normal tissues, and found that circ_0078619 expression was up-regulated in OSCC, but its biological function and underlying mechanism in OSCC warrant further investigation.

In OSCC, microRNA (miRNA, miR)-142-5p expression is reduced, and miR-142-5p can restrain OSCC cell growth, migration, and invasion (14). Additionally, Rho-associated coiled-coil containing protein kinase 1 (ROCK1) is highly expressed in OSCC and promotes OSCC cell proliferation (15). In the present study, we report that circ_0078619 can promote OSCC cell growth, migration, and invasion, and suppress apoptosis, via modulating the miR-142-5p/ROCK1 axis.

MATERIALS AND METHODS

Human tissue collection

The 45 cases of OSCC and para-cancerous tissues used in the experiments were all selected from the surgically removed tumorous and non-tumorous tissues in Beijing Shijitan Hospital. The isolated tissues were maintained in liquid nitrogen within 30 min after surgery. None of the participants had undergone radiotherapy, chemotherapy, or other anti-cancer therapies prior to surgery. All subjects

signed an informed consent, and the study was approved and guided by the Ethics Committee of Beijing Shijitan Hospital.

Cell culture

OSCC cell lines (Fadu, Tca8113, SCC-25, and CAL-27) and normal human oral epithelial cell line (Hs 680.Tg) were bought from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. When grown to the logarithmic growth phase, the cells were collected for the subsequent experiments.

Cell transfection

CAL-27 and SCC-25 cells were transferred at 1×10^6 cells/ml to 60 mm culture dishes and then cultured at 37°C in 5% CO₂ for 24 h, and subsequently, cell transfection was performed. The pcDNA empty vector (NC), small interfering RNA against circ_0078619 (si-circ_0078619-1 and si-circ_0078619-2) and their negative controls (si-NC), miR-142-5p inhibitors, miR-142-5p mimics and their controls inhibitor NC, and mimic NC were bought from RiboBio Co., Ltd. (Guangzhou, China). The procedures were carried out following the instructions for Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was used, after 24 h of transfection, to detect the transfection efficiency.

qRT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was employed for total RNA extraction from tissues or cultured cells. The reverse transcription of RNA into cDNA was carried out with a Reverse Transcription Kit (Takara, Dalian, China). qRT-PCR was conducted with a SYBRGreen Master Mix kit (Takara, Dalian, China) in the ABI7500 real-time PCR system (Applied Biosystems, San Francisco, CA, USA). The 2^{-ΔΔCt} method was adopted for calculating the relative expressions of circ_0078619, miR-142-5p, and ROCK1 mRNA, with GAPDH and U6 as internal references. Primer sequences are shown in Table I.

Cell counting kit-8 (CCK-8) assay

The cells of each group during exponential growth were chosen to make a single-cell suspension.

After counting the number of the cells, the cell density was adjusted, and cells were then transferred to 96-well plates with 3,000 cells per well. The next day, each well was added with 90 μ L of medium and 10 μ L of CCK-8 solution (Dojindo Molecular Technologies, Dojindo, Japan). After 2 h of incubation, a microplate reader was utilized for measuring the absorbance at 450 nm of each well. The cell viability was detected after 24, 48 and 72 h, respectively. The cell growth curve was drawn, with time as the x-coordinate and OD 450 nm as the y-coordinate.

5-bromo-2'-deoxyuridine (BrdU) assay

CAL-27 and SCC-25 cells were transferred at 2.5×10^5 cells/well to a 24-well plate (with a cover slide in it). After 24 h of culture, BrdU solution (Beyotime Biotechnology, Shanghai, China) was added, with which the cells were incubated for 4 h. Subsequently, the cells were fixed for 30

min with 4% cold paraformaldehyde. After the supernatant was removed, each well was added with 100 μ L of 1 $\times$ Apollo for staining for 30 min. Again, the supernatant was removed, and each well was added with 100 μ L of 1 $\times$ Hoechst 33342 reaction solution for incubation at room temperature for 20 min in the dark. After the cells were rinsed with PBS, the number of BrdU-positive cells and the total number of cells in 10 random fields were counted under a microscope, and the rate of BrdU-positive cells was calculated.

Transwell assays

In the cell migration assay, CAL-27 and SCC-25 cells during logarithmic growth were transferred to 24-well plates, cultured for 24 h at 37°C in 5% CO₂, and resuspended in the medium without serum. The upper and lower compartments of the Transwell chamber (Corning Incorporated, Corning, NY, USA) were added with 200

Table I. Primers used for qRT-PCR

Genes	Primers
Circ_0078619	F: TCCTTGCAAAGGGCACGTAT
	R: GCTCGGATCTTTTTCTCCTGCT
miR-142-5p	F: CCGGTCATAAAGTAGAAAGC
	R: GTGCAGGGTCCGAGGT
ROCK1	F: AACATGCTGCTGGATAAATCTGG
	R: TGTATCACATCGTACCATGCCT
U6	F: GCCATCACGCCACAGTTTC
	R: TTCACGAATTTGCGTGTCAT
GAPDH	F: ACAACTTTGGTATCGTGGAAGG
	R: GCCATCACGCCACAGTTTC

F: forward; R: reverse

μL of the cell suspension and 500 μL of complete medium with 10% FBS, respectively. Then the cells were cultured for 24 h. After washing the cells twice with PBS, a cotton

swab was utilized to gently wipe the non-migrated cells in the upper compartment. After that, the cells were fixed for 15 min with 4% paraformaldehyde, washed 3 times with

Table II. *Correlation between clinicopathological features and circ_0078619 expression in OSCC*

Pathological	Number	circ_0078619 expression			
Parameters	(n = 45)	High (n=22)	Low(n=23)	χ^2	p-Value
Gender				1.0792	0.2989
Male	28	12	16		
Female	17	10	7		
Age (years)				0.5841	0.4447
< 60	12	7	5		
≥ 60	33	15	18		
Tumor size				1.7935	0.1805
< 5	18	11	7		
≥ 5	27	11	16		
TNM stage				6.4209	0.0113*
I-II	25	8	17		
III-IV	20	14	6		
Differentiation				8.3180	0.0039*
Low, medium	17	13	4		
High	28	9	19		
Lymph node metastasis				2.0208	0.1552
Yes	17	6	11		
No	28	16	12		

* $P < 0.05$

PBS, and then stained for 10 min with 0.1% crystal violet. After being rinsed with PBS again, the stained cells were counted in 5 random fields. In the cell invasion assay, the Transwell upper compartment was coated with Matrigel before adding the cells, and the rest of the procedures were the same as those in the migration assay.

Flow cytometry

Annexin V-FITC and propidium iodide (PI) double staining kit (Southern Biotechnology, Birmingham, AL, USA) was used for cell apoptosis detection. The cells during logarithmic growth were trypsinized and then transferred at 1×10^5 cells/well to 6-well plates, where they were subsequently cultured at 37°C for 24 h. Then, in 200 μ L of binding buffer, the cells were resuspended, and PI staining solution (5 μ L) and Annexin-V-FITC staining solution (10 μ L) were added into the suspension. They were mixed gently and incubated away from light for 15 min at room temperature. Ultimately, a flow cytometer was employed for detecting the apoptosis rate within 1 h.

Dual-luciferase reporter gene assay

TargetScan (http://www.targetscan.org/vert_72/) and circular RNA interactome database (<https://circinteractome.nia.nih.gov/index.html>) were adopted for predicting the binding sites between miR-142-5p and circ_0078619, as well as between ROCK1 mRNA 3'UTR and miR-142-5p. The wild-type circ_0078619 (WT circ_0078619), wild-type ROCK1 (WT ROCK1), mutant circ_0078619 (MUT circ_0078619), and mutant ROCK1 (MUT ROCK1) dual-luciferase reporter vectors were designed and constructed. The above-mentioned reporter vectors, together with miR-142-5p mimics, mimics NC, miR-142-5p inhibitors and inhibitors NC, were co-transfected into SCC-25 and CAL-27 cells, respectively. After 48 h, following the manufacturer's instructions, the luciferase activity was measured with a dual-luciferase reporter gene detection kit (Promega Corp., Madison, WI, USA).

RNA immunoprecipitation (RIP) assay

Following the manufacturer's instructions, a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was adopted for verifying the combination between circ_0078619 and miR-142-5p. RIP lysis buffer was utilized for lysing SCC-25 and CAL-27 cells, and 100 μ L of cell lysate was incubated with

magnetic beads conjugated with human anti-Argonaute2 (Ago2) antibody or negative control IgG in RIP buffer. The immunoprecipitate was then incubated with proteinase K to remove the proteins. Subsequently, RNA was extracted. Next, qRT-PCR was conducted to detect miR-142-5p and circ_0078619.

Western blot

The OSCC cells were lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors. After high-speed centrifugation, the supernatant was collected and heated for 10 min in a 100°C water bath to denature the proteins. After protein quantification by the BCA method, SDS-PAGE was conducted and the proteins were transferred onto the PVDF membrane. After being washed with TBST, the membrane was incubated overnight with rabbit anti-ROCK1 antibody (Abcam, Shanghai, China, ab134181, 1:1000) and rabbit anti-GAPDH antibody (Abcam, Shanghai, China, ab9485, 1:1000) at 4°C, followed by rinsing with TBST and incubation with goat anti-rabbit IgG H&L (HRP) (Abcam, Shanghai, China, ab205718, 1:2000) for 1 h at room temperature. After rinsing the membrane with TBST again, a hyper-sensitive ECL kit (Biosharp, Hefei, China) was used for chemiluminescence development.

Statistical analysis

All experimental data were processed by SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA) and expressed as mean $\pm$ standard deviation. Student's *t*-test was utilized for the comparison between two groups, and the comparison of means among multiple groups was performed through the one-way analysis of variance. Enumeration data were represented by a contingency table, and χ^2 test was performed to analyze the difference between the two groups. When $P < 0.05$, the difference was of statistical significance.

RESULTS

Circ_0078619 expression is up-regulated in OSCC tissues

First of all, the expression characteristics of circRNA expression profile in OSCC tissues were analyzed through GSE131182. 190 circRNAs were up-regulated and 54 circRNAs were down-regulated

in OSCC tissues (Fig. 1A). Some of the up-regulated circRNAs, among circ_0078619 are shown in Fig. 1B. Subsequently, qRT-PCR was performed to detect circ_0078619 expression in OSCC tissues, and consistently, it was revealed that circ_0078619 expression in OSCC tissues was dramatically up-regulated compared with that in adjacent tissues (Fig. 1C). A *Chi*-squared test was carried out to analyze the association between the clinicopathological indicators of OSCC patients and circ_0078619 expression in OSCC tissues, and it was revealed that high circ_0078619 expression was closely associated with advanced TNM stage and poor differentiation of OSCC tissues (Table II).

Effects of circ_0078619 on OSCC cell growth, migration, invasion, and apoptosis

To decipher the biological functions of circ_0078619 in OSCC, qRT-PCR assay was employed for detecting circ_0078619 expression in OSCC cell lines and normal oral epithelial cell line (Fig. 2A), and the results showed that in OSCC cell lines, circ_0078619 was up-regulated. CAL-27 and SCC-25 cells were transfected with si-circ_0078619-1 and si-circ_0078619-2 to knock down circ_0078619

(Fig. 2B). Then the changes in cell proliferation were detected by CCK-8 assay, which showed that circ_0078619 knockdown significantly restrained the viability of OSCC cells (Fig. 2C). BrdU assay indicated that compared to the control group, the percentage of BrdU-positive cells in OSCC cells with circ_0078619 knockdown was significantly reduced (Fig. 2D). Next, Transwell assays were utilized to detect the effects of circ_0078619 on migration and invasion of OSCC cells, and it was revealed that circ_0078619 knockdown repressed the metastatic potential of OSCC cells (Fig. 2 E, F). Additionally, flow cytometry showed that knocking down circ_0078619 significantly induced OSCC cell apoptosis (Fig. 2G).

Circ_0078619 directly targets miR-142-5p

To pinpoint the downstream targets of circ_0078619, we searched Circular RNA Interactome database and discovered that miR-142-5p might be one of the functional targeted miRNAs of circ_0078619 (Fig. 3A). To further verify the targeted relationship between miR-142-5p and circ_0078619, the subcellular localization of circ_0078619 in CAL-27 and SCC-25 cells

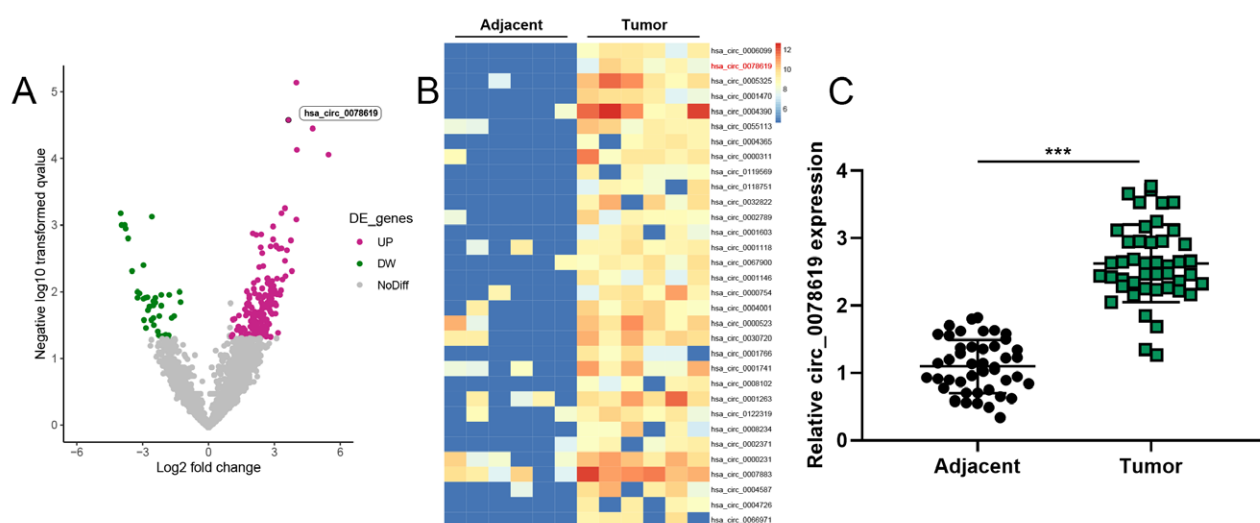


Fig. 1. Circ_0078619 expression was up-regulated in OSCC tissues. **A)** GSE131182 dataset was downloaded from the GEO database, and the volcano plot was used to analyze the differentially expressed circRNAs in OSCC tissue samples compared with normal tissues. **B)** A heat map was used to show some up-regulated circRNAs in GSE131182. **C)** Circ_0078619 expression in 45 cases of OSCC tissues and adjacent tissues was detected by qRT-PCR. *** $P < 0.001$.

was identified through qRT-PCR. It was indicated that circ_0078619 was primarily distributed in the cytoplasm (Fig. 3B). Subsequently, a dual-luciferase reporter assay was performed to explore the interaction between miR-142-5p and circ_0078619. The findings revealed that the transfection of miR-

142-5p mimics inhibited the luciferase activity of the WT circ_0078619 reporter, and transfection of miR-142-5p inhibitors worked oppositely, whereas the transfection of miR-142-5p mimics or miR-142-5p inhibitors did not significantly affect the luciferase activity of MUT circ_0078619 reporter (Fig. 3C).

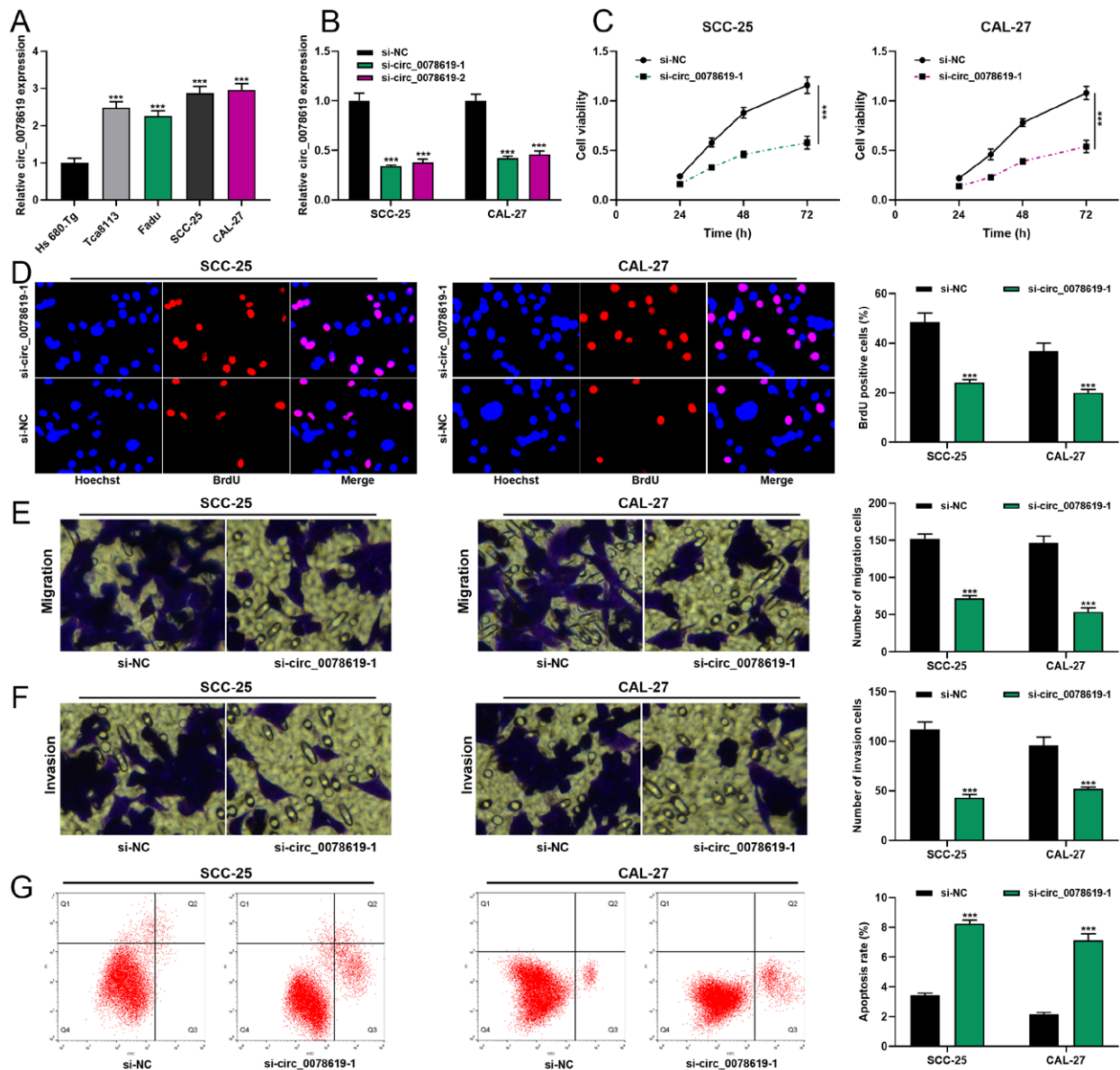


Fig. 2. Effects of circ_0078619 on OSCC cell proliferation, migration, invasion, and apoptosis. **A)** Circ_0078619 expression in normal human oral epithelial cell line (Hs 680.Tg) and OSCC cell lines (Tca8113, Fadu, SCC-25, and CAL-27) was detected by qRT-PCR. **B)** The transfection efficiency of si-circ_0078619-1 and si-circ_0078619-2 in SCC-25 and CAL-27 cells was detected by qRT-PCR. **C, D)** CCK-8 and BrdU assays were used to detect the effects of circ_0078619 knockdown on SCC-25 and CAL-27 cell proliferation. **E, F)** Transwell assays were used to detect the effects of circ_0078619 knockdown on SCC-25 and CAL-27 cell migration and invasion. **G)** Flow cytometry was used to detect the effects of circ_0078619 knockdown on SCC-25 and CAL-27 cell apoptosis. *** $P < 0.001$.

Furthermore, RIP assay proved that circ_0078619 and miR-142-5p were highly enriched in the Ago2 group compared to the control group, which indicated that there was a direct interaction between them (Fig. 3D). Subsequently, it was revealed that knocking down circ_0078619 markedly elevated miR-142-5p expression (Fig. 3E); in comparison with adjacent tissues, miR-142-5p expression in OSCC tissues was markedly lowered, and circ_0078619 and miR-142-5p expressions were inversely correlated (Fig. 3 F, G). These results suggested that circ_0078619 was a molecular sponge for miR-142-5p.

Circ_0078619 up-regulates ROCK1 expression by adsorbing miR-142-5p

To further elaborate on the downstream regulatory mechanism of miR-142-5p, the TargetScan database (http://www.targetscan.org/vert_72/) was utilized

for predicting the downstream targets of miR-142-5p, and it was discovered that ROCK1 was a downstream target of miR-142-5p (Fig. 4A). Dual-luciferase reporter gene assay suggested that the transfection of miR-142-5p mimics inhibited luciferase activity of WT ROCK1 reporter, and the transfection of miR-142-5p inhibitors promoted it, while the transfection of neither miR-142-5p mimics nor miR-142-5p inhibitors had a significant effect on the luciferase activity of MUT ROCK1 reporter (Fig. 4B). Western blot showed that knocking down circ_0078619 could down-regulate ROCK1 expression at protein level, while the transfection of miR-142-5p inhibitors could partially abolish this effect (Fig. 4C). qRT-PCR showed that ROCK1 mRNA was significantly highly expressed in OSCC as opposed to adjacent tissues (Fig. 4D); miR-142-5p and ROCK1 mRNA expressions were inversely

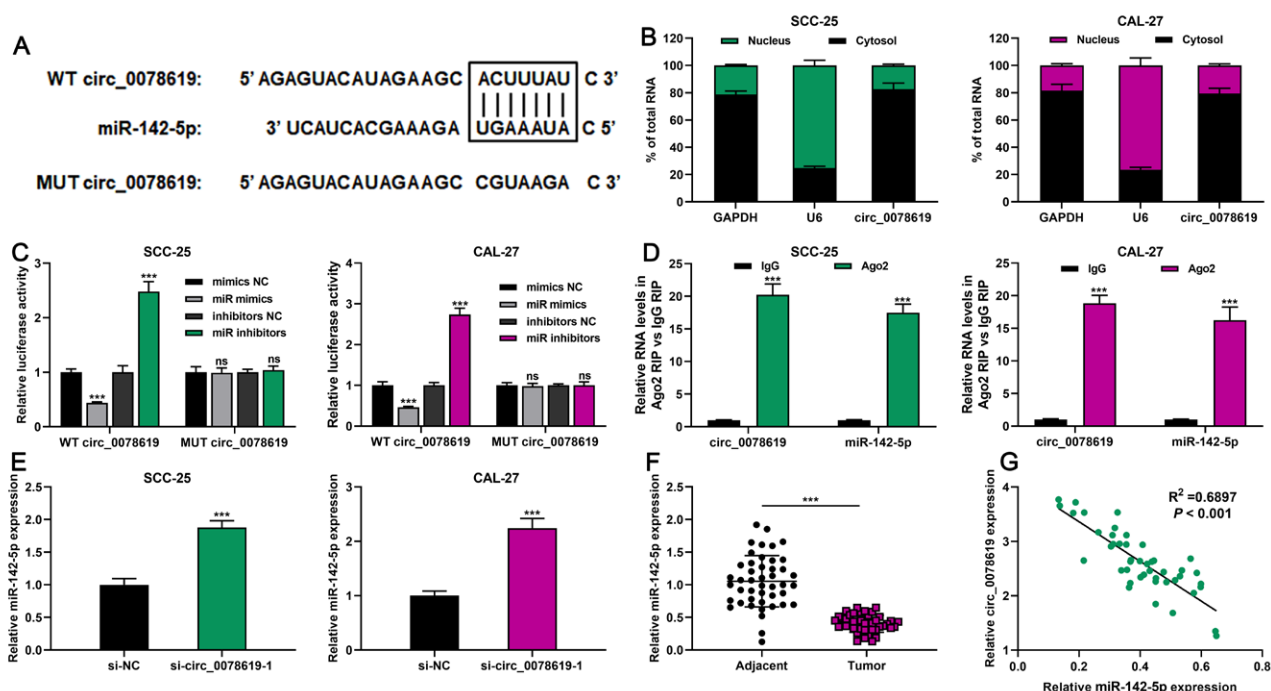


Fig. 3. Circ_0078619 directly targeted miR-142-5p in OSCC cells. **A)** The online database Circular RNA Interactome was used to predict the binding site between circ_0078619 and miR-142-5p. **B)** Subcellular localization of circ_0078619 was examined by qRT-PCR after nucleocytoplasmic separation. **C)** Luciferase activities of WT circ_0078619 reporter and MUT circ_0078619 reporter were detected by a dual-luciferase reporter gene system after the transfection of miR-142-5p mimics or inhibitors. **D)** RIP assay was used to validate the direct interaction between circ_0078619 and miR-142-5p. **E)** Effects of circ_0078619 knockdown on miR-142-5p expression in OSCC cells were detected by qRT-PCR. **F)** MiR-142-5p expression in 45 pairs of OSCC tissues and adjacent tissues was detected by qRT-PCR. **G)** Pearson correlation analysis of the correlation between miR-142-5p and circ_0078619 expressions in OSCC tissues. *** $P < 0.001$.

correlated, while circ_0078619 and ROCK1 mRNA expressions were positively correlated (Fig. 4 E, F).

Effects of miR-142-5p and circ_0078619 on OSCC cell growth, migration, invasion, and apoptosis

To further investigate the regulatory effects of circ_0078619/miR-142-5p axis on OSCC cell growth, migration, invasion, and apoptosis, SCC-25 and CAL-27 cells were co-transfected with si-circ_0078619-1 and miR-142-5p inhibitors, and qRT-PCR results showed that the transfection was successful (Fig. 5A). Subsequently, through CCK-8, BrdU, Transwell, and flow cytometry assays, it was found that compared with the control group, knocking down circ_0078619 remarkably suppressed OSCC

cell growth, migration and invasion, and induced the apoptosis, while the transfection of miR-142-5p inhibitors counteracted these effects (Fig. 5 B, F).

DISCUSSION

CircRNAs are originally thought to be viroids in plants (16). With the advancement of bioinformatics, high-throughput sequencing can be used to screen out differentially expressed circRNAs in disease progression, and the relationship between circRNAs and the development of various diseases and their regulatory mechanisms have been gradually unveiled (17, 18). CircRNAs, through functioning as competing endogenous

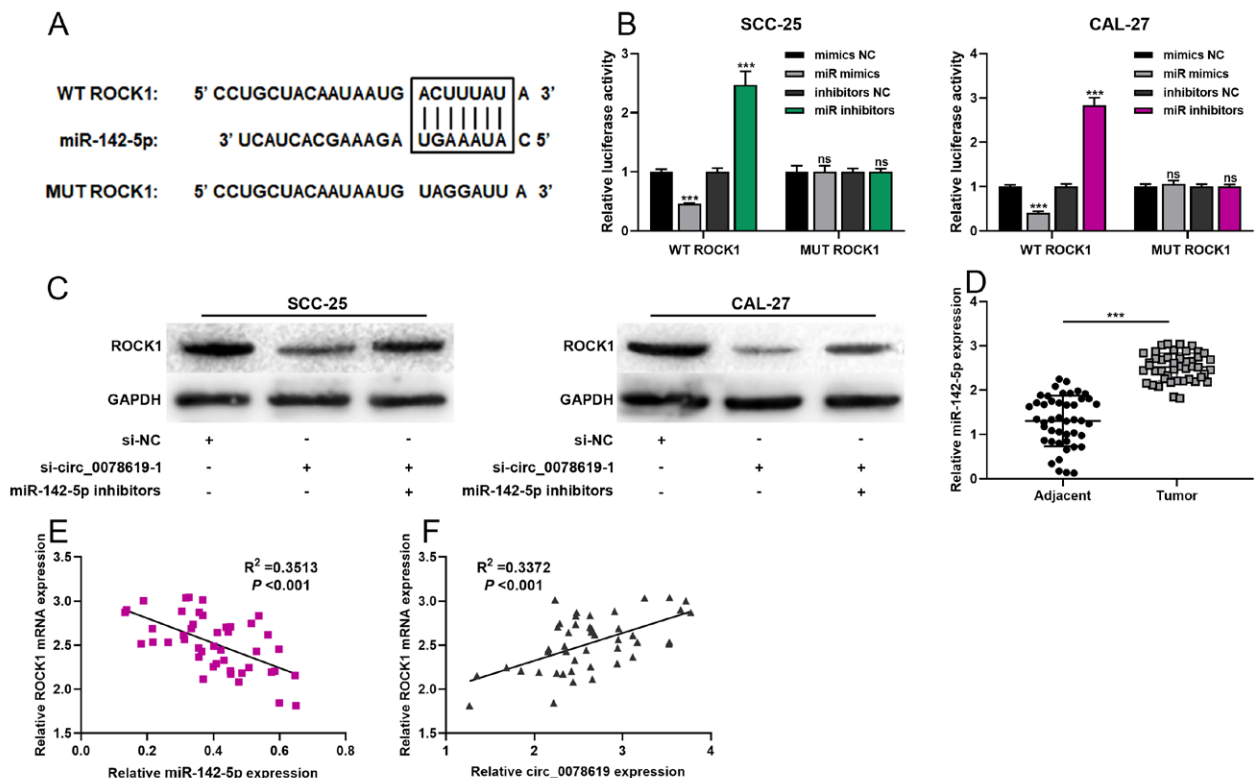


Fig. 4. Circ_0078619 up-regulated ROCK1 expression by adsorbing miR-142-5p. **A)** The online database TargetScan was used to predict the binding site between miR-142-5p and ROCK1 mRNA 3'UTR. **B)** Luciferase activities of WT ROCK1 reporter and MUT ROCK1 reporter were detected by a dual-luciferase reporter gene system after the transfection of miR-142-5p mimics or inhibitors. **C)** Western blot was used to detect the effects of circ_0078619 and miR-142-5p on ROCK1 protein expression. **D)** ROCK1 mRNA expression in 45 cases of OSCC tissues and adjacent tissues was detected by qRT-PCR. **E, F)** Pearson correlation analysis of the correlation among circ_0078619, miR-142-5p, and ROCK1 mRNA expressions in OSCC tissues. *** $P < 0.001$, and ns $P > 0.05$.

RNAs (ceRNAs) or interacting with RNA-binding proteins to exert biological functions, regulate cell cycle, proliferation, migration, angiogenesis, apoptosis, and other biological processes (19, 20). For example, circ-PKD2 can sponge miR-204-3p to up-regulate adenomatous polyposis coli 2 (APC2) expression to restrain OSCC cell growth, migration, and invasion, and induce cell cycle arrest and apoptosis (21); circ_0007059 can inhibit OSCC cell growth, migration, and invasion, and induce apoptosis by regulating the AKT/mTOR signal pathway (22). This study found that expression of circ_0078619 in OSCC tissues was significantly enhanced, which was associated with unfavorable

pathological characteristics. Additionally, knocking down circ_0078619 remarkably restrained OSCC cell growth, migration, and invasion, and facilitated apoptosis. The aforementioned results proved that circ_0078619 may be a therapeutic target for OSCC.

MiRNA is a recognized single-stranded non-coding RNA with a length of 19-22 nucleotides that mainly induce the translation inhibition or degradation of target mRNAs through binding to the 3'UTR and regulating gene expression at the post-transcriptional level, thus participating in multiple biological processes, including cell cycle regulation, epigenetic regulation, cell differentiation regulation (23-25). Recently, emerging studies showed that

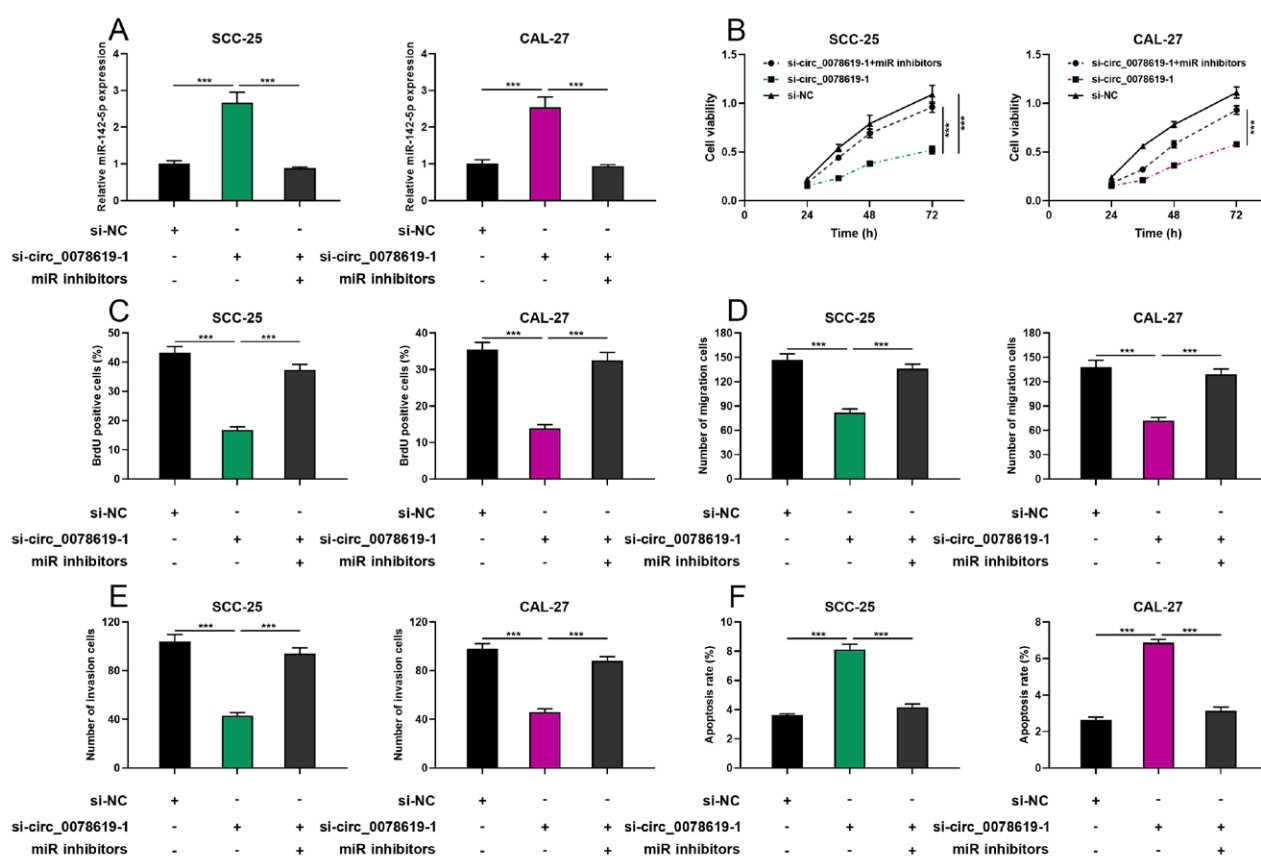


Fig. 5. The effects of circ_0078619/miR-142-5p axis on OSCC cell proliferation, migration, invasion, and apoptosis. **A)** The transfection efficiency of si-circ_0078619-1 and miR-142-5p inhibitors in SCC-25 and CAL-27 cells was detected by qRT-PCR. **B, C)** CCK-8 and BrdU assays were used to detect the effects of si-circ_0078619-1 and miR-142-5p inhibitors co-transfection on OSCC cell proliferation. **D, E)** Transwell assays were used to detect the effects of si-circ_0078619-1 and miR-142-5p inhibitors co-transfection on SCC-25 and CAL-27 cell migration and invasion. **F)** Flow cytometry was used to detect the effects of si-circ_0078619-1 and miR-142-5p inhibitors co-transfection on OSCC cell apoptosis. *** $P < 0.001$.

miRNAs feature prominently in OSCC occurrence and development. For instance, miR-155-5p can promote OSCC cell growth, migration, and invasion via reducing AT-rich interactive domain 2 (ARID2) expression, and highly expressed miR-155-5p is connected with the patient's TNM stage, tumor size, lymph node metastasis, and histological grade (26); miR-650 can promote OSCC cell growth, migration, and invasion via inhibiting growth factor independent-1 (Gfi1) expression (27). In addition, miRNA expression can be regulated by circRNA. For instance, circ_0005576 can serve as a sponge to decoy miR-153-3p to up-regulate kinesin family member 20A (KIF20A) expression, thereby promoting cervical carcinoma cell growth, migration, and invasion (28); circ-PVT1 up-regulates phexokinase II (HK2) expression by adsorbing miR-106a-5, thus promoting OSCC cell proliferation, metastasis, and glycolysis (29). Our study found that circ_0078619 directly targeted miR-142-5p. Further research suggested that circ_0078619 promoted OSCC cell growth, migration, and invasion, and inhibited apoptosis, via adsorbing miR-142-5p. The present study, for the first time, reported that miR-142-5p could be adsorbed by circ_0078619, which was a new explanation for abnormal miR-142-5p expression in OSCC.

Rho-associated kinase (ROCK) is a serine/threonine-protein kinase with a molecular weight of about 160 kDa, and is made up of approximately 1300 amino acid residues; it is expressed in various tissues and is classified into two subtypes, ROCK1 and ROCK2 (30-32). Reportedly, ROCK1 is implicated in the tumorigenesis and development of various tumors. For instance, ROCK1 can promote non-small cell lung carcinoma cell migration and invasion through modulating the PTEN/PI3K/FAK pathway (33); down-regulating ROCK1 expression can inhibit ovarian carcinoma cell migration and invasion (34). In OSCC, ROCK1 expression is remarkably elevated in OSCC tissues, and up-regulating ROCK1 expression *in vitro* facilitates OSCC cell growth, migration, and invasion (15, 35). This work revealed that miR-142-5p could bind to ROCK1 mRNA 3'UTR to negatively regulate its expression, and circ_0078619 could positively

regulate ROCK1 expression. Therefore, it could be concluded that circ_0078619 up-regulates ROCK1 expression by adsorbing miR-142-5p, thus facilitating the malignant progression of OSCC. Our findings further explain how ceRNA mechanism regulates the expression of ROCK1 in OSCC progression.

To sum up, for the first time, our study finds that expression of circ_0078619 is increased in OSCC, and high circ_0078619 expression is closely associated with the poor clinicopathological indicators of OSCC patients; in terms of mechanism, it is confirmed that circ_0078619 facilitates OSCC cell growth, migration, and invasion, and suppresses the apoptosis, via modulating the miR-142-5p/ROCK1 axis. The present study may provide new insights into early diagnosis, therapy, and prognosis judgement of OSCC.

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CircRNA (circ_0008057) promotes uremic serum-mediated proliferation and migration of vascular smooth muscle cells via miR-370/PLK1 signaling pathway

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In order to explore the mechanism of gefitinib-acquired resistance in lung cancer, a new biomarker has been developed for early clinical diagnosis and intervention; human NSCLC (Non-Small Cell Lung Cancer) cell lines H292 (denoted as H292S) and PC9 (denoted as PC9S) were used to establish gefitinib-resistant NSCLC cell lines H292 and PC9 models. CCK-8 (Cell Counting Kit-8) method was used to test the drug resistance of the cells. circRNAs (circular RNAs) that were differentially expressed before and after resistance were screened by RNA sequencing technology. The effects of circSETD3 overexpression and interference on the sensitivity of gefitinib was observed to analyze the nuclear localization of circSETD3 and verify the interaction between circSETD3-miR-520h-ABCG2. The results showed that the most significant change in differential expression of human NSCLC cell lines before and after drug resistance was hsa_circ_0000567, that is, circSETD3, which is mainly present in the cytoplasm. In H292S and PC9S, compared with the negative control group, the cell proliferation ability of the overexpression group was significantly increased, and the apoptosis ability was significantly decreased. In H292R and PC9R, compared with the negative control group, the proliferation ability of the interference group was significantly decreased, and the apoptosis ability was significantly increased. Overexpression of circSETD3 to H292S and PC9S, the expression of ABCG2 increased significantly. Also, the expression of ABCG2 decreased significantly after transfection with miR-520h mimics. H292R and PC9R interfered with circSETD3, the expression of ABCG2 decreased significantly. Moreover, the expression of ABCG2 increased significantly after transfection with miR-520h inhibitor. In conclusion, circSETD3 can be used as a novel biomarker for lung cancer. It relieves miR-520h degradation of the transporter ABCG2 by down-regulating the miR-520h expression, causing gefitinib to be pumped out of the cell.

End-stage renal disease (ESRD) is a global health problem. Severely impaired renal function leads to a series of metabolic disorder syndromes (1). Although the popularization and development of blood purification technology have prolonged lifespan of the patients for nearly half a century, their quality of life and long-term survival rate are

still seriously threatened (2). In recent years, studies on hemodialysis patients have found that the main pathological basis of calcification in the middle layer of arterial walls of patients is the differentiation of vascular smooth muscle cells (VSMCs) caused by the progressive deterioration of renal function (4). VSMCs are the main components of blood vessel

Key words: vascular smooth muscle cells; circ_0008057; miR-370; PLK1; uremic serum

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walls and have high plasticity in terms of proliferation, migration and transformation (5). Many studies have shown that the pathophysiological process of cardiovascular disease is often accompanied by changes in gene expression profiles. Advancing basic research has paid greater attention to circular RNA (circRNA), which has a stable closed-loop structure and is widely expressed in human cells (7). As a molecular sponge of microRNA (miRNA), it regulates the transcription and expression of parental genes. However, the expression profile of circRNA and its regulatory mechanism on the proliferation and migration of VSMCs are still unclear.

In this study, gene chip technology was used to investigate the effect of uremic serum on the expression profile of circRNA in smooth muscle cells. It also confirmed the role and mechanism of related circRNA and its downstream target signaling pathways in the proliferation and migration of vascular smooth muscle cells, thereby providing new theoretical and experimental basis for the role of catheter-related lesions and potential therapeutic targets.

MATERIALS AND METHODS

Culture and treatment of vascular smooth muscle cells

Human aortic smooth muscle cells (HASMCs) were purchased from ATCC company, USA. DMEM complete culture medium containing 50ml FBS + 5ml streptomycin double antibody + 445ml DMEM culture medium (Thermo Fisher, USA), was evenly mixed (aseptic operation), and divided into 50-ml sterile EP tubes and stored at 4°C. 1/10 of the final volume of the uremic serum and healthy serum was prepared, evenly mixed, and stored at 4°C. The 10% uremic serum was used as stimulation, and the healthy serum was used as a control.

Gene chip analysis

In this study, the arraystar human circRNA microarray was used to analyze the expression profile of human circRNA globally. About 5,780 types of circRNAs can be detected by this chip.

RNA labeling and hybridization with microarray

The samples were labeled and hybridized according to the Agilent Monochrome Microarray-based Gene Expression Analysis Protocol (Agilent Technologies,

Table I. *The sequence of primers*

Primers	Sequences (5'-3')
circ_0008057 forward	CTGGTGGAAATTGGCAGAGAG
circ_0008057 reverse	CGACCCACCTGGCTCTTCTC
β-actin forward	CGCTCTCTGAACCTCCTGTTC
β-actin reverse	ATCCGTTGCCGCCGACCTTCAC

Table II. *The primer sequences*

Primers	Sequences (5'-3')
si_circ_0008057-1	CACGCTAATTAACCTGTTGATA
si_circ_0008057-2	CCGCCCTTAGACAATAAGGTTG
NC	TTCTCCGAAATTCTCACGTTT

Santa Clara, California, USA). mRNA was isolated and purified from total RNA. Each sample was then enriched with random primers and transcribed into fluorescent cRNA without 3'offset. The labeled cRNAs were purified with Rnasy-Mini kit (Qiagen, Germany), and the concentration and activity of each cRNA were measured with a Nanodrop ND-1000 spectrophotometer. To each labeled cRNA, 5 sets of 1x0 blocking solution and lpl25x fragmentation buffer were added, followed by heating at 60°C for 30 min, and addition of hybridization buffer. The mixture was poured onto a padded glass slide. The slide was incubated for 17 h in an Agilent hybridization incubator at 65°C. Finally, an Agilent DNA microarray scanner was used to clean, fix, and scan the hybridization chip. Agilent feature extraction software (version 11.0.1.1) was used to analyze the chip image. Quantiles were standardized by the genespring gxxv11.5.1 software package (Agilent Technologies, Santa Clara, California, USA), and the subsequent data were processed. After standardizing the raw data, mRNAs with particular limit values (all target values) were selected for further data analysis. The filtration method was changed several times to determine the differentially expressed mRNAs. The Agilent-genespringx software (version 11.5.1) was used to draw heat maps and hierarchical clustering.

Extraction of total RNA from VSMCs

RNA was extracted from the cells once the fusion degree of the cells in the culture dish reached 80-90% or more. The cells were digested with 0.25% trypsin (Solarbio, China) and centrifuged at 1,000r, for 3 min. Later, the cells were washed with 1 ml PBS solution (Solarbio, China), and centrifuged again. The procedure was repeated twice, and finally the cells were transferred to an enzyme-free centrifuge tube. About 1 ml of triazole lysate (Solarbio, China) was added to the enzyme-free centrifuge tube to dissolve the cell pellet, and the shaker flask centrifuge tube was inverted. To the centrifuge tube 200 μ L of chloroform (J&K Chemicals, China) were added. The tube was shaken for 15 s and left at room temperature for 5 min. Later, the tube was cooled to 4°C and centrifuged in a pre-cooled ultracentrifuge for (12,000r, 15min, 4°C). After centrifugation, the liquid in the centrifuge tube formed three layers, with RNA located in the upper layer. The upper colorless aqueous solution was carefully and slowly absorbed using a pipette,

and transferred to an enzyme-free centrifuge tube for subsequent experiments.

The RNA concentration was measured with a ND-1000 spectrophotometer (Nanodrop, USA). Reverse transcription kit (Takara, Japan) was used to reversely transcribe RNA into cDNA. The specific primers were designed and synthesized using NCBI primers (Invitrogen, China) with β -actin as the internal parameter. Fluorescence quantitative PCR (Nanodrop, USA) was used to detect the expression of circRNA in osteosarcoma cells and normal cells. The Step-one-plus system was used to amplify the fluorescent quantitative PCR, and a 10- μ L reaction system was configured. With p-actin as the internal parameter, the corresponding CT value (threshold cycle number) was obtained by PCR amplification curve. The relative expression levels of genes were compared by calculating the corresponding $2^{-\Delta CT}$. The primer sequences are shown in Table I.

SiRNA transfection by lentivirus

The circRNA and Negative Control (NC) siRNA sequences used in this study were designed and synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd., China. The VSMCs in the logarithmic growth phase were selected and digested with trypsin and centrifuged to obtain cell pellets. The cells were resuspended in culture medium and seeded on 6-well plates with a cell density of 5×10^6 . About 2 ml of complete medium was added to the plates and incubated for 24 h. The original medium was drawn and the cell pellet was washed with PBS, and 5pg/mL puromycin was added to the pellet. At the same time, the lentivirus suspension (MOI=100) was added to the 6-well plate and stored for 24 h. Later, the original medium was aspirated and the 6-well plate was washed with PBS. A fresh McCoy's 5a medium containing 10% fetal bovine serum (FBS) was added to the 6-well plate. After culturing for 3-5 days, a stable circRNA knockout cell line was selected. The medium containing puromycin was added to the 6-well plate, and the cells were collected for other functional experiments. The primer sequences are shown in Table II.

CCK-8 method was used to detect cell viability

The cell counting kit (CCK-8) was purchased from Thermo Fisher Scientific. The transfected cells in logarithmic growth phase were digested with 0.25%

trypsin solution and centrifuged to obtain a cell pellet. The cell pellet was resuspended in culture medium and the cells were counted with a cell counting plate. Based on the results of cell counting, the cell suspension was diluted to about 5,000 cells/100 μ L. The diluted cell suspension was added to each well of the 96-well plate. The 96-well plate was incubated in a 37°C constant temperature incubator for an appropriate time (24, 48, 72, 96 h, respectively). 100 μ L of 10 % CCK-8 solution was added to each well of the 96-well plate. The plate was placed in a 37°C constant temperature incubator for 1-4 h. The SpectryMaxPlus34 (Molecular Devices, USA) continuous spectrophotometer was set to 450 nm wavelength to measure and record the absorbance of each well.

Transwell assay was used to detect cell migration and invasion

After the adherent cells became round or free, the cells were centrifuged in a centrifuge tube at 1,000 r for 3 min. Later, the cells were resuspended with basic medium, the number of cells was counted by cell counting plate, and the cell density was diluted to 3×10^5 cells/mL. 100 μ L of the diluted cell suspension was added into a Transwell chamber, and 500 μ L of 20% fetal bovine serum (FBS) McCoy's 5A medium was added into the lower chamber. The cells were cultured in a constant temperature incubator for another 24 h. The liquid in the upper chamber was drained. The upper chamber was taken out, and the cells were wiped with a cotton swab. The cells were moved into another well and fixed with methanol for 30 min. The dye was first washed with fresh water (about 30 μ L) and then with dry water for about 1 min. After cleaning, the polycarbonate film at the bottom was cut off with a surgical blade and placed on a clean glass slide. A drop of neutral resin was added on the polycarbonate film and the cover glass was placed. Finally, the treated polycarbonate film was observed under an inverted microscope and photographed.

Western blot assay

About 4-20% precast gel (Thermo Fisher, USA) was placed in the electrophoresis buffer and the sample was filled with 30 μ g protein per well. Each group had 3 composite holes. The power was turned on after connecting the positive and negative poles, the voltage was adjusted to 120V, and the electrophoresis was stopped

when it reached close to the bottom of the prefabricated rubber. The corresponding PVDF membrane was cut to a particular size and placed into methanol with preformed glue to wet and clean the membrane. It was soaked in electric buffer for further use. The pre-formed glue was taken out and cut into a suitable size. Then the pre-formed glue, sponge, filter paper and PVDF membrane were used to make an electric rotating sandwich. After each layer was laid, the wires were clamped. It was placed correctly in the electric rotating tank and stored in the refrigerator, and the power was turned on. The current was adjusted to 380 mA and the time was set to 100 min. Later the PVDF film was transferred in 5% skimmed milk powder and sealed for 45 min. The film was rinsed with pure water and the diluted primary antibody (PLK1) was added. It was shaken overnight in the incubator at 4°C. PVDF membrane was washed twice, 15 min each time, with PBST. The secondary antibody (Abcam, USA) was diluted with skimmed milk powder and incubated for 1 h at room temperature. It was washed twice, 15 min each time, with PBST. The ECL solution A and B were mixed at a ratio of 1:1. The PVDF membrane was taken out and the PBST was drained. The ECL luminescent liquid was added evenly on the membrane, the PVDF membrane was covered with a plastic wrap and imaged in a dark room. The film was scanned onto a computer using a scanner (E-Gel Imager, Thermo Fisher, USA) and the optical density was measured with Image-J analysis software.

Statistical analysis

SPSS 20.0 (IBM, USA) and Graphpad Prism 7.0 (GraphPad Software, USA) were used to analyze the relevant data in this study, in which the mean $\pm$ standard deviation was used to represent the analyzed data. *Chi*-squared test, F test and analysis of variance were used to analyze the data. $p < 0.05$ indicated significant difference.

RESULTS

Expression profile of circRNAs in VSMCs

In this study, uremic serum was used *in vitro* on pre-cultured human VSMCs to simulate the injury of VSMCs observed in uremic patients under hyperglycemia. At the same time, the healthy serum culture group was used as a control for the study. After 48 hours of culture, the gene chip was used to

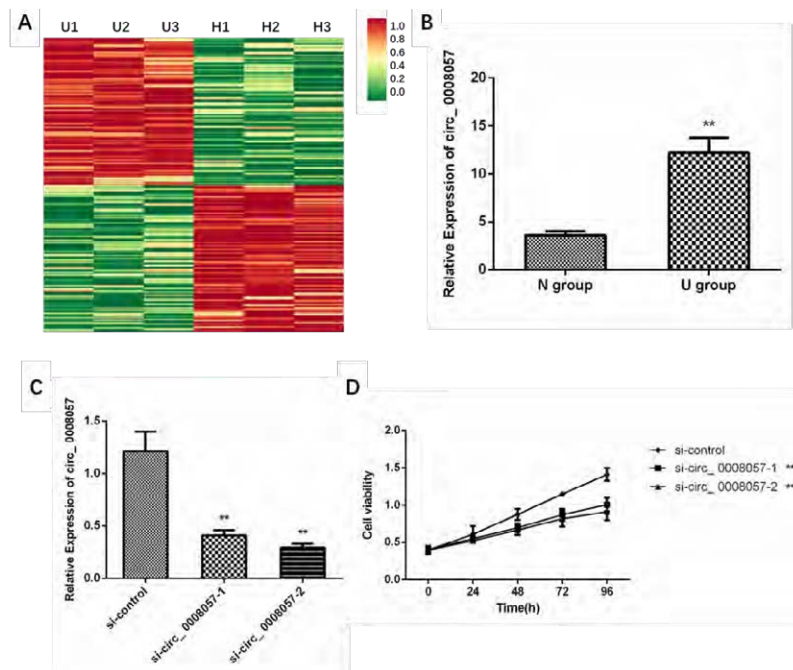


Fig. 1. *A*) Expression of circRNA was detected by microarray. The results of uremic serum group (U) and healthy serum group (H) were shown in thermogram; *B*) RT-PCR showed that compared with normal serum group, the group of VSMCs intervened by uremic serum showed significantly upregulated expression of circRNA. The ordinate is the relative expression of circ_0008057. ** $P < 0.01$; *C*) uremic serum induces circRNA expression in siRNA transfected VSMCs; *D*) MTT assay showed that the number viable of cells in knockdown group decreased significantly.

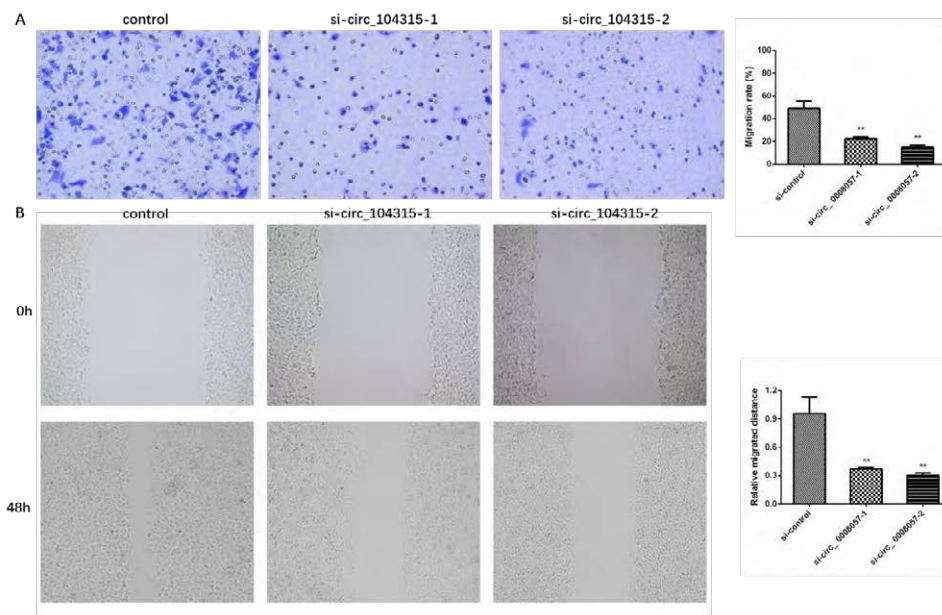


Fig. 2. *A*) Transwell chamber migration experiment. The percent of migration rate in the control group and circ_0008057 knockdown group. The vertical coordinates of the three groups indicated the number of cells in each field, *B*) The cell migration distance was observed by scratch test in control group and circ_0008057 knockdown group. The vertical coordinate indicated the distance of cell migration.

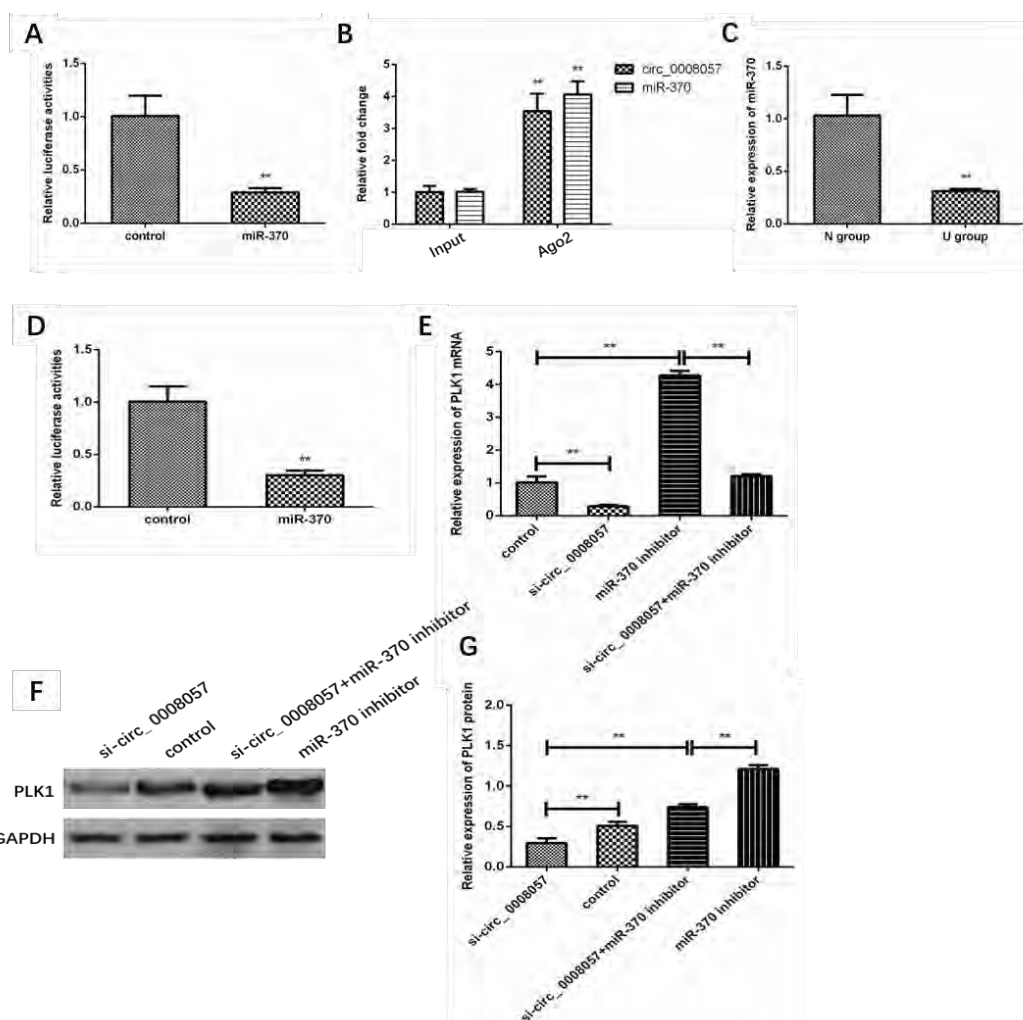


Fig. 3. *A)* Luciferase assay showed that luciferase activity was regulated by wild-type or mutant circ_0008057 and miR-370; *B)* Rip assay showed that circ_0008057 and miR-370 were bound to Ago2; *C)* The expression of miR-370 was detected by RT-PCR in VSMC of normal glucose group and high glucose group. *D)* Luciferase assay showed that luciferase activity was regulated by PLK1 and miR-370; *E)* The expression of PLK1 mRNA was detected by RT-PCR; *F,G)* The expression of PLK1 protein was detected by Western blot.

analyze the expression profile of circRNA in the two groups. The results showed that after the treatment with uremic serum, there were 925 differentially expressed circRNAs in the uremic serum intervention group, including 450 up regulated and 475 down regulated circRNAs (Fig. 1A). The circ_0008057 was selected as the subject of this study because it was the up-regulated factor of about 5 times of the control group ($P < 0.01$) (Fig. 1B).

Effects of circ_0008057 on the survival number of VSMCs

In the cultured VSMCs treated with uremic

serum, circ_0008057 showed a significant up-regulation trend, which suggested that circ_0008057 might play an important role in regulation of the proliferation and migration of VSMCs. In this study, a specific siRNA was synthesized to knock down circ_0008057 which reduced the circRNA by more than 60% ($P < 0.01$) (Fig. 1C). The above-mentioned hypothesis was verified in reverse. si-circ_0008057 was transfected in the uremic serum treated culture group of VSMCs. The expression of circ_0008057 was significantly decreased. However, MTT assay showed that the expression of circ_0008057 from the second day of culture was significantly lower

than that of the control group. The knockdown of circ_0008057 could reduce the number of viable VSMCs ($P < 0.05$) (Fig.1D).

Effects of circ_0008057 on cell migration of VSMCs

The effect of circ_0008057 on the migration of VSMCs was investigated using Transwell migration test and scratch test. In VSMCs transfected with siRNA, the number of migration cells was significantly reduced by about 55% compared with the control group ($P < 0.01$) (Fig. 2A). Further scratch test showed that the knockdown of circ_0008057 reduced the migration distance of VSMCs by about 60% ($P < 0.01$) (Fig. 2B). Therefore, circ_0008057 inhibited the migration of VSMCs.

circ_0008057 targeted miR-370

Bioinformatics analysis showed that circ_0008057 contained a binding site of miRNA (miR-370). The luciferase activity measured by luciferase assay showed that compared with the normal control group, the luciferase activity of VSMCs decreased significantly by 57% ($P < 0.01$) in uremic serum treated VSMCs (Fig.3A). However, further RNA immunoprecipitation showed that circ_0008057 and miR-370 were significantly enriched about 3-fold ($P < 0.01$) by binding with Ago2 (Fig. 3B). The RNA sequencing data of Ago2 precipitation indicated that circ_0008057 was bound to Ago2 and acted as a miRNA sponge with differential expression of circRNA. The expression of miR-370 in the VSMCs group treated with uremic serum was significantly down-regulated by 69% ($P < 0.01$), compared with the normal group (Fig. 3C), therefore, circ_0008057 attached to the binding site of miR-370 and played a regulatory role.

The regulation pathway of circRNA circ_0008057/miR-370/PLK1 in VSMCs

Bioinformatics analysis predicted a binding site of PLK1 on miR-370 gene. Luciferase reporter gene analysis showed that luciferase activity was regulated by PLK1 and miR-370. The activity of luciferase was significantly decreased ($P < 0.05$), suggesting that miR-370 and PLK1 can bind directly (Fig. 3D). The results of the present study found that

compared with the control group, the expression of PLK1 mRNA in VSMCs transfected with siRNA was significantly decreased ($P < 0.01$). Compared with the control group, the expression of PLK1 mRNA was significantly increased after adding miR-370 inhibitors ($P < 0.01$) (Fig. 3E). Hence, miR-370 inhibitors significantly decreased the expression of PLK1 protein ($P < 0.01$) in VSMCs after circ_0008057 knockdown (Fig. 3 F, G). This study found that knockdown of circ_0008057 decreased the expression of PLK1, which could be reversed by miR-370 inhibitors, suggesting that miR-370 mediated the regulation of PLK1 by circ_0008057.

DISCUSSION

CircRNA is a special non-coding RNA which was discovered recently. It is an important member of the RNA family like relay miRNA and long non-coding RNA. CircRNA has recently become a research hotspot in many fields and many studies on the relationship between circRNA and vascular diseases have been reported (9). CircRNAs are involved in a variety of biological behaviors such as cell proliferation, differentiation, senescence, and tumor formation (10). In the hyperuremia serum state of uremic patients, atherosclerosis is more likely to occur and progress. The changes in expression of circRNAs in VSMCs may be related to a variety of biological behaviors of those cells (11). This study screened the differential expression of circRNAs between the group of human VSMCs intervened by uremic serum and normal serogroup cells through high-throughput chip analysis. The chip analysis data of circRNA expression was verified by RT-PCR, and predicted the difference through bioinformatics analysis. The potential targets of circRNA expression were further screened for the differential expression of circ_0008057, and its possible target miR-370 was used in functional experiments. In VSMCs, circ_0008057 can target PLK1 through miR-370 and regulate various cell behaviors such as cell cycle progression, cell proliferation, and cell migration. This study explored the regulatory role of circRNA in human VSMCs, elucidated the innovative molecular mechanism of the development of atherosclerotic

disease in patients with advanced uremia, and clarified the pathogenesis and therapeutic targets of circRNA-related vascular disease. Therefore, the results of this study provide novel theoretical basis and experimental support.

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LETTER TO THE EDITOR

Early rehabilitation program in SARS-CoV-2 hospitalized patients during the first lock-down: an Italian experience

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To the Editor,

During the first lockdown period (March 9-June 3, 2021), following the COVID-19 pandemic (CoronaVirus Disease 2019), admission rates to an Intensive Care Unit (ICU) were approximately 5% of all patients who were admitted with COVID-19 (1). About 42% of patients required oxygen-therapy. Based on the emerging data, the individuals at highest risk of developing severe COVID-19 disease, requiring hospitalization and/or ICU support, are older, male, with at least one coexisting comorbidity, scores of higher severity (measured by SOFA scores), elevated D-dimer levels and/or lymphocytopenia (1). The percentage of patients in ICU reported daily in Italy between March 1 and 11, 2020, was consistently between 9% and 11% of infected patients (1).

The importance of rehabilitation after COVID-19 has been widely highlighted in the literature (2, 3). There are some specific aspects different from the traditional rehabilitation process such as the need to receive patients from acute care wards and at the same time, discharge patients to home earlier

than usual. Thus, it is imperative that rehabilitation begins in the acute care ward and continues after transfer to the rehabilitation ward (2-5). Pulmonary-rehabilitation, for a personalized and progressive treatment plan for the individual patient, should include early mobilization with frequent changes in posture, active, passive and active-assisted mobilization, training to achieve a sitting-standing position and simple exercises to be performed in bed (2-5).

Some specific aspects of rehabilitation activities can lead to difficulties in harmonizing therapeutic needs with the measures required to protect professionals and prevent the spread of the infection (6). A systematic review has mainly emphasized the heterogeneity of targeted populations that were offered early exercise and lack of precision regarding eligibility criteria, program modalities, start times, and progression and discontinuation criteria (7). Usually, for the Italian rehabilitation organization, patients transferred from the acute intensive care unit must always be clinically stable and, therefore,

Key words: Covid-19; rehabilitation; subacute care; infectious disease

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based on the diagnosis and functional evaluation, return to specific hospital settings for post-acute rehabilitation. In the period of the Covid emergency, in order to guarantee a rapid recovery of the patient and a rapid home discharge, in consideration of the high demand for beds, rehabilitation was also provided in the medical departments.

Starting from these premises, the purpose of this clinical study is to describe an Italian rehabilitation experience of a rehabilitation path dedicated to patients who have just been stabilized and transferred from the ICU to the infectious disease division during the COVID emergency.

MATERIALS AND METHODS

We referred to the single-subject design model, which is suitable for research in rehabilitation in respect to a retrospective observational study (8). Assessments were conducted at the Sub-Intensive Care Unit (sICU) or Infectious Diseases Division (IDD) division, during the patients' hospitalization during the first lock down period. Patients were consecutively recruited from the sICU and the IDD of the S.Spirito Hospital (Pescara, Italy). Inclusion criteria were adults (≥ 18 years) admitted to hospital in sICU or IDD, whose clinical condition allowed for a rehabilitation exercise program based on an ICU physician decision in accordance with the physical rehabilitation physician. The exclusion criteria were history of severe trauma (head, spine, lower limbs or pelvis) and musculoskeletal, neurological conditions or cognitive impairment that did not allow the patient to follow the proposed rehabilitation program. This study was performed according to the Helsinki Declaration on human experimentation and was approved by the Departmental Committee of Oral Medical Science and Biotechnology of University of Chieti (Italy) (protocol number 3439-CI 13). All patients signed informed consent forms after receiving detailed information about the study's aim and procedures. The rights of human subjects who were involved in the study were protected.

Rehabilitative proposal while in hospital

The standardized early exercise program began after clinical stabilization and continued until discharge (six days/week; 30-60 min each). Specifically, during the first

week, patients underwent a 30-minute training session; from the second week the duration of the session was gradually increased to 60 minutes, with rest breaks based on the patient's tolerance conditions and, if necessary, dividing the session into a morning and an afternoon session of 30 minutes each.

Rehabilitative Plan

Phase 1. Postural changes in lateral/prone position and in a sitting position on the bed; deep breathing and respiratory exercises (exercises aimed at making breathing conscious and promoting the activation of the diaphragm muscle through phases of deep exhalation); weaning from the ventilator; Active exercises to promote venous return; passive/active assisted mobilization.

Phase 2. Postural passages in a sitting position on the bed and chair and in standing position; low intensity calisthenics exercises; deep breathing and breathing exercises (exercises aimed at making breathing conscious and promoting the activation of the diaphragm muscle through phases of deep exhalation); active exercises to promote venous return; passive mobilization and assisted activity.

Phase 3. Postural passages in sitting position on bed and chair and in standing position; low intensity rhythmic gymnastic exercises; deep breathing and breathing exercises (exercises aimed at making breathing conscious and promoting the activation of the diaphragm muscle through phases of deep exhalation); active exercises to promote venous return; passive mobilization and assisted activity; exercises for balance; assisted walking for short distances.

Suspension criteria for exercise

Criteria for stopping the rehabilitation session included changes in hemodynamic parameters, tachycardia or bradycardia, heart rhythm or ECG abnormalities such as to suggest the suspension of rehabilitation training, changes in ventilation parameters, paradoxical/asynchrony breathing, excessive respiratory effort, agitation or hemoptysis. All the rehabilitation procedures respected the contact isolation measures of the COVID guidelines (9).

Outcome measures

The Short Physical Performance Battery (SPPB) and the Clinical Frailty Scale (CFS) were used to evaluate functioning in older people and to predict the outcomes of elderly people hospitalized with acute illness, respectively (10).

Statistical analysis

Wilcoxon's non-parametric test for paired samples was used to evaluate the differences between the parameters in the two times (T0, T1). The results were presented in the form of median and range of variation (minimum, maximum).

RESULTS

Data from twenty-eight patients (53.5% females, 46.5% males; mean-age: 68.07 ± 17.42 years) are reported (Table I). Upon admission to the COVID medical division, patients presented with ventilated-mask assisted respiration (65%) or non-invasive ventilation (NIV) (25%), while 10% of patients presented with cannula-assisted respiration

tracheostomy. Upon discharge, 86% of patients were discharged spontaneously into ambient air, while 14% were discharged with a prescription to continue oxygen therapy at home. Furthermore, on average during hospitalization, patients lost 1.5 ± 0.8 Body Mass Index (BMI) points; 68% of patients had hypertension (48% hypertensive heart disease), 30% had diabetes mellitus, 26% had an association of hypertension and diabetes mellitus. and 12% had chronic obstructive pulmonary disease.

Compared to the T0 rating scales, patients reported a bed rest and functional deconditioning condition for median SPPB of 0 (0-3) and for CFS of 8 (6-8), with a good response after rehabilitation and Overall physician care ($p < 0.001$) for SPPB at T1

Table I. *Characteristics of patients*

Characteristics of the patients	Value
Age (mean±standard deviation)	68.07 ± 17.42
Sex (%)	53.5 (F); 46.5 (M)
Days of hospitalization (mean±standard deviation)	43.9 ± 14.75
Body Mass Index (mean±standard deviation)	22.17 ± 2.4 (T0); 20.58 ± 1.2 (T1)
Days of rehabilitation (mean±standard deviation)	32.5 ± 10.21
Respiratory devise (%)	25% NIV, 65% ventimask, 10% tracheostomy

F: female; M: male; NIV: non-invasive ventilation

Table II. *Wilcoxon test*

Parameters	T0	T1	p value
Balance	0 (0-1)	4 (0-4)	<0.001
Step	0 (0-1)	1 (0-4)	<0.001
5 STS	0 (0-1)	2 (0-4)	<0.001
SPPB	0 (0-3)	6 (0-12)	<0.001
CFS	8 (6-8)	5 (3-7)	<0.001

SPPB: Short Physical Performance Battery; CFS: Clinical Frailty Scale; 5 STS: Five Times Sit to Stand Test

with a median of 6 (0-12) and a median of 5 (3-7) for CFS, respectively (Table II).

DISCUSSION

The purpose of our consultation was to assess the progress of these patients' daily functioning after discharge from ICU. According to the literature, an important rehabilitation profile, which emerged with respect to our results, was that patients need all the respiratory-rehabilitation procedures and to be gradually reconditioned to a functional physical activity for assuming the sitting position, and, subsequently, to walking. However, the functional recovery data that patients showed at discharge indicate the need to continue the "*ad personam*" rehabilitation process in the home environment, where outpatient rehabilitation, in the immediate lockdown, was not easy to organize (11, 12). Then, a physician expert in rehabilitation should be taken into consideration and a dedicated intervention set up after thorough assessment of the patient's clinical condition, in collaboration with all rehabilitation team professionals.

Patients on discharge, although no longer in bed and weaned from assisted breathing, showed a median CFS score of 5, indicating greater vulnerability to external stressors and the need for care at discharge (as a moderately fragile). Similarly, on the SPPB scale, patients showed good but not complete recovery, with a median score of 6, continuing to experience problems with balance, uncertainty in walking at reduced speed and often needing a walker, and difficulty in walking (12).

Thereafter, patients received advice to gradually increase their daily living activities and were given functional physical exercises to be performed at home with the supervision and assistance of the home physiotherapist (hospital therapist who supervises the patient at home on a scheduled basis). Furthermore, the patients were contacted by telephone (by the physiotherapist and/or by the physician specializing in physical and rehabilitation medicine) about two weeks after discharge, waiting to start the correct home rehabilitation-treatment.

A limitation of our work, linked to the emergence

of the situation, was the lack of information collected in terms of evaluation scales on the psycho-emotional sphere, which deeply affected patients in the most fragile age group, but from which, as narrative medicine, we have gained experience in the daily stories of the patient throughout the care process. In conclusion, it must be considered that patients in the post-critical phase following COVID-19 may experience signs and symptoms related to prolonged bed rest, previous and current respiratory problems as well as the cognitive-emotional sphere. Furthermore, prolonged bed rest can cause musculoskeletal deconditioning, which is the main cause of exertional dyspnea which, in patients with previous respiratory diseases, can be even more serious. Therefore, the need for rehabilitation as soon as possible for these fragile patients during hospitalization is emphasized to continue in an outpatient or home rehabilitation environment according to a "tailor-made rehabilitation".

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LETTER TO THE EDITOR

Plasma brain natriuretic peptide levels in children with idiopathic epilepsy treated with long-term sodium valproate and oxcarbazepine monotherapyG. Vartzelis¹, A. Attilakos², C. Tsentidis¹, I. Kalimeraki¹, D. Maritsi¹, A. Marmarinos¹ and A. Garoufi¹

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To the Editor,

Brain natriuretic peptide (BNP) was first discovered in hypothalamus but it is mainly secreted by the heart in response to increased ventricular wall stress associated with heart failure and it is recognized as an important and reliable biomarker for cardiac dysfunction (1, 2). BNP has been studied extensively in adults and has been shown to increase significantly in relation to cardiovascular, renal and pulmonary disorders (3). Children and infants with structural cardiac defects, dilated cardiomyopathy, pulmonary hypertension and sepsis, have all been found to have increased levels of BNP (2, 4).

Recent studies postulate that epileptic activity may trigger BNP secretion in the short term, either directly by the cerebral tissue or indirectly by the heart, as a result of the cardiovascular overactivity that accompanies epileptic seizures (5-8). Nevertheless, several aspects of BNP concentrations in epileptic patients remain unanswered such as their long-term changes after the seizures or the possible effects of antiepileptic drug treatment. Antiepileptic drugs may have an impact on autonomic cardiac function and/or cardiovascular risk factors and, therefore, a possible effect on BNP secretion (9).

This prospective study aimed to evaluate BNP

plasma concentrations in children with idiopathic epilepsy, during long-term treatment with sodium valproate (VPA) or oxcarbazepine (OXC) monotherapy.

MATERIALS AND METHODS

The study was carried out in a tertiary pediatric referral center in Athens, Greece. Subjects were derived from patients treated for idiopathic epilepsy. Written informed consent was obtained from all parents before participation and the study was approved by the local ethics committee.

Inclusion criteria were age between 4 and 15 years with a diagnosis of idiopathic epilepsy (focal or generalized). Exclusion criteria were previous use of any antiepileptic medication, chronic illness, history of cardiac disease, chronic use of other medication during the study period, developmental deficits and abnormal MRI findings. All patients underwent a brain MRI apart from those with a certain diagnosis of epileptic syndromes (childhood absence, juvenile myoclonic and epilepsy with centrotemporal spikes). All patients who required at some point add-on therapy with a second antiepileptic drug (AED) were also excluded from the study.

After applying the exclusion criteria, the final study population comprised 59 children (29 boys),

Key words: anti-epileptic drugs; brain natriuretic peptide; children; convulsions.

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aged 4 to 15 years (mean age 8.9 ± 2.9 years, median age 8.8 years). Patients were treated with VPA ($n=32$) or OXC ($n=27$) monotherapy based on clinical and electroencephalographic criteria. The maintenance dose was gradually titrated to the usual therapeutic doses (25-40 mg/kg/day for VPA and 25-35 mg/kg/day for OXC). Patients were followed for a period of 20 months. One hundred and forty three healthy children (70 boys, 4 to 15 years, mean age 10.9 ± 2.8 years, median age 10.8 years) were used as controls.

In all children, Body Weight (BW), Height (H) and Blood Pressure (BP) were measured and Body Mass Index (BMI) was calculated using the standard Quetelet formula [$BMI = BW (Kg) / H^2 (m^2)$]. For the classification in normal weight, overweight and obese, international growth charts

were used and Standard Deviation Scores (SDS-BMI, z-scores) were derived for the study population.

Complete blood count and basic chemistry panel were investigated in fasting morning blood venous samples. For the assessment of BNP levels, venous blood samples were collected in EDTA tubes and were placed in ice and immediately centrifuged at 4000 rpm for 10 min at 4°C. The extracted plasma was stored at -60° C until measurement. Brain natriuretic peptide fragment was investigated by enzyme immunoassay (EIA) using a commercially available kit ("Biomedica Gruppe", Austria). The Intra Assay and Inter Assay Variations were 4% CV and 3.8% CV, respectively and the Detection Limit 5 fmol/ml.

In the epileptic group the investigations were performed

Table I. Study population characteristics

mean $\pm$ SD, median (range)	overall	patients (n=59)	controls (n=143)	
age	10.29 $\pm$ 2.96, 10.25 (4.16, 16.83)	8.83 $\pm$ 2.98, 8.8 (3.7, 15.25)	10.89 $\pm$ 2.75, 10.83 (5.83, 16.83)	P<0.001*
Gender (boys/girls)	99/103	29/30	70/73	
SDS BMI (z-scores)	0.56 $\pm$ 1.06, 0.48 (-1.69, 3.21)	0.80 $\pm$ 1.25, 0.94 (-1.54, 3.21)	0.47 $\pm$ 0.97, 0.34 (-1.69, 2.62)	P=0.031*

*ANOVA between patients and controls. BMI: Body Mass Index

Table II. Plasma brain natriuretic peptide (BNP) levels (mean $\pm$ SD) in children receiving OXC or VPA (group A) and in healthy controls (group B)

	BNP1 (8 months) fmol/ ml	BNP2 (20 months) fmol/ ml	BNP1 vs BNP2
Group A (n=59)	545.62 $\pm$ 191.91	645.01 $\pm$ 227.64	p=0.0019*
Group B (n=143)	694.58 $\pm$ 273.82		
Group A vs Group B	p=0.001*	p=0.12*	

*ANCOVA adjusted for gender, age and BMI effect. BNP: Brain Natriuretic Peptide; OXC: Oxcarbazepine; VPA: Valproate

at 8 and 20 months (time 8 and time 20, respectively) of VPA (n=32) and OXC (n=27) monotherapy. In the control group, the investigations were performed once. At the time of testing, all subjects were free of acute illness and at least 2 months seizure-free.

Statistical analysis

Data analysis was performed using STATA for Windows v 11.2, (Stata Corp, Texas, USA, 2009). Age, SDS-BMI and BNP values exhibited normal distribution, so parametric analysis was performed. SDS-BMI and age values were compared with analysis of variance (ANOVA), while BNP values between groups were compared with analysis of covariance (ANCOVA), adjusted for the effect of gender, age and SDS-BMI with Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

RESULTS

During the 20-month period all patients had an absolute response to the treatment, remaining seizure-free after 6 months of therapy.

Study population characteristics and SDS-BMI distribution are described in Table I. Patients differed when compared with controls, regarding age and SDS-BMI. No correlation was found between BNP levels and gender ($p=0.44$), age ($p=0.45$), SDS-BMI ($p=0.56$) or pubertal status ($p=0.6$) of the subjects. Since previous studies reported differences in BNP

values after gender, age and BMI stratification, we performed analysis of BNP levels with gender, age and BMI as covariates.

When compared with controls, epileptic children had significantly lower BNP levels at time 8 ($p=0.001$), but similar levels to controls at time 20 ($p=0.12$). Compared to levels at 8 months, BNP levels were significantly increased at 20 months of therapy ($p=0.0019$) (Table II). There were no significant differences in BNP levels between the VPA and OXC group at any time during the study (8 and 20 months, respective p values 0.21 and 0.32) (Table III). BNP levels had no significant correlation with BMI in healthy children and children with epilepsy at 8 and 20 months of treatment. Twenty month post-therapy BNP levels were lower in children with generalized epilepsy compared to those with focal seizures ($p=0.01$) (Table IV).

DISCUSSION

In the present study, plasma BNP levels were lower than controls at the 8th month of OXC or VPA monotherapy after adjustment for gender, age and BMI. However, the long-term use of the above antiepileptic drugs was associated with an increase of BNP levels at 20th months of treatment, similar to control group levels. There were no significant differences in BNP levels between the VPA and OXC group at 8 or 20 months of treatment. Moreover,

Table III. Plasma brain natriuretic peptide (BNP) levels (mean±SD) in children receiving OXC, VPA

	BNP1 (8 months) fmol/ml	BNP2 (20 months) fmol/ml	BNP1 vs BNP2
OXC (n=27)	566.8±168.8	628.4±202.6	$p=0.06^*$
VPA (n=32)	527.17±211.0	657.58±247.6	$p=0.0024^*$
OXC vs VPA	$p=0.21^*$	$p=0.32^*$	

*ANCOVA adjusted for gender, age and BMI effect. BNP: Brain Natriuretic Peptide; OXC: Oxcarbazepine; VPA: Valproate

Table IV. Plasma brain natriuretic peptide (BNP) levels (mean±SD) in children regarding seizure type, generalized or focal

	BNP1 (8 months) fmol/ml	BNP2 (20 months) fmol/ml	BNP1 vs BNP2
Generalized epilepsy (n=29)	500.7±119.0	558.2±164.9	p=0.036*
Focal epilepsy (n=30)	558.4±176.5	672.4±173.2	p=0.009*
Generalized vs Focal epilepsy	p=0.07*	p=0.01*	

*ANCOVA adjusted for gender, age and BMI effect. BNP: Brain Natriuretic Peptide

children suffering from generalized seizures had significantly lower BNP levels at 20 months post-therapy, compared to those with focal seizures.

BNP secretion after convulsive seizures has been related to transient myocardial ischemia and/or brain dysfunction that returns to normal after 24-48 hours to 7 days (5-8, 10). The lower BNP levels 8 months after the initiation of VPA or OXC monotherapy, the elevation of BNP in the epileptic group at 20 months of treatment at levels similar to the ones of controls, and the differences in BNP levels between children with generalized compared to focal seizures, indicate a possible role of antiepileptic drugs and/or epilepsy itself. Antiepileptic drugs may have an impact on autonomic cardiac function and/or cardiovascular risk factors and, therefore, a possible effect on BNP secretion (9). Considering the controversies that exist in respect to the paradox of low BNP levels in individuals with metabolic cardiovascular risk factors (11), the transient decrease in BNP levels at 8 months of VPA or OXC monotherapy might be associated with the cardiometabolic effect of antiepileptic drug treatment. On the other hand, optimized seizure control may be related to lower BNP levels, since epileptic activity in the brain can activate BNP secretion in both the heart and the brain (5-8).

Limitations of our study are the small number of patients enrolled, the absence of BNP measurement in the first hours after the seizure attack and the absence of a group with uncontrolled seizures.

The advantages are the prospective design and the homogeneity of the study groups.

In conclusion, long term use of VPA or OXC monotherapy in children with idiopathic epilepsy was associated with an increase of BNP at levels similar to the ones of controls. Further long-term prospective studies are required in order to clarify the clinical significance of the above observation and whether this is the direct result of the antiepileptic medication or the result of epilepsy itself and/or the optimized seizure control.

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LETTER TO THE EDITOR

Effect of CRISPR/Cas9 system-mediated NF- κ B knockdown on CNE-2 immune function in nasopharyngeal carcinoma

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To the Editor,

The HLA-I antigen processing machinery (APM) plays a crucial role in anti-cancer immune responses (1). Loss of surface-expressed HLA-I molecules is particularly important, as these enable tumor cells to evade recognition and lysis by cytotoxic T-lymphocytes (2). Transcriptional control of APM genes is regulated by the nuclear factor kappa-B (NF- κ B). BamH1 fragment C rightward-reading frame No. 1 (BCRF-1) is an Epstein–Barr virus (EBV) homolog of human interleukin (IL)-10 (hIL-10), and is known as viral IL-10 (vIL-10), which shares various immune suppression similarities, but lacks the immune-stimulatory effect when compared with hIL-10 (3).

It is known that the downregulation of TAP-1, TAP-2, LMP-2, LMP-7, and HLA-I in nasopharyngeal carcinoma (NPC) induces immunosuppression (4). Previous studies suggest that the inhibition of NF- κ B activity may be an important mechanism for the vIL-10 suppression of APM (TAP-1, TAP-2, LMP-2, LMP-7,

and HLA-I) gene transcription in CNE-2 cells.

CRISPR/Cas is a prokaryotic cell-specific immune system that targets exogenous genetic material. In CRISPR/Cas technology, exogenous DNA is cleaved and degraded, and mediated by sequence-specific RNA. These exogenous genetic materials include phage or exogenous plasmids (5). Compared with ZFN and TALEN technology, the targeting efficiency of CRISPR/Cas (up to 80%) is relatively higher than both of these (6) and is flexible and convenient to design the target site. Therefore, a stable cell line with the knockdown of NF- κ B p65 by CRISPR/Cas, a stable cell line overexpressing BCRF-1, a stable cell line with the knockdown of NF- κ B p65 by CRISPR/Cas and the overexpression of BCRF-1 were constructed, in order to verify whether vIL-10 inhibits the expression of “operon,” which is processed and presented by major histocompatibility complex-I (MHC-I) class antigens through the NF- κ B pathway.

Key words: nasopharyngeal neoplasms; nuclear factor kappa-B (NF- κ B); viral interleukin-10 (vIL-10); CRISPR/Cas9

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MATERIALS AND METHODS

Human poorly differentiated NPC cell line CNE-2 was purchased from the Cancer Institute of Yunnan Cancer Hospital. NF- κ B p65 CRISPR/Cas9 KO plasmid was purchased from Santa Cruz (USA). NF- κ B p65 antibody was purchased from Cell Signaling Technology (USA). TAP-1 polyclonal antibody, TAP-2 antibody, LMP-2 antibody, and LMP-7 polyclonal antibody were all purchased from Elabscience (China). Rabbit anti-HLA class I (HLA) polyclonal antibody was purchased from Proteintech (USA). Goat anti-mouse IgG secondary antibody (unconjugated) and goat anti-rabbit IgG secondary antibody HRP (conjugated) were purchased from Signalway Antibody LLC (USA). This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval of the Ethics Committee of Third Affiliated Hospital of Kunming Medical University.

CNE-2 is undifferentiated human NPC cell line. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, America) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, America), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin. Cells were cultured at 37°C in a humidified incubator with 5% CO₂. Cells in the logarithmic growth phase were used in all experiments. Prior to treatment, cells were seeded in a serum-free medium overnight.

NF- κ B p65 CRISPR/Cas9 KO plasmid transfecting CNE2 cells: 24 h before transfection, 0.6–2.4 $\times 10^6$ cells were inoculated in 4 mL antibody-free medium and transferred onto a 60 mm petri dish. The fusion degree of cells was 80–90% upon transfection. Eight μ g of NF- κ B p65 CRISPR/Cas9 KO plasmid DNA was diluted with 500 μ g of Opti-MEM. The tube was lightly rotated to mix the transfection reagent Lipofectamine™ 2000, and 20 μ L of Lipofectamine™ 2000 was diluted with 500 μ L of Opti-MEM. The transfection reagent was mixed with NF- κ B p65 CRISPR/Cas9 KO plasmid DNA diluent. The transfection complex was added to the cell culture dish. After 6 h of transfection, the medium was replaced with fresh medium. Two plasmids, BCRF1 (T plasmid) and PCMV-N-Flag, were digested, and the target fragments were recycled for DNA ligation: No. 1: BCRF-1; No. 2 and No. 3: BCRF-1 after EcoRI and BamHI cleavage; No. 4: PCMV-N-Flag; No. 5 and No. 6: PCMV-N-Flag after

EcoRI and BamHI cleavage (Fig. 1). The successfully constructed BCRF-1 overexpressing vector was used to transfect the CNE-2 cells. Monoclonal cells were selected for colony PCR validation. Successfully transfected monoclonal cells were selected.

Cell sorting and single-cell clone selection

Cells were digested with 0.25% trypsin 48 h after transfection and were collected. CNE-2 cells that were not transfected with fluorescent plasmids were used as controls. Cells were processed by flow cytometry, in order to sort cells transfected with the NF- κ B p65 CRISPR/Cas9 KO plasmid (with fluorescence).

The selected cells with green fluorescence were added to 10 mL of CNE-2 medium (DMEM/F12 containing 10% fetal bovine serum and 1 $\times$ penicillin-streptomycin double-antibodies) and placed onto 10 mm culture plates. After one week of cultivation, the single clones of cells were manually picked up to the 24-well plates and allowed to culture for three days. The single clones on the 24-well plates were then digested, respectively passaged, and proliferated. Afterward, the cells were collected for protein extraction. In addition, cells of each clone were cryopreserved.

Synthesis of the BCRF-1 gene

The CDS region (red label) was synthesized, and EcoRI and BamHI restriction sites were added at both ends (linked to T vector). BCRF1: BCRF1 protein precursor (Human herpesvirus 4 [Epstein–Barr virus]) Gene ID: 3783689, updated on 20 May 2015. Human [gi|82503188|ref|NC_007605.1|:9535-10356](https://www.ncbi.nlm.nih.gov/nuccore/82503188) Human herpesvirus 4 complete wild type genome.

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CCCAGGAACCCGAAACAGTGTTCCTCCAGAGCTGTAAAAATAGAACGCCC
TGGAAGTGCCTCAGTGTGCAATGCAGCTTTAGCCATGCCATGCTCTATAAA
TCACTTCCCTATCTCAGGTAGGCTGCACACCTTAGGTATGGAGCGAAGGT
TAGTGGTCACTCTGCACTGCTGGTGTCTTACCTGGCACCTGAGTGTG
GAGGTACAGACCAATGTGACAATTTCCCAAATGTTGAGGGACCTAAGAG
ATGCCCTCAGTCGTGTAAAAACCTTTTCCAGACAAAGGACGAGGTAGATA
ACCTTTTGCTCAAGGAGTCTCTGCTAGAGGACITTAAGGGCTACCTTGGAT
GCCAGGCCCTGTGAGAAATGATCCAATCTACCTGGAGGAAGTCATGCCAC
AGGCTGAAAACCCAGGACCCTGAAGCCAAAGACCATGTCAATTCTTTGGGT
GAAAATCTAAAGACCCTACGGCTCCGCTCGCAGGTGCCACAGGTTCTCT
GCCGTGTGAGAACAGAGTAAAGCTGTGGAACAGATAAAAAATGCCCTTA
ACAAGCTGCAGGAAAAAGGAATTTACAAAGCCATGAGTGAATTTGACATTT
TTATTAACATACATAGAAGCATACATGACAATTAAGCCAGGTGATAATCCA
TACCTTGGAAGCAGGAGATGGGTGCATTTACCCCAACCCCTTTTCGAC
TGTCAITTTACAATAAAATGAAACCTTTTATCTTGATTGCCTCTTGTTCTT
GCCGCCAGGTACCTTCTGTGTTCTCCCAACGGGAAAAAGATAGCTTCT
GCAGAA
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For construction of plasmid overexpressing BCRF1, the synthesized CDS region was connected to the

eukaryotic expression vector (pCMV-N-Flag vector). The mRNA was detected by agarose gel electrophoresis, and the concentration of the RNA was determined using a spectrophotometer. For each sample, 2 µg of mRNA was obtained for reverse transcription.

For removing the genomic DNA from RNA, a total volume of 10 µL of the reaction solutions (Full RNA: 2 µg; 10 × DNase x Buffer: 1 µL; Recombinant DNase I: 0.5 µL; DEPC-treated water: up to 10 µL) was prepared in a microcentrifuge tube. This was left standing at 37°C for 20–30 min, added with 0.5 µL of EDTA, and heated at 80°C for 2 min.

Synthesis of cDNA

Reverse transcription reaction solutions were prepared from the following components (the preparation methods were the same in each group, all were on ice), left standing at 70°C for 5 min, and preserved at 4°C. After the end of this step, the reagents (5 × First Sstrand Buffer: 4 µL; M-MLV: 1 µL; Toal: 20 µL) were added into the PCR tube. GAPDH was used as an internal control, and the agarose gel electrophoresis of the PCR product was performed to determine whether the product was the target gene.

Real-time fluorescent quantitative polymerase chain reaction amplification

The mixture of all components, except for the template, was prepared according to the PCR reaction system. During each reaction, 19 µL of the mixture was added to each well of the 96-well PCR plate. One uL of sample template (cDNA) was added to each reaction container, and the reaction tube/plate was covered or sealed. The

mixture was gently shaken, to ensure all components were concentrated at the bottom of the reaction container. The reaction system was placed in the gene amplification instrument set, as mentioned previously. The data were collected, and the results analyzed. The real-time fluorescent quantitative polymerase chain reaction (RT-qPCR) reaction procedure (two-step method, annealing, and extension were merged into one step) (Table I).

Relative quantitative analysis

In the present study, the $\Delta\Delta Ct$ relative quantification method was used to express the changes in gene expression. Before the $\Delta\Delta Ct$ method was used for quantitation, a feasibility test was performed to verify whether the amplification efficiency of the target gene and internal control were basically equal. With GAPDH as an internal control, a series of fluorescent quantitative PCR reactions were carried out. The reaction system and reaction conditions were the same as the above-mentioned reaction. The difference in amplification slope between the target gene and internal control was less than 0.1, and $\Delta\Delta Ct$ was used for the quantitative analysis. The standard was not necessary for the absolute quantitation or relative standard curve quantitative method. The formula for calculating the relative expression of target genes in samples was: $F = 2^{-\Delta\Delta Ct}$; $\Delta Ct = \text{mean Ct value of the target gene} - \text{mean Ct value of housekeeping gene}$; $\Delta\Delta Ct = \Delta Ct (\text{sample to be tested}) - \Delta Ct (\text{control sample})$. The relative expression calculated by $2^{-\Delta\Delta Ct}$ was expressed as the fold of the expression of the target gene in the sample to be measured against that in the control sample.

Cells were collected and lysed, the total protein was extracted according to the method provided by the kit, and the protein concentration was determined using bicinchoninic acid (BCA). An equal volume (25 µg) of the protein sample was obtained and subjected to 10% polyacrylamide gel electrophoresis for 1 h, followed by wet electric transmembrane, blocking with 5% skim milk at room temperature for 1 h, the addition of the first antibody, incubation at room temperature for 30 min, and cultured overnight at 4°C. The secondary antibody was then added, and incubated at room temperature for 1.5 h, developed and fixed.

The gray analysis of the protein bands was carried out using ImageJ software. The experimental data were statistically analyzed using statistical software SPSS 17.0.

Table I. Real-time fluorescence quantitative PCR amplification

Primer	Primer sequence
HLA-A-F	GTCACAGACTGACCGAGTGG
HLA-A-R	CGACCCACGCCATACATT
LMP7-F	CTTACGGGTGAACAAGGTGA
LMP7-R	GCTGCCGACACTGAAATACG
PSMB9/LMP2-F	TGCACGAGCGCATCTACT
PSMB9/LMP2-R	TTCCTCCCAGGGTTCCAT
TAP1-F	ACCGCCTTCGTTGTCAGTTAT
TAP1-R	TATCGGCTGAGCCATCTTGTAG
TAP2-F	TTGCGAAGCCTGGTGAAAGT
TAP2-R	CCTGATGGCGGGTGTGTGA

The means were compared among groups using univariate analysis of variance. $P < 0.05$ was considered statistically significant. $P < 0.05$ was denoted as “*”.

RESULTS

Cells were observed under a fluorescent microscope 48 hours after CNE-2 cells were

transfected with the NF- κ B p65 CRISPR/Cas9 KO plasmid. Cells with green fluorescence were CNE-2 cells transfected with the NF- κ B p65 CRISPR/Cas9 KO plasmid (Fig. 2A). Western blot was used to detect the expression of NF- κ B p65 in monoclonal cells (Fig. 2B). The results revealed that NF- κ B p65 was completely knocked down in some cloned cells. Cloned cells with completely knocked down NF- κ B

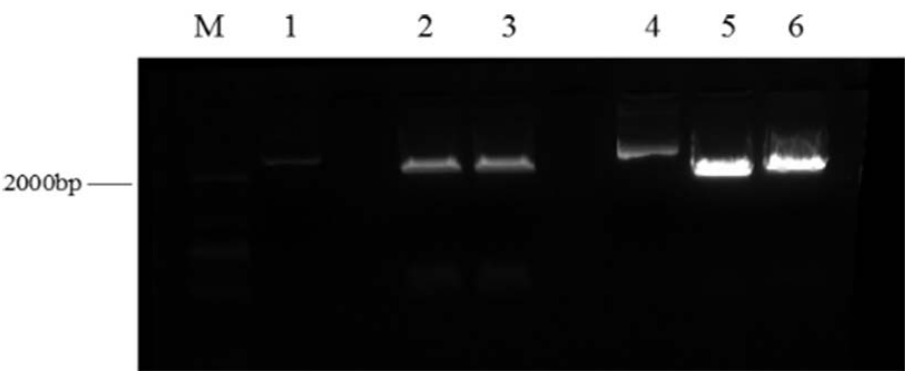


Fig. 1. The cleavage map of *BCRF1* (T plasmid) and *PCMV-N-Flag* plasmid

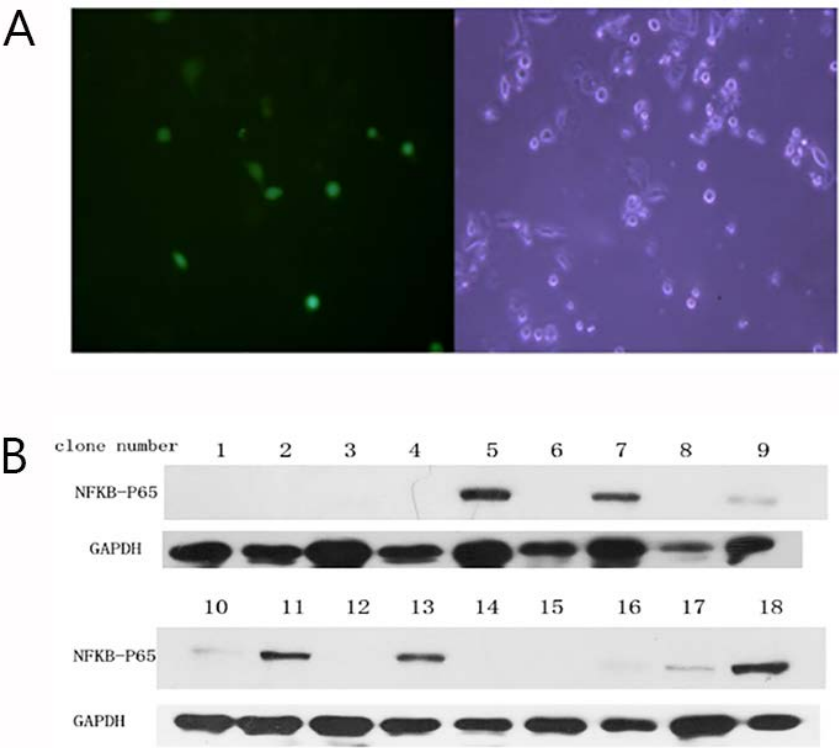


Fig. 2. A) At 48 hours after the transfection of CNE-2 cells under a fluorescent microscope; B) The expression of NF- κ B p65 in the monoclonal antibody: clones 1, 2, 3, 4, 6, 8, 12, 14 and 15 were completely knocked down.

p65 were selected for the following experiments.

As shown in Fig. 3, at the mRNA level, differences in the expression of TAP-1, TAP-2, LMP-2, and LMP-7 between the CNE2-PCMV-BCRF1 group (BCRF1 overexpressing group) and CNE2-PCMV group (empty vector group) were not statistically significant ($P > 0.05$), while the expression of HLA-I was significantly lower in the CNE2-PCMV-BCRF1 group (BCRF1

overexpressing group) when compared with the CNE2-PCMV group (empty vector group, $P < 0.05$). Furthermore, the expression of TAP-1, TAP-2, LMP-2, and LMP-7 was significantly lower in the CNE2-KO-NF- κ B-PCMV-BCRF1 group (NF- κ B knockdown and BCRF1 overexpressing group) when compared with the CNE2-PCMV-BCRF1 group (BCRF1 overexpressing group, $P < 0.05$), while differences in

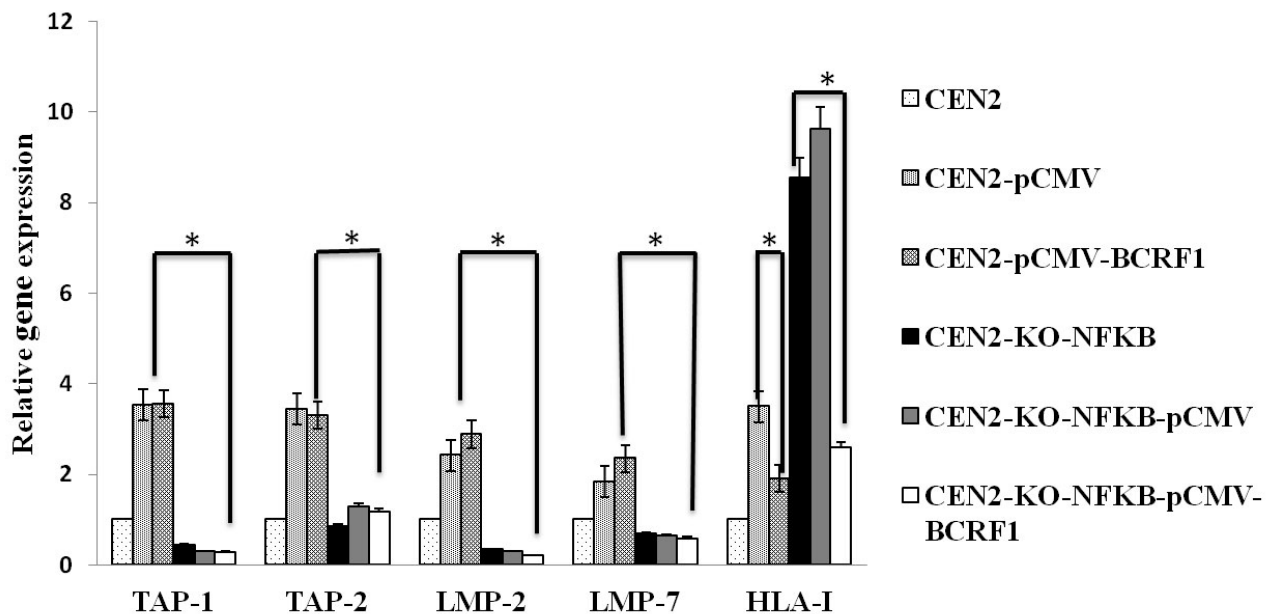


Fig. 3. The relative mRNA expression of TAP-1, TAP-2, LMP-2, LMP-7 and HLA-I in CNE-2 cells

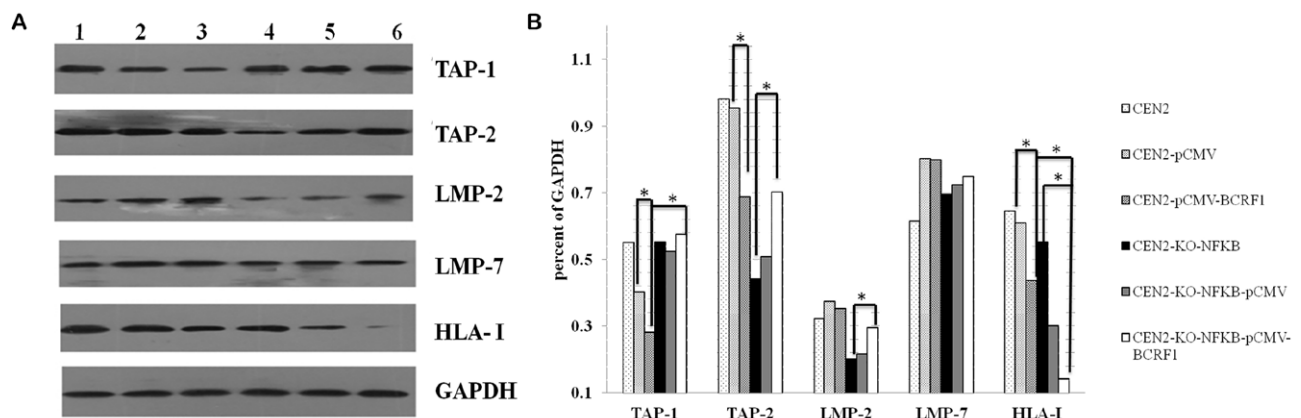


Fig. 4. Changes in the protein expression of TAP-1, TAP-2, LMP-2, LMP-7 and HLA-I in CNE-2 cells. **A)** Western blot results of TAP-1, TAP-2, LMP-2, LMP-7 and HLA-I in CNE-2 cells after knockdown of the NF- κ B gene by CRISPR/Cas9. 1: CNE-2 group (untreated group), 2: CNE2-PCMV group (empty vector group), 3: CNE2-PCMV-BCRF1 group (BCRF1 overexpressing group), 4: CNE2-KO-NF- κ B group (NF- κ B knocking out group), 5: CNE2-KO-NF- κ B-PCMV group (NF- κ B knocking out + empty vector group), 6: CNE2-KO-NF- κ B-PCMV-BCRF1 group (NF- κ B knockdown and BCRF1 overexpressing group). **B)** Development bands: ImageJ software was used to analyze the ratio of the gray level analysis results to GAPDH gray values. * $P < 0.05$.

the expression of HLA-I between these two groups were not statistically significant ($P > 0.05$). Between the two groups, differences in the expression of TAP-1, TAP-2, LMP-2, and LMP-7 between the CNE2-KO-NF- κ B-PCMV-BCRF1 group (NF- κ B knockdown and BCRF1 overexpressing group) and CNE2-KO-NF- κ B group (NF- κ B knockdown group) were not statistically significant ($P > 0.05$), while the expression of HLA-I statistically decreased ($P < 0.05$).

As shown in Fig. 4, at the protein level, the expression of TAP-1, TAP-2 and HLA-I were significantly lower in the CNE2-PCMV-BCRF1 group (BCRF1 overexpressing group) than in the CNE2-PCMV group (empty vector group, $P < 0.05$). Although LMP-2 and LMP-7 decreased, the differences were not statistically significant ($P > 0.05$). The expression of TAP-1 and TAP-2 was higher in the CNE2-KO-NF- κ B-PCMV-BCRF1 group (NF- κ B knockdown and BCRF1 overexpressing group) than in the CNE2-PCMV-BCRF1 group (BCRF1 overexpressing group), and the difference in TAP-1 expression was statistically significant ($P < 0.05$). In the second group, the expression of LMP-2, LMP-7, and HLA-I decreased, but only the difference in HLA-I expression was statistically significant ($P < 0.05$). Furthermore, the expression of TAP-1, TAP-2, LMP-2, and LMP-7 was higher in the CNE2-KO-NF- κ B-PCMV-BCRF1 group (NF- κ B knockdown and BCRF1 overexpressing group) than in the CNE2-KO-NF- κ B group (NF- κ B knockdown group), and the differences in TAP-2 and LMP-2 expression were statistically significant ($P < 0.05$), while the decrease in expression of HLA-I was also statistically significant ($P < 0.05$).

DISCUSSION

The previous study conducted by the investigators revealed that vIL-10 could inhibit APM by downregulating the activation and translocation of NF- κ B p65 and its ability to bind to DNA and promoting NPC cells to escape immune surveillance (7). This theory is further validated by the present study. In the present study, the results revealed that the overexpression of BCRF-1 did not affect the structure and expression of the TAP-1 and TAP-

2 genes. However, after knocking out NF- κ B p65, the expression of the TAP-1 and TAP-2 genes significantly decreased. This verifies that NF- κ B p65 can bind to the TAP's promoter to regulate gene expression. At the protein level, after the overexpression of BCRF-1, the expression of both TAP-1 and TAP-2 genes decreased, suggesting that vIL-10 has a negative regulatory effect on TAP-1 and TAP-2. Furthermore, NF- κ B p65 was further knocked down, and the negative regulatory effect of vIL-10 on TAP-1 and TAP-2 decreased, suggesting that the negative regulatory effect of vIL-10 on TAP-1 and TAP-2 was exerted through NF- κ Bp65. In addition, our results revealed that vIL-10 has no direct regulatory effect on LMP-2 and LMP-7, and LMP2 and LMP7 may not play decisive roles in antigen presentation (8).

In humans, the decrease in MHC-I antigen expression is one of the links of transformation from normal cells to abnormal forms, and the degree of the reduction is positively correlated with the malignancy and metastasis of tumors (9). The present study also revealed that vIL-10 can directly inhibit the expression of the HLA-I gene, which does not require the mediation of the signal of NF- κ B p65.

In conclusion, the present study verified the role of vIL-10 in the regulation of APM in NPC cells. The investigators consider that vIL-10 can inhibit APM expression directly or through the NF- κ B pathway and promote NPC cells to escape immune surveillance. This provides a new idea for exploring the immunotherapy of NPC.

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LETTER TO THE EDITOR

Silenced fatty acid-binding protein 4 suppresses epithelial-mesenchymal transition of endometriosis via the phosphatidyl inositol 3-kinase/protein kinase B axisY. Zhang^{1*}, A. Hou², XR. Zhuang¹, XJ. Gao¹ and GX. Zhang¹¹Department of Gynecology, the Affiliated Hospital of Chengde Medical College, Chengde, Hebei Province, China; ²Department of Gynecology, the University of Chinese Academy of Sciences Shenzhen Hospital, Shenzhen, Guangdong Province, China*Received January 26, 2021 – Accepted July 5, 2021*

To the Editor,

Endometriosis (EMS) is a mechanically estrogen-dependent inflammatory disorder occurring in extrauterine glands and endometrium tissues, which is often responsible for subsequent high infertility rate and severe pelvic pain (1). Epithelial-mesenchymal transition (EMT) is a crucial participator in EMS progression (2). Therefore, there is an urgent need for biomarkers and novel therapeutic targets for EMS.

According to Sun and his colleagues, fatty acid binding protein (FABP4) is a reliable biomarker in predicting gestational diabetes mellitus and designing clinic intervention (3). Uehara et al. indicated that FABP4 could upregulate the phosphatidyl inositol 3-kinase/protein kinase B (PI3K/AKT) axis to exacerbate the development of prostate cancer (4). Fabio et al. reported that the PI3K/AKT axis is strongly expressed in EMS, and it regulates EMS cell viability, angiogenesis and molecular resistance to progestins (5). All in all, we are encouraged to postulate that the crosstalk between FABP4 and the PI3K/AKT axis might affect the progression of EMS. Therefore, this study was designed to confirm this hypothesis.

MATERIALS AND METHODS

Research subjects

From July 2018 to July 2020, 20 ovarian EMS patients (aged from 29-52 years, with a median age of 43.25±5.98) treated and undergoing laparoscopic surgery at the obstetrics and gynecology department in our hospital were enlisted in this study for the collection of ectopic endometrium (the ectopic group) and eutopic endometrium (the eutopic group). These patients were assigned according to revised American Fertility Society EMS classification into the I-II stage (n=9) and the III-IV stage (n=11). To ensure an accurate result, the patients received no hormone drugs 3 months prior to surgery. All patients were diagnosed with EMS, which was then confirmed via pathological examination. Normal endometria from 20 cervical intraepithelial neoplasia patients without EMS (aged from 30-50 years, with a median age of 42.70±4.95) were used as the control group. No control subjects had received steroid hormones for 3 months prior to surgery or were complicated with diseases including EMS, uterine fibroid, cervical polyp and endometrial cancer. Endometrioma tissues and endometrium tissues were obtained in the operating room, immersed in Dulbecco's

Key words: fatty acid-binding protein 4; endometriosis; PI3K/AKT axis; epithelial-mesenchymal transition; migration; invasion

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modified Eagle's medium (DMEM)-F12 containing 2% double antibody (iced bath), and were rapidly sent to the laboratory. Then, tissues were fully washed with autoclaved phosphate buffer saline (PBS) after removing blood, foreign matters, and necrotic tissues, and divided into two parts: one was selected for primary cell culture after the fresh lesion tissues were separated, and the other was stored in a centrifuge tube and maintained at -80°C for later extraction of RNA, DNA, and proteins.

This study was approved and supervised by the ethics committee of our hospital. All the subjects signed the informed consents, and all procedures were strictly conducted in accordance with the code of ethics.

Separation and culture of primary endometrial stromal cells (ESCs)

Primary ESCs were separated as recorded in a previous study(18). In brief, ectopic endometrium tissues were shattered and digested in Hank balanced salt solution consisting of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25 mmol/mL), 1% penicillin/streptomycin, collagenase (1 mg/mL, 15 U/mg) and deoxyribonuclease (0.1 mg/mL, 1500 U/mg) at 37°C for 60 min. Dispersed endometrial cells were filtered and separated through a $40\text{ }\mu\text{m}$ cell filter. ESCs were placed in 75-cm^2 Falcon tissue culture flask (BD Biosciences, MA, USA) and cultivated in DMEM/Ham's F12 (1:1) consisting of 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, Maryland, USA) and antibiotics (100 $\mu\text{g/mL}$ penicillin and streptomycin) in a 37°C incubator with 5% CO_2 . Cell purification was evaluated by immunohistochemical vimentin staining (Techno, Co., Ltd, Tokyo, Japan). Primary cultured ESCs were analyzed by a flow cytometer (BD Accuri C6 Plus, BD Biosciences, Franklin Lakes, NJ, USA). Cultured ESCs between passages 3 and 5 were used.

Cell transfection and treatment

A total of 100 μL cells (1×10^5 cells/mL) were seeded into each well of 96-well plates for 24-h cultivation. After that, small interfering (si) RNAs (si-negative control (NC), si-FABP4-1, si-FABP4-2 and si-FABP4-3) (20 μM , Shanghai GenePharma Co, Ltd, Shanghai, China) were transfected into cells with compliance to the instructions of Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) (target sequences of these siRNAs are listed in Table I). Cells were then exposed to differential medium

with or without LY294002 (PI3K/AKT inhibitor, 15 μM , R&D System Inc., Minneapolis, MN, USA) or insulin-like growth factor (IGF)-1 (PI3K/AKT activator, 50 μM , Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 30-min cultivation.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Trizol reagent (Invitrogen) was utilized to extract the total RNA from tissues or cells. Then, total RNA was quantified by Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total RNA (2 μg) was reversely transcribed to cDNA via the Bestar qPCR RT kits (DBI@Bioscience, Shanghai, China). Afterwards, cDNA was applied to conduct RT-PCR by the DBI Bestar® SybrGreen qPCR Master Mix kits (DBI@Bioscience). With the assistance of Stratagene RT-PCR detection system (Mx3000P, Agilent, Santa Clara, CA, USA), 40 cycles of amplification and detection were performed including denaturation at 94°C for 2 min, heating at 94°C for 20 s and annealing at 58°C for 20 s. The value of relative FABP4 mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). $2^{-\Delta\Delta\text{Ct}}$ method was applied for the quantification of the data. The primer sequence is shown in Table II.

Western blot analysis

After 48 h of transfection, total protein was separated from tissues and cells by radio-immunoprecipitation assay buffer (Biotechnology Co., Ltd, Shanghai, China) containing protease inhibitor. Subsequently, protein concentration was determined by bicinchoninic acid protein analysis kits (Pierce, Rockford, IL, USA). Next, the same amount of proteins were transferred onto the polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, USA) after being separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Subsequently, the membranes were sealed by tris-buffered saline consisting of 5% bovine serum albumin for 2 h and then incubated with the primary antibodies (all from Cell Signaling Technology, Beverly, MA, USA): FABP4 (#2120), E-cadherin (#3195), N-cadherin (#13116), Vimentin (#5741), PI3K (#4255), AKT (#9272), p-AKT (#4060) and GAPDH (#5174) at 4°C overnight. Membranes were rinsed by 0.1% tris-buffered saline-tween for 3 times, and incubated with horseradish peroxidase-combined

goat anti-rabbit secondary antibody (#7074, 1:2500, Cell Signaling Technology) at room temperature. Protein bands were observed by chemiluminescence kits (Pierce) and quantified by imaging software image Lab (BioRad, Inc., Hercules, CA, USA). GAPDH was employed as the internal control of western blot analysis.

Cell counting kit-8 (CCK-8)

At the end of transfection, cells (5×10^3 cells/well) were seeded into a 96-well plate. Subsequently, cell proliferation was analyzed using CCK-8 reagent (Bimake, Shanghai, China) supplemented into each well at different time points (24, 48, 72 and 96 h) for the incubation of cells at 37°C with 5% CO₂ for 2 h. Eventually, optical density at 490 nm was observed and measured using an all-purpose microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Transwell assay

Transfected cells in each group were removed by Transwell chamber (8 µm aperture) with microporous membrane of polycarbonate and seeded into chamber, with 200 µL serum-free DMEM/F12 medium (1×10^5 cells/mL) added into the apical chamber and 600 µL complete medium containing 20% FBS into the basolateral chamber. Each step was repeatedly performed in 3 different wells. After 36 h of incubation, chambers were taken out, fixed by PBS with 4% paraformaldehyde for 30 min, washed with PBS and stained using crystal violet solution at room temperature for 30 min. Afterwards, chambers were repeatedly washed by PBS till PBS appeared to be pellucid. Cells located at the apical chamber that did not pass through the microporous membrane were wiped away carefully using a clean cotton swab. Stained migrated cells at the basolateral chamber were photographed under

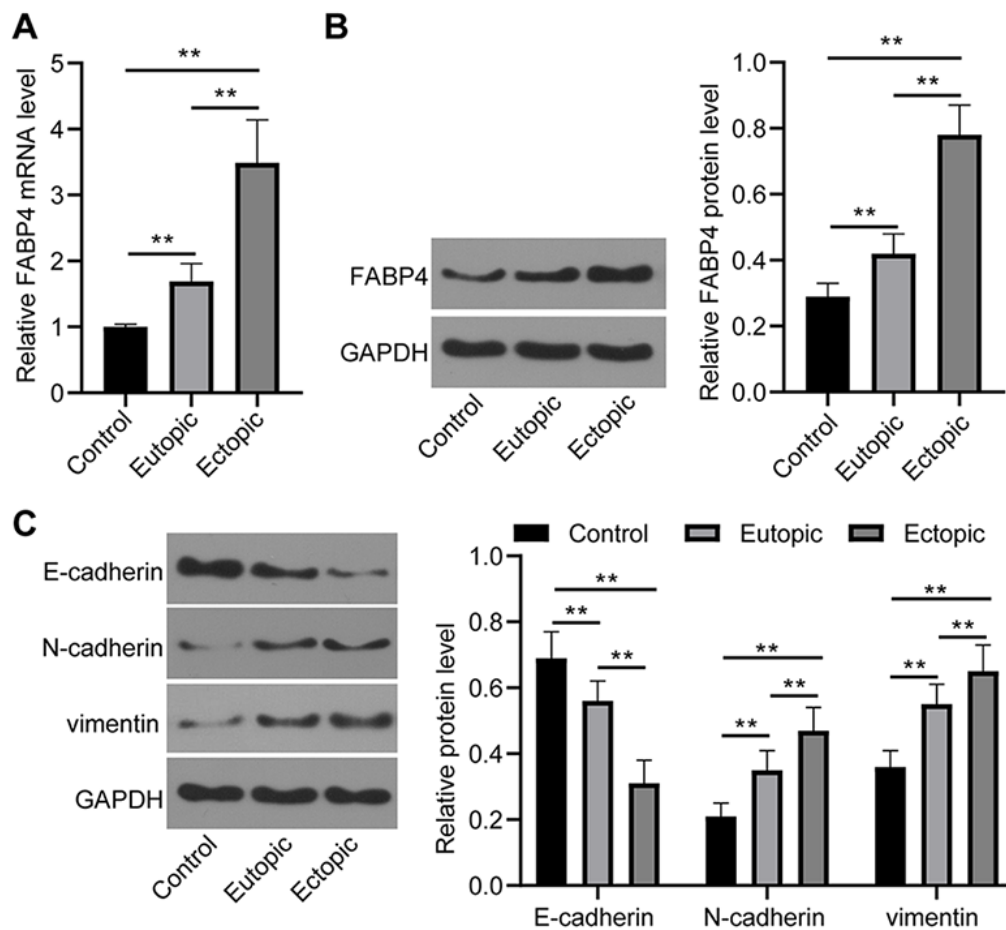


Fig. 1. FABP4 is strongly expressed in EMS. **A, B**) FABP4 mRNA and protein levels in each group measured by qRT-PCR (**A**) and western blot analysis (**B**). **C**) protein levels of EMT markers in each group detected by western blot analysis. N=20, the student's t-test and paired t-test were used to analyze data. ** $p < 0.01$.

a microscope (Nikon Instruments, Chiyoda-ku, Tokyo, Japan). Five visual fields were randomly selected under 200 $\times$ light microscope to calculate the number of cells passing through the microporous membrane, to assess migrant index via the mean number of these cell number (cell number/visual field). When cell invasion was analyzed, cells were treated in the identical manner; except the apical

chamber was coated by serum-free DMEM/F12 diluted medium gel (BD, Franklin Lakes, NJ, USA).

Statistical analysis

Graphpad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was employed for data verification. The data were in normal distribution. Student's *t*-test or paired

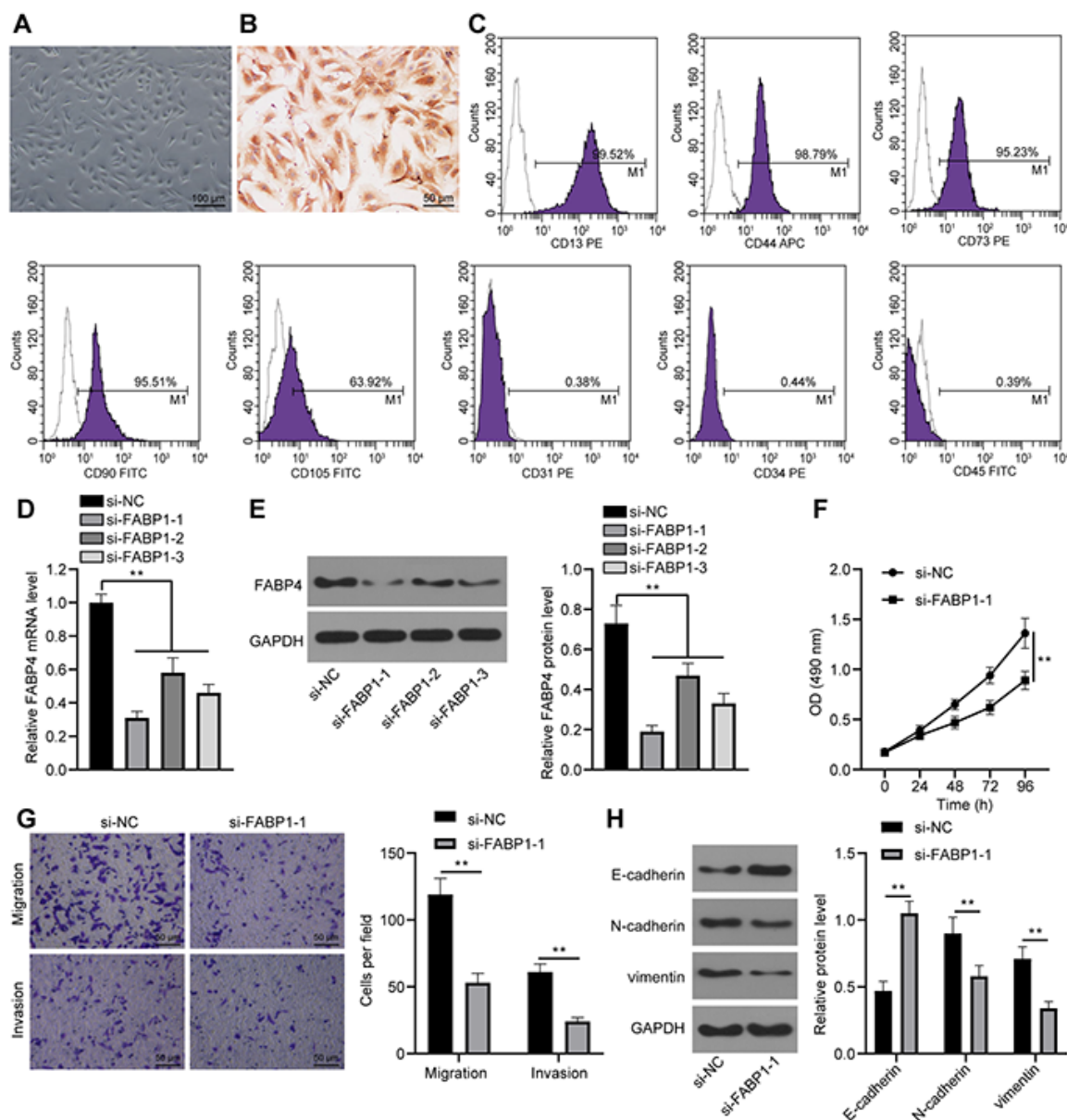


Fig. 2. Silencing of FABP4 inhibits ESC growth and EMT. **A)** ESCs displayed long and thin and shuttle-shaped under an inverted microscope (100 $\times$). **B)** ESCs > 95% in strong Vimentin staining observed by immunohistochemistry (200 $\times$). **C)** Expression patterns of mesenchymal cell surface markers analyzed by flow cytometry. **D, E)** FABP4 mRNA and protein levels with si-FABP4 in each group analyzed by qRT-PCR (**D**) and western blot analysis (**E**). **F)** ESC proliferation with si-FABP4 detected by CCK-8 method. **G)** ESC migration and invasion with si-FABP4 measured by Transwell assay. **H)** protein levels of EMT markers with si-FABP4 evaluated by western blot analysis. *N*=3, Student's *t*-test was used to analyze data. ** *p*<0.01.

t-test was applied for analysis of comparisons between two groups, one-way analysis of variance (ANOVA) for comparing different groups, and Tukey's post-hoc multiple comparisons test for pairwise comparisons after ANOVA. The *p*-value was attained using a two-tailed test and *p*<0.05 indicated a significant difference.

RESULTS

FABP4 is strongly expressed in EMS

This research included ectopic endometrium tissues and eutopic endometrium tissues (20 pairs of each) from 20 EMS patients and 20 pairs of

normal endometrium tissues from 20 non-EMS patients, which served as the control group. FABP4 expression in different endometrium tissues was assessed by qRT-PCR and western blot analysis, from which it was revealed that FABP4 expression was the highest in the ectopic group; while the eutopic group had higher FABP4 expression than the control group (Fig. 1 A, B) (*p*<0.05). Protein levels of EMT markers (E-cadherin, N-cadherin and Vimentin) were detected by western blot analysis (Fig. 1C), and the result showed that compared with the control group, both the ectopic group and the eutopic group exhibited decreased E-cadherin level and increased

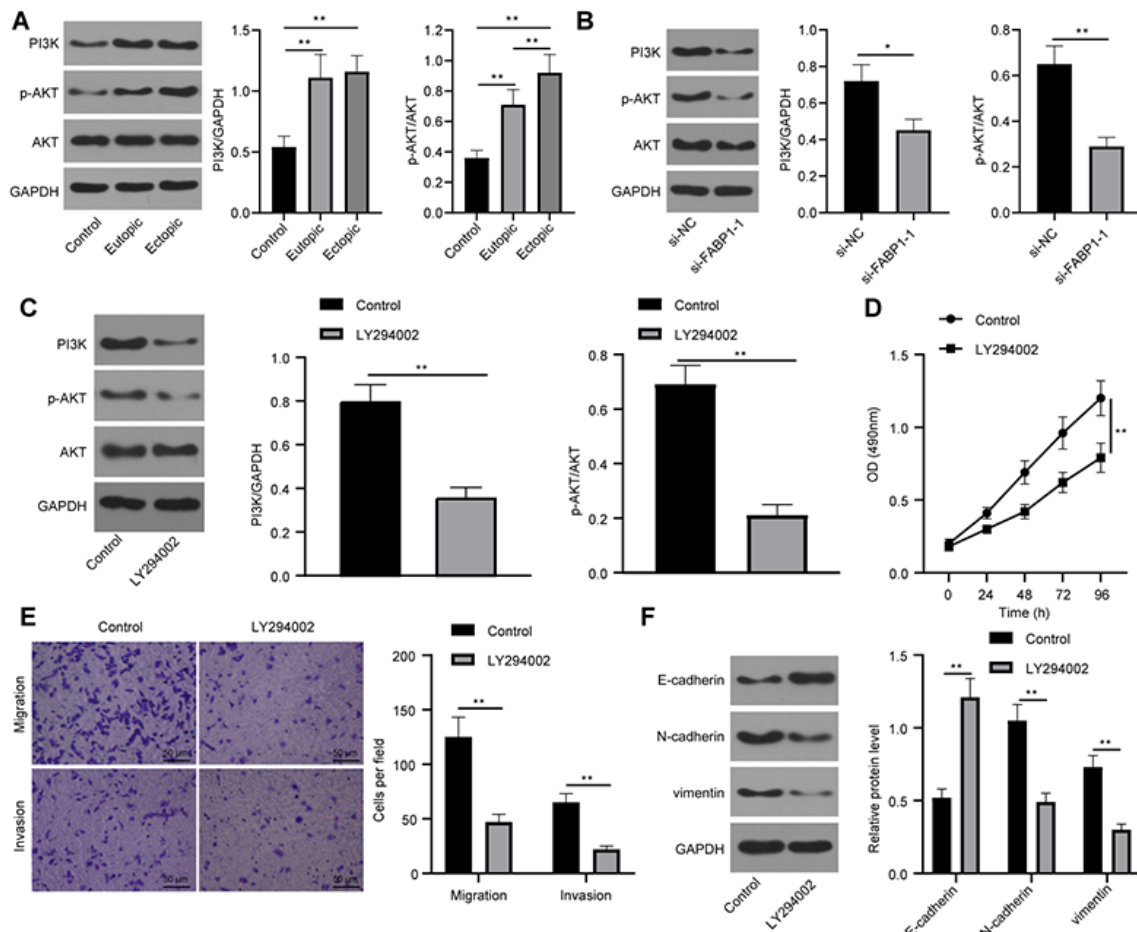


Fig. 3. The effect of LY294002, inhibitor of the PI3K/AKT axis on the function of ESCs. **A)** levels of the PI3K/AKT axis-associated proteins in each group measured by western blot analysis, *n*=20. **B)** Levels of the PI3K/AKT axis-associated proteins with si-FABP4 in each group assessed by western blot analysis. **C)** Levels of the PI3K/AKT axis-associated proteins with LY294002 treatment measured by western blot analysis. **D)** Effect of LY294002 treatment on ESC proliferation detected by CCK-8 method. **E)** Effect of LY294002 treatment on ESC migration and invasion verified by Transwell assay. **F)** Effect of LY294002 treatment on levels of EMT markers (E-cadherin, N-cadherin and Vimentin) analyzed by western blot analysis, *n*=3. Student's *t*-test and paired *t*-test were used to analyze data. **p*<0.05, ***p*<0.01.

levels of N-cadherin and Vimentin; while there was also apparently difference in the levels of these EMT markers between the ectopic group and the eutopic group (all $p < 0.05$).

Silencing of FABP4 inhibits ESC growth and EMT

Primary ESCs were extracted with the purpose of probing the potential effect of FABP4 on EMS (Fig. 2 A, B). Results from flow cytometry found that expression of mesenchymal cell surface markers showed that mesenchymal cell markers CD13, CD44, CD73, CD90 and CD105 were positively expressed, while CD31, CD45 and HLA-DR were negatively expressed (Fig. 2C). Cells were transfected with si-NC and si-FABP4, with si-FABP4-1, which displayed the highest

efficiency, chosen for subsequent performance (Fig. 2 D, E) (all $p < 0.05$). Afterwards, the effect of FABP4 on cell proliferation was measured by CCK-8 method, and the result found that silencing of FABP4 quenched ESC proliferation (Fig. 2F) (all $p < 0.05$). Likewise, Transwell assay, which was conducted to detect cell migration and invasion, revealed that silenced FABP4 could restrict ESC migration and invasion (Fig. 2G) (all $p < 0.05$). To further confirm whether FABP4 reduced ESC migration and invasion via EMT, western blot analysis was carried out to determine the levels of EMT markers. Then it was found that silenced FABP4 upregulated E-cadherin level but reduced N-cadherin and Vimentin levels (Fig. 2H) (all $p < 0.05$). In summary, silenced FABP4 contributed

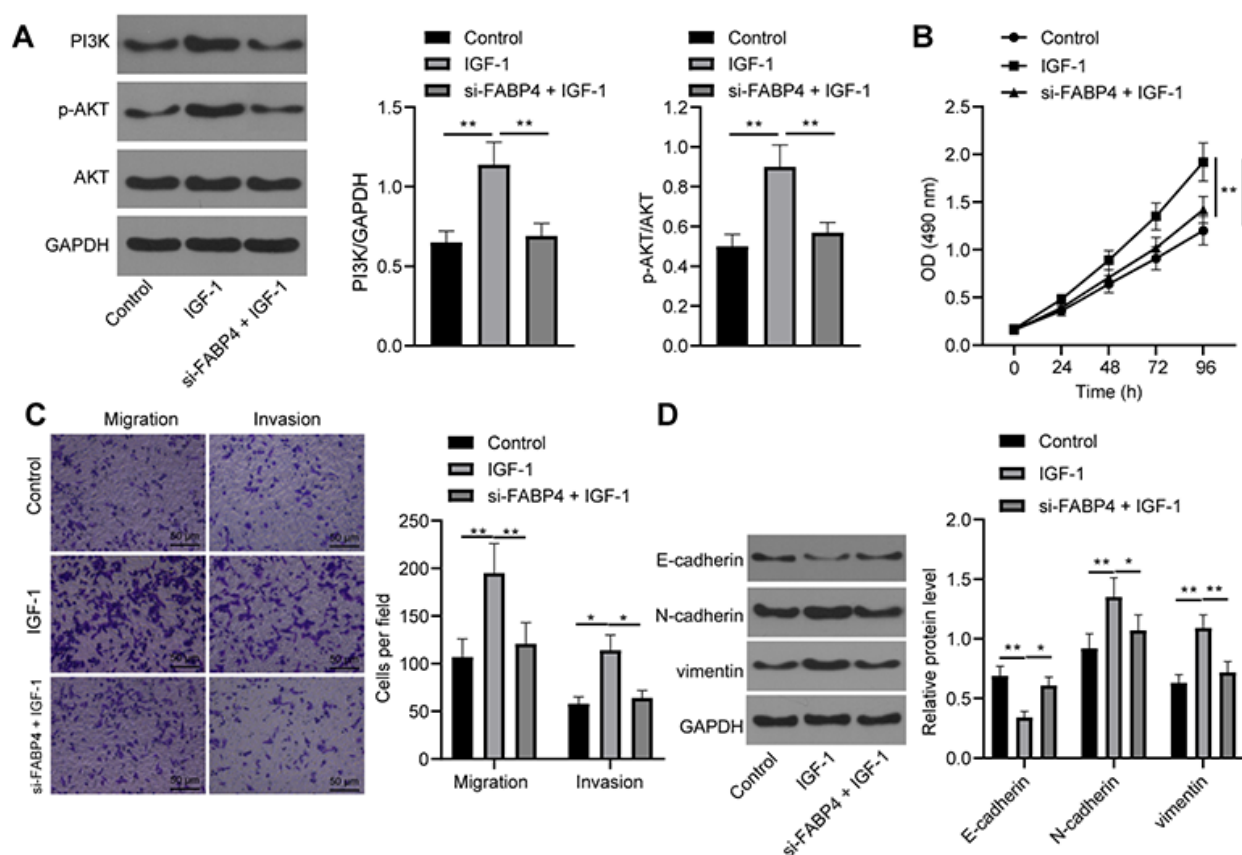


Fig. 4. Activation of the PI3K/AKT axis reverses the inhibitory role of silenced FABP4 on ESC proliferation and EMT. **A)** Levels of the PI3K/AKT axis-associated proteins treated with IGF-1 measured by western blot analysis. **B)** Effect of IGF-1 on ESC proliferation detected by CCK-8 method. **C)** Effect of IGF-1 on ESC migration and invasion verified by Transwell assay. **D)** Effect of IGF-1 on levels of EMT markers (E-cadherin, N-cadherin and Vimentin) analyzed by western blot analysis. $N=3$, the student's t -test was used to analyze data. Tukey's multiple comparisons test was applied for post hoc test. * $p < 0.05$, ** $p < 0.01$.

to the suppression of EMS by reducing cell proliferation, migration, invasion and EMT.

FABP4 activates the PI3K/AKT axis, and downregulation of the PI3K/AKT axis inhibits ESC proliferation and EMT

Levels of the PI3K/AKT axis-associated proteins in each group were evaluated by western blot analysis, and resulted that the ectopic group possessed higher levels of PI3K and p-AKT/AKT than the normal group (Fig. 3A) (all $p < 0.05$). It was further discovered that silencing of FABP4 could reduce levels of PI3K and p-AKT/AKT as detected by western blot analysis of the effect of si-NC and si-FABP4 on the PI3K/AKT axis (Fig. 3B) (all $p < 0.05$). These data demonstrated that silenced FABP4 could alleviate EMS by downregulating the PI3K/AKT axis. To explore the role of the PI3K/AKT axis in ESCs, LY294002 was applied to inactivate

the levels of PI3K and p-AKT/AKT (Fig. 3C) (all $p < 0.05$). Next, CCK-8 method and Transwell assay were performed to measure the effect of the PI3K/AKT inhibitor LY294002 on ESC proliferation, migration and invasion. Then, it was found that LY294002 discouraged ESC proliferation, migration and invasion (Fig. 3 D, E) (all $p < 0.05$). Similarly, western blot analysis of the levels of EMT markers revealed that LY294002 brought about elevated E-cadherin level and declined N-cadherin and Vimentin levels (Fig. 3F) (all $p < 0.05$); this showed that inactivation of the PI3K/AKT axis quenched ESC growth, migration, invasion and EMT.

Activation of the PI3K/AKT axis reverses the inhibitory role of silenced FABP4 on ESC proliferation and EMT

To ascertain that FABP4 affected EMS EMT via the PI3K/AKT axis, functional rescue assay

Table I. Target sequence of si-FABP4

Gene	Sequence (5'–3')
si-FABP4-1	CCTGGTACATGTGCAGAAA
si-FABP4-2	GGAAAGTCAAGAGCACCAT
si-FABP4-3	GCATGGCCAAACCTAACAT

Note: si: small interfering; FABP4: fatty acid-binding protein 4

Table II. Primer sequence of qRT-PCR

Gene	Sequence (5'–3')
FABP4	Forward: AGAAGTGGGAGTTGGCTTCG
	Reverse: ACTCTCTGACCGGATGACGA
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC
	Reverse: TGGTGAAGACGCCAGTGA

Note: qRT-PCR: quantitative real-time polymerase chain reaction; FABP4: fatty acid-binding protein 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

was conducted where ESCs were transfected by FABP4 siRNA for 24 h, followed by the exposure to differential medium with or without IGF-1 for 30 min for the following functional analysis. Compared with the control group, the IGF-1 group expressed increased levels of PI3K and p-AKT/AKT; whereas the si-FABP4+IGF-1 group showed lower levels of PI3K and p-AKT/AKT than the IGF-1 (Fig. 4A) (all $p<0.05$). Cell migration and invasion were assessed by CCK-8 method and Transwell assay, and revealed that the IGF-1 group exhibited higher ESC proliferation, migration and invasion than the control group; while the si-FABP4+IGF-1 group had restricted ESC proliferation, migration and invasion compared with the IGF-1 group (Fig. 4 B, C) (all $p<0.05$). Eventually, when levels of EMT markers were analyzed by western blot analysis, the result suggested that compared with the control group, the IGF-1 group exhibited declined E-cadherin level and augmented levels of N-cadherin and Vimentin; while compared with the IGF-1 group, the si-FABP4+IGF-1 group expressed elevated E-cadherin level and dampened N-cadherin and Vimentin levels (Fig. 4D) (all $p<0.05$). The above findings indicated that upregulation of the PI3K/AKT axis reversed the suppressive role of silenced FABP4 on ESC proliferation and EMT.

DISCUSSION

In a previous study, it was found that FABP4 could be a candidate indicator of EMS as it was strongly expressed in ectopic endometrium as compared with that in eutopic endometrium (6). On the other hand, EMT, a process that the epithelial cells transdifferentiated into motile mesenchymal cells, was an essential player in wound healing, cell growth and stem cell activities, as well as pathological development of fibrosis and carcinoma (7). Robustly expressed FABP4 reduced E-cadherin and improved levels of Vimentin and N-cadherin in cervical squamous cell carcinoma (8). All in all, silencing of FABP4 reversed severe EMS progression.

When FABP4 was underexpressed, the PI3K/AKT axis was also downregulated, illustrating that they were positively related. Importantly, FABP4

could upregulate the PI3K/AKT axis to augment severity and prognosis of a wide range of chronic diseases caused by obesity (9). The PI3K/AKT axis accelerated ESC dissemination and strengthened ESC viability in ectopic endometrium of EMS patients (10). Hyperactivation of the PI3K/AKT axis could enhance endometrial cancer cell aggressiveness and accelerate EMT (11). Generally speaking, the simultaneous downregulation of FABP4 and the PI3K/AKT axis brought about therapeutic benefits to EMS.

In summary, our study unearthed that FABP4 depletion inhibited ESC growth and EMT through the downregulation of the PI3K/AKT axis. These results are promising in prospect of promoting future treatment of EMS. Though our findings offer therapeutic option in EMS treatment, the experimental results and effective application into clinic practice still need in-depth validation.

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LETTER TO THE EDITOR

Management of recurrent cystitis in clinical practice: a nationwide surveyG. Ciprandi¹, S.E. Aragona² and Study Group on Urinary Infections*¹Allergy Clinic, Casa di Cura Villa Montallegro, Genoa, Italy;²Center of Regenerative Medicine, Humanitas Mater Domini, Castellanza (VA), Italy*Received March 25, 2021 – Accepted July 5, 2021*

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To the Editor,

Uncomplicated cystitis is defined as an infection in a healthy person who is not pregnant or has not recently had local medical devices inserted, and without anatomical or functional urinary tract abnormalities. Isolated cystitis cases are generally well tolerated by patients, but on several occasions, recurrent cystitis (defined as two infections in 6 months or three or more infections in 1 year) has a detrimental impact on their quality of life. Recurrent uncomplicated cystitis (RUC) occurs in approximately 35-53% of women treated within one year (1).

Because of the formation of bacterial biofilms, unfortunately, antibiotic treatment results are often not satisfying. The infection is not always eradicated, and bacteriuria and recurrences may persist. Biofilms have recently been advocated as playing a possible role in the pathogenesis of RUC, mainly concerning persistent colonization by harmful or pathogenic bacteria (2). Moreover, antibiotics are burdened by a high prevalence of resistance that significantly affects their effectiveness. Consequently, new strategies have been prospected to solve these issues. In this regard, local dysbiosis is a relevant pathogenic factor associated with infection recurrence (3). *Escherichia coli* is the most prevalent etiologic agent for RUC,

but it may be counteracted by *Lactobacilli* (4).

The most recent guidelines do not apply the use of a non-antibiotic strategy (5). However, there is growing evidence regarding the importance of probiotics and herbal medications in the prevention and treatment of RUC (6). Probiotics, mainly *Lactobacilli*, antagonize pathogens, dropping local pH, inhibiting adherence, and producing antibacterial and anti-inflammatory agents (7). Also, d-mannose interferes with bacterial adhesion, as highlighted by a recent systematic review (8). Vitamin D3 regulates the immune system and modulates the response to infections (9). *Urtica dioica* extracts impair bacterial survival, mainly of *Escherichia coli*, reduce virulence, and inhibit biofilm formation (10). *Uva-ursi* extracts exert anti-infective and anti-inflammatory activities, useful in preventing RUC (11). Moreover, quercetin, a flavonoid, resolves inflammation and has anti-oxidative activity, both of which are useful in preventing RUC (12).

Therefore, a nutraceutical (Cistiduo[®], Aurora Biofarma, Milan, Italy) containing these components has been recently proposed to prevent and treat urinary tract infections. For this reason, a nationwide survey investigated its aptitude in managing RUC by a group of Italian gynecologists.

Key words: recurrent cystitis; survey; clinical practice; Lactobacilli; d-mannose; vitamin D; quercetin; bearberry; nettle

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MATERIALS AND METHODS

The survey was conducted in gynecological clinics, distributed across Italy, ensuring extensive and complete national coverage during 2020. Gynecologists recruited all consecutive outpatients with RUC. The inclusion criteria were RUC history and adulthood. Exclusion criteria were having acute cystitis, comorbidities, or concomitant medications, mainly antibiotics, that could interfere with the interpretation of the results. All participants signed informed consent. The procedure was conducted in a real-world setting.

The patients were subdivided into two groups. Group A (active group) was treated with oral Cistiduo®: one sachet in the morning and one in the evening for seven days, then another course for additional seven days after one-month suspension. Group B (control group) did not take any medications. Patients were evaluated immediately before treatment (T0) and after treatment (T1).

Cistiduo® contains d-mannose 800 mg, nettle dry extract 100 mg, quercetin 85 mg, bearberry dry extract 50 mg, vitamin D3 200 IU, *Lactobacillus acidophilus* 10⁹ CFU/g, and *Lactobacillus rhamnosus* 0.5x10⁹ CFU/g. Notably, Cistiduo® has an innovative packaging, as the sachet is internally divided into two separate compartments, which inhibit the interaction of the components until taking it.

The evaluated parameters included: fever (expressed as % of patients with fever), burning and pain during micturition (assessed by a visual analogic scale: 0=no symptom, 10=very severe symptom), pollakiuria (scored as absent, mild, moderate, and severe), and hematuria (expressed as % of patients having it).

Statistical analysis was performed using the GraphPad Prism software (version 8.0.1 for Windows; GraphPad Software®, San Diego, CA, USA). For all tests, the accepted minimum significance limit was 0.05.

RESULTS

The current survey included 61 patients (mean age 55±15 years) with RUC: 55 in Group A and 6 in Group B. The considered parameters were not statistically different between groups at baseline, so the two groups were homogeneous.

At T1, the percentage of patients with fever decreased from 12.7% to 1.8% in Group A, and from 33.3% to 0% in Group B, without any statistical

intergroup difference. VAS for burning decreased from 6 (4-7) to 1(1-2) in Group A, and from 7 (6-7) to 2.5 (2-6) in Group B; the intergroup comparison showed a significant difference (p=0.013) as reported in Fig. 1. VAS for pain decreased from 5 (3-6) to 1 (1-2) in Group A, and from 6 (6-7) to 2 (1-6) in Group B; there was a significant intergroup difference (p=0.028) as reported in Fig. 2. Pollakiuria was mild in 50.9%, moderate in 36.4%, and severe in 7.3% of patients in Group A at baseline; pollakiuria was absent in 90.9% and mild in 9.1% at the end of the treatment. Group B patients had mild pollakiuria in 16.7% and moderate in 66.7% at baseline. At the end of the study, 50% had no pollakiuria, 33.3% mild, and 16.7% moderate. The intergroup analysis showed a significant difference (p=0.002), as reported in Fig. 3. Hematuria was reported only by four patients in Group A at T0, but nobody had hematuria at T1. The nutraceutical was safe and well-tolerated by all patients.

DISCUSSION

Recurrent uncomplicated cystitis is a bothersome problem in women and involves a relevant social burden. Anatomic and behavioral factors promote the recurrence of urinary infections. Intestinal bacteria, mainly *Escherichia coli*, could persistently colonize the bladder when defensive mechanisms are impaired, mainly concerning eubiosis, such as a balanced microbiota.

RUC is usually persistent, and unfortunately, there is no standard therapy (5, 7). Gynecologists commonly prescribe oral antibiotics courses, but they are usually scarcely effective, mainly due to antibiotic resistance, and bacteria produce biofilms to defend from antibiotics. Moreover, intestinal and urogenital dysbiosis exert an important pathogenic role in persisting cystitis.

Consequently, probiotics have been tested for inhibiting cystitis recurrence. In particular, Lactobacilli species are the predominant component of vaginal and urinary tract microflora. Lactobacilli produce an abundant amount of lactic acid, dropping pH and consequently inhibiting pathogen proliferation. In this regard, there is evidence that probiotics could prevent RUC (3, 4). In particular,

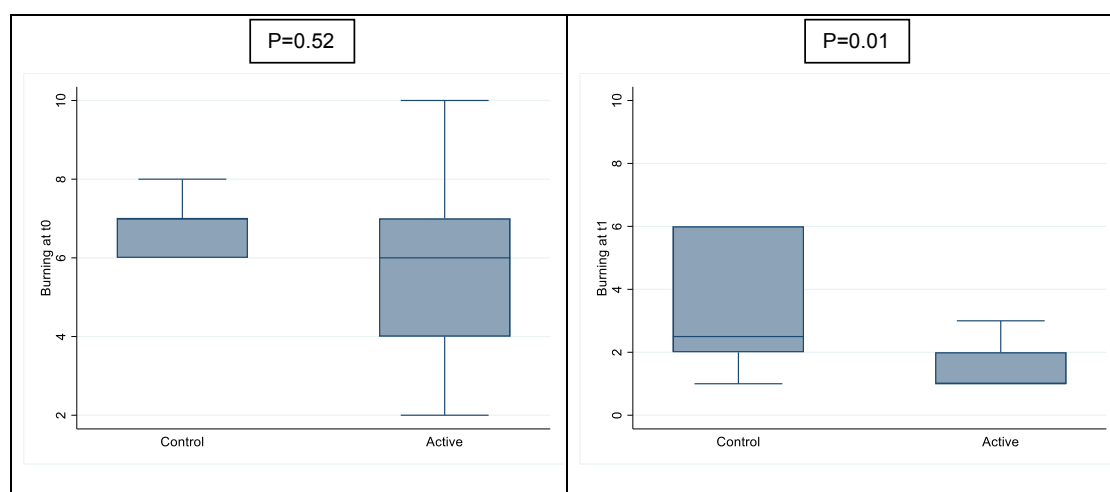


Fig. 1. Severity of burning during micturition at baseline and after treatment in controls and actively-treated patients.

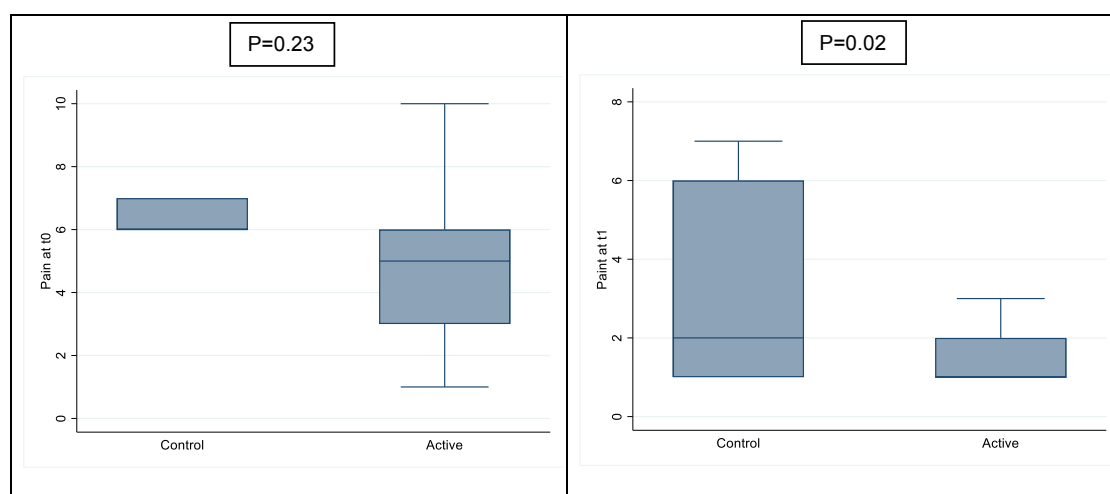


Fig. 2. Severity of pain during micturition at baseline and after treatment in controls and actively-treated patients.

L. acidophilus and *L. rhamnosus* were promising species. Also, d-mannose may antagonize pathogens, inhibiting bacterial adhesion to the bladder mucosa. Vitamin D stimulates the immune response to contrast pathogens. Moreover, herbal compounds contain several active ingredients useful in the prevention of infections, including cystitis. There is accumulating evidence that the dry extracts of uva-ursi and nettle may effectively prevent RUC (6). In particular, quercetin is an agent with relevant anti-inflammatory and anti-infective activities. Thus, the nutraceutical Cistiduo®, containing these components, could

represent an attractive preventive strategy option. The present survey investigated this issue in a real-world setting, such as gynecological clinics.

The outcomes showed that two courses with this nutraceutical provided a significant reduction of the perception of burning and pain micturition and improved pollakiuria. The multicomponent composition could explain its positive efficacy as it may act on several aspects involved in the infectious and inflammatory reaction present in recurrent cystitis. On the other hand, the present survey has some limitations, including the open design, the lack

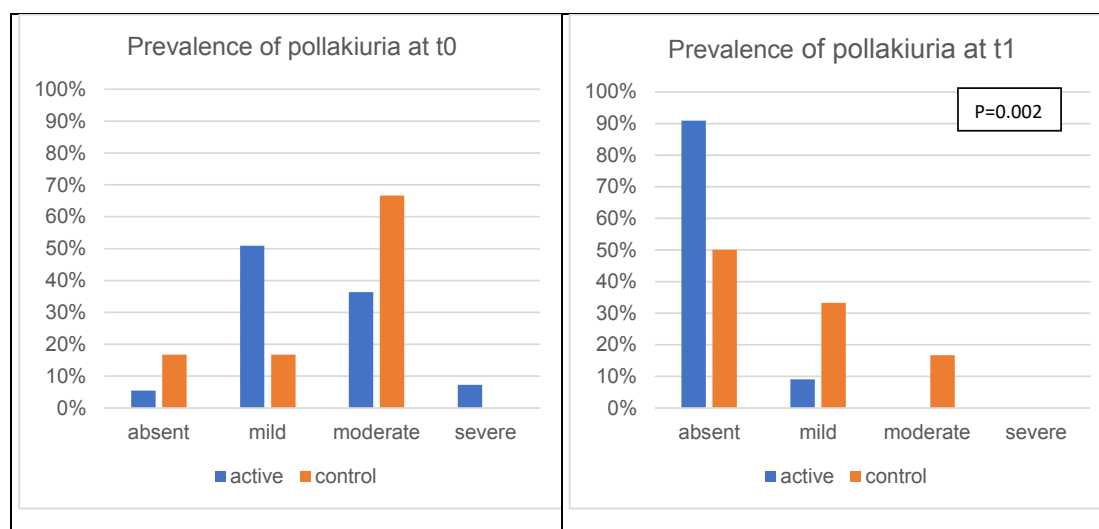


Fig. 3. Percentage distribution of subjects with absent, mild, moderate, and severe pollakiuria at baseline and after treatment in controls and actively-treated patients.

of objective parameters, and pathogen documentation. Of course, further rigorous studies should be conducted to confirm these preliminary results.

In conclusion, the present survey showed a multicomponent nutraceutical containing Lactobacilli, d-mannose, vitamin D3, quercetin, nettle, and uva-ursi, could be a promising option in preventing cystitis recurrence.

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LETTER TO THE EDITOR

Effects of rehabilitation on reducing dyskinesias in a Parkinson's disease patient abusing therapy with levodopa-carbidopa intestinal gel: a paradigmatic case report and literature review

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To the Editor,

Dyskinesias are frequent complications occurring in the long-term management of patients affected by Parkinson's disease (PD) (1) that might cause disability, and negatively affect health-related quality of life (HRQoL) (2), with a significant adverse impact on the activities of daily living (ADL) (3). In the complex management of PD patients, a key role might be played by levodopa-carbidopa intestinal gel (LCIG) infusion therapy directly in the duodenum, allowing a continuous delivery by a portable pump, thus resulting in smoother levodopa plasmatic concentrations (4). A continuous LCIG infusion has shown to be related to a reduction in motor complications and improvement in HRQoL compared with the standard oral formulation of levodopa in patients with advanced PD (5, 6). However, LCIG therapy has several side effects, including the development of motor complications, especially dyskinesias (7, 8).

Rehabilitation might play a key role in patients affected by neurological disorders to reduce their disability level (9, 10), therefore a specific rehabilitative program including therapeutic exercise and fall prevention strategies could contribute to preserve functional ability and autonomy in ADL in PD patients (9). Furthermore, it has been demonstrated

that a patient-tailored intensive rehabilitation protocol could be effective in reducing intensity and duration of levodopa-induced dyskinesias, allowing a reduction in the drug dosage of oral levodopa, without worsening of symptoms (1). This phenomenon might be explained by the attenuation of dyskinesias (due to stimulation of dopaminergic receptors) by performing the correct movements of the rehabilitative treatment (1). Moreover, Meloni et al. (11) showed the controversial manifestation of LCIG-induced dyskinesias according to the drug dose, highlighting the need of rehabilitation in PD patients. These dyskinesias represent an issue in the management of PD patients. Indeed, to date, to the best of our knowledge, there is still a lack of evidence on the effects of rehabilitation in reducing LCIG-induced dyskinesias in drug-abusing PD patients.

Therefore, by the present paradigmatic case report, we aimed to describe the key role that a specific rehabilitation plan might play in terms of functioning in a PD patient with dyskinesias, due to an abuse of LCIG therapy.

Case presentation

A 77-year-old male PD patient, with a body mass index of 25.18 kg/m², referred to our Rehabilitation Unit in December 2020, due to frequent and disabling

Key words: Parkinson's disease; dyskinesias; functioning; balance; rehabilitation

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dyskinesias. He referred to undergo LCIG whenever he feels tired and slow in movement without any control over the daily dose, resulting in dyskinesias due to this abuse. The participant carefully read and signed an informed consent and researchers provided to protect the privacy, and the procedures in this study were in accordance with the Declaration of Helsinki.

In 2011, he had a neurological diagnosis of PD. The first treatment administered was levodopa/benserazide therapy per os, with partial benefit. Then, the patient reported to have suffered from several side effects, including motor blockages, oral, four-limb and trunk dyskinesias, and mental confusion. Thus, in 2017, at a follow-up visit, the neurologist prescribed LCIG into the duodenum, through a percutaneous endoscopic gastrostomy (PEG). However, the following year, he developed LCIG-induced dyskinesias, with ballistic movements prevalent in the lower limbs that negatively influence gait and independence in ADL. In the previous two years, the patient had undergone a combined therapy with LCIG plus amantadine, with insufficient effects, considering that from August 2020 the dyskinesias had started to gradually worsen. A morning bolus dose, a continuous maintenance dose, and extra bolus doses of LCIG were then administered over 16 hours/day. However, the patient had abused the LCIG without respecting the recommended daily dose, with consequent increase in the number of dyskinesias. According to his daily diary, the patient

reported that in the 20 days prior to the first physiatric evaluation he assumed a mean LCIG dosage of 43.6 ± 4.8 ml/day.

The patient referred to our Outpatient Department of Physical Medicine and Rehabilitation in ON phase in December 2020. At the physiatric clinical evaluation, we noticed: uncertain walking with forward displacement of the body's center of gravity; camptocormic attitude; adequate trunk control; presence of dyskinesias, especially in the lower limbs with a consequent difficulty in walking; limitation of the right ankle joint Range Of Motion (ROM) in dorsiflexion. As a result, he walked increasing the left stride length to counteract the right-side impairment, with an altered duration of the phases of the gait cycle. In addition, walking so fast, he often stumbled and was afraid of falling, resulting in a noteworthy limitation of autonomy in the ADL. Fig. 1 depicts the patient undergoing the gait assessment.

Therefore, to reduce the daily infusion of LCIG, we planned a 20-day rehabilitation protocol, consisting of 5 sessions/week for 3 weeks; each session lasting 90 minutes consisting of 5 different activities: i) warm-up activities and muscle stretching exercises; ii) passive and active kinesiotherapy exercises, prescribed to improve ROM, with particular attention to the right tibio-tarsal joint and to act on postural changes; iii) exercises for improving the gait cycle using the Sensory Treadmill (Gait Trainer Treadmill Biodex, BTS Bioengineering Spa, Garbagnate M.se, Milano, Italy) with auditory and visual cues; iv) balance exercises, using a stabilometric platform with a visual cue, to correct the forward displacement of the body's center of gravity; v) balance exercises, performed in front of a mirror to control involuntary and exaggerated movements (1). The patient adequately performed the rehabilitation plan without any interruption for the entire study protocol and without any side effects, testifying the safety and the tolerability of rehabilitation in such complex patients.

To evaluate the effectiveness of the rehabilitation protocol on our PD patient, we assessed several outcome measures, including: Movement Disorders Society Unified Parkinson's Disease Rating Scale



Fig. 1. The Parkinson's disease patient at the first physiatric evaluation.

Table I. *MDS-UPDRS parts III and IV assessed at the baseline (T0), at the end of treatment (T1), and at 1 month after the end of treatment (T2)*

	T0	T1	T2
MDS-UDPRS part III			
3.1. Speech impairment	1 (minimum)	1 (minimum)	1 (minimum)
3.2. Facial mimic impairment	2 (mild)	1 (minimum)	1 (minimum)
3.3. Stiffness			
Stiffness of neck	0 (absent)	0 (absent)	0 (absent)
Stiffness of right upper limb	0 (absent)	0 (absent)	0 (absent)
Stiffness of left upper limb	0 (absent)	0 (absent)	0 (absent)
Stiffness of right lower limb	3 (moderate)	2 (mild)	1 (minimum)
Stiffness of left lower limb	3 (moderate)	2 (mild)	2 (mild)
3.4. Impairment in movements of hand fingers			
Right hand fingers	0 (absent)	0 (absent)	0 (absent)
Left hand fingers	1 (minimum)	1 (minimum)	1 (minimum)
3.5. Impairment in hand movements			
Right hand	0 (absent)	0 (absent)	0 (absent)
Left hand	1 (minimum)	1 (minimum)	1 (minimum)
3.6 Impairment in hand pronosupination Movements			
Right hand	0 (absent)	0 (absent)	0 (absent)
Left hand	1 (minimum)	1 (minimum)	1 (minimum)
3.7 Impairment in toes repeated movements			
Right toes	1 (minimum)	0 (absent)	0 (absent)
Left toes	2 (mild)	1 (minimum)	1 (minimum)
3.8 Impairment in leg agility			
Right leg	1 (minimum)	0 (absent)	0 (absent)
Left leg	2 (mild)	1 (minimum)	1 (minimum)
3.9 Impairment in getting up from a chair	2 (mild)	1 (minimum)	1 (minimum)
3.10 Impairment in walking	2 (mild)	1 (minimum)	0 (absent)
3.11 Freezing	2 (mild)	1 (minimum)	1 (minimum)
3.12 Alteration in postural stability	2 (mild)	1 (minimum)	1 (minimum)
3.13 Postural alteration	2 (mild)	2 (mild)	1 (minimum)
3.14 Impairment in movement spontaneity	2 (mild)	1 (minimum)	1 (minimum)
3.15 Postural tremor of the hands			
Right hand	0 (absent)	0 (absent)	0 (absent)
Left hand	0 (absent)	0 (absent)	0 (absent)
3.16 Kinetic tremor of the hands			
Right hand	0 (absent)	0 (absent)	0 (absent)
Left hand	0 (absent)	0 (absent)	0 (absent)
3.17 Amplitude of tremor at rest			
Right arm	0 (absent)	0 (absent)	0 (absent)
Left arm	0 (absent)	0 (absent)	0 (absent)
Right leg	0 (absent)	0 (absent)	0 (absent)
Left leg	0 (absent)	0 (absent)	0 (absent)
Lips/jaw	0 (absent)	0 (absent)	0 (absent)
3.18 Continuity of the tremor at rest	0 (absent)	0 (absent)	0 (absent)
HOEHN & YAHR	3	2	2
MPD UDPRS part IV			
4.1. Time spent with dyskinesias	3 (moderate)	2 (mild)	2 (mild)
Awake timing (hours)	14	14	14
Hours spent with dyskinesias	8	6	5

Percentage of dyskinesias	57.1%	42.8%	35.7%
4.2. Functional impairment due to dyskinesias	3 (moderate)	2 (mild)	2 (mild)
4.3. Time spent in OFF phase	2 (mild)	1 (minimum)	1 (minimum)
Awake timing (hours)	14	14	14
Hours spent in OFF phase	4	2.4	2.4
Percentage of OFF phase	28.5%	17.1%	17.1%
4.4 Functional impact of motor fluctuations	2 (mild)	2 (mild)	2 (mild)
4.5 Complexity of motor fluctuations	1 (minimum)	1 (minimum)	1 (minimum)
4.6 Painful Dystonia during OFF phase	0 (absent)	0 (absent)	0 (absent)

MDS-UPDRS: Movement Disorders Society-Unified Parkinson's Disease Rating Scale

(MDS-UPDRS) in its parts III and IV (including the Dyskinesias percentage), trunk control test, Berg Balance Scale (BBS), Functional Independence Measurement (FIM), inertial sensor gait analysis, time up and go (TUG), and stability index, through the platform Balance System Biodex (BTS Bioengineering Spa, Garbagnate M.se, Milano, Italy). All the above-mentioned outcomes were assessed at baseline (T0), at the end of treatment (T1), and at one month after the end of treatment (T2). Lastly, we evaluated the LCIG dosage self-administered by the patient in the 20 days during the rehabilitation treatment, comparing these findings with data reported by the patient in the 20 days prior to the psychiatric evaluation.

After the 20-day rehabilitative intervention, there was a reduction in both MDS-UPDRS parts III and IV, as shown in Table I. In addition, the percentage of dyskinesias considerably reduced from 57.1% (T0) to 42.8% (T1). Both BBS (from 36 to 46) and FIM had a noteworthy improvement (from 95 to 107) at T1. Furthermore, the patient showed an improvement in gait analysis parameters at T1. After the treatment, the gait quality index of the right lower limb increased (from 68.3 ± 9.3 to 92.4 ± 3.3), obtaining a rebalancing between the right and left sides, both reaching the normal ranges. Lastly, the test execution time of TUG resulted considerably reduced (25.8 vs 14.1 s). After discharge, the patient continued to perform some exercises learnt during hospitalization (warm-up activities, muscle

stretching exercises, and balance exercises) with intriguing results considering that all outcomes maintained similar results to the 1-month follow-up evaluation (T2) (see Table II for further details).

As shown in Fig. 2, there was a noteworthy decrease in the LCIG daily dose, considering that LCIG dose/day before admission was 43.6 ± 4.8 ml/day and 37.6 ± 2.8 ml/day at T1. Moreover, the trend of daily doses in the 20 days during rehabilitative treatment reached lower values, with an evident further decline in the last week (see Figure 2 for further details).

DISCUSSION

Dyskinesias in PD are considered as common complications of dopaminergic treatment (12). Meloni et al. (11) tried to manage LCIG-induced dyskinesias by increasing morning LCIG flow and report that an increase in dopaminergic stimulation may improve dyskinesias in the short term, probably through the shortening of time necessary to reach the useful therapeutic threshold. In the present case report, a specific intensive rehabilitation reduced duration and frequency of dyskinesias in the PD patient, improving his motor performance and autonomy in ADL. We also observed an increase in the walking quality index and a change in oscillation phase of the gait cycle.

According to MDS-UPDRS part III-IV score, the patient was no longer limited by dyskinesias at

discharge. However, he carried out all the ADL and took part in all social interactions, even in periods of dyskinesias. As the dyskinesias decreased, there was an improvement in gait and balance that was maintained at the 1-month follow-up evaluation.

To the best of our knowledge, only Morgante et al.

(7) have described the abuse of LCIG infusion, with the onset of dyskinesias, as a side effect. The Authors affirmed that, albeit the self-administration of LCIG may lead to a risk of an abuse of dopaminergic drugs, a valuable LCIG infusion might be achieved in PD patients. In the literature, Frazzitta et al. (12)

Table II. Secondary outcomes assessed at the baseline (T0), at the end of treatment (T1), and at 1 month after the end of treatment (T2)

	T0	T1	T2
Berg Balance Scale	36 (Need of assistance)	46 (independent)	43 (independent)
FIM	95/126	107/126	105/126
Eating	6	6	6
Grooming	6	7	7
Bathing	6	7	7
Dressing upper body	3	5	5
Dressing lower body	3	5	4
Toilette	6	6	6
Bladder management	4	5	5
Bowel management	7	7	7
Bed/chair/wheelchair transfers	5	6	6
Toilet	5	6	6
Bath/shower	5	6	5
Walking/wheelchair	5	5	5
Stairs	5	5	5
Auditory/visual comprehension	6	6	6
Verbal/ non-verbal expression	5	6	6
Social interaction	6	7	7
Problem solving	6	6	6
Memory	6	6	6
Inertial sensor gait analysis			
Gait quality index left	96.6 ± 2.5	92.2 ± 4.4	89.1 ± 6.4
Gait quality index right	68.3 ± 9.3	92.4 ± 3.3	90.8 ± 5.7
Flight phase duration left (sec)	0.41 ± 0.1	0.55 ± 0.4	0.52 ± 1.70
Flight phase duration right (sec)	0.24 ± 0.5	0.45 ± 0.5	0.43 ± 1.3
Stance phase duration left (sec)	0.63 ± 0.6	0.71 ± 0.4	0.8 ± 0.18
Stance phase duration right (sec)	0.78 ± 0.9	0.8 ± 0.4	0.65 ± 0.11
Average cadence (semi-steps/min)	117.7 ± 8.6	97.2 ± 4.2	95.4 ± 2.6
TUG analysis			
TUG (sec)	25.8	14.1	14.1
A-P acceleration (standing) (m/s ²)	2.7	1.7	2.0
A-P acceleration (sitting) (m/s ²)	3.30	1.1	1.6
Lateral acceleration (standing) (m/s ²)	1.9	1.0	1.2
Lateral acceleration (sitting) (m/s ²)	4.9	2.0	2.2
Vertical acceleration (standing) (m/s ²)	3.4	1.9	2.3
Vertical acceleration (sitting) (m/s ²)	3.5	3.2	2.1
Average rotation speed (intermediate rotation) (°/s)	75.5	45.6	54.9
Average rotation speed (final rotation) (°/s)	80.7	49.6	76.7

Data are expressed as absolute values or as means ± standard deviations. FIM: Functional Independence Measurement; TUG: Timed up and go test.

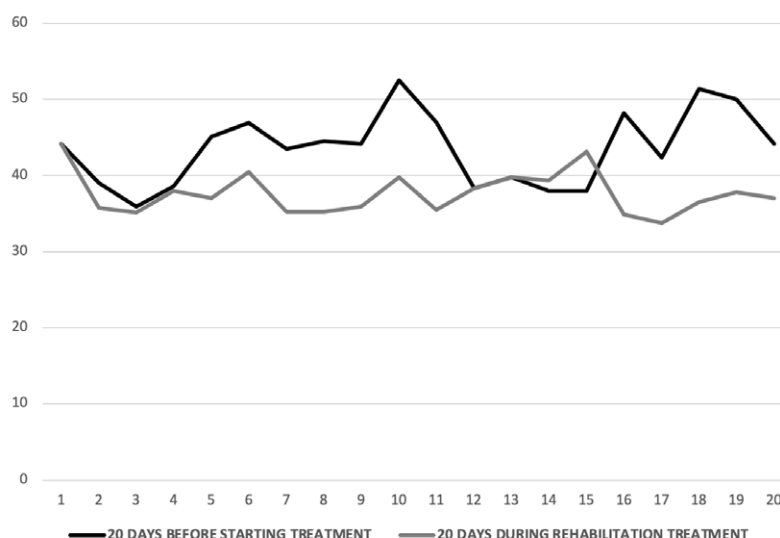


Fig. 2. Differences in levodopa-carbidopa intestinal gel dosage self-administered by the patient in the 20 days prior to the beginning of rehabilitation (grey line) and in the 20 days during the rehabilitation treatment (black line).

also suggested that it is possible to act on dyskinesias in Parkinsonian patients with an adequate intensive rehabilitation protocol, allowing a reduction in drug dosage and related adverse effects.

To date, no rehabilitation treatment has been described in the literature to reduce LCIG-induced dyskinesias and to avoid levodopa abuse. Indeed, this case report firstly investigated the role of physiotherapy in such paradigmatic patients. This case report has, however, some study limitations: firstly, a single case is insufficient to draw conclusions and provide the literature with important influences; secondly, absence of data on long-term effects.

The patient-oriented rehabilitation treatment led to a lower number of dyskinesias, inducing the patient to adhere to a correct LCIG infusion protocol. This finding suggests that an individual rehabilitation plan might represent an effective approach in such a paradigmatic and complex disease. Further studies are needed to confirm the effects of rehabilitation on dyskinesias reduction in PD patients abusing LCIG therapy.

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LETTER TO THE EDITOR

Identification of circRNA-mediated competing endogenous RNA network in the development of bladder urothelial carcinomaSP. Shao^{1,2#}, RJ. Zhao^{1#}, S. Lu^{3#}, LP. Wen², JJ. Ni⁴, KM. Zhu⁵ and WD. Han^{1*}

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To the Editor,

Bladder urothelial carcinoma (BLCA) has a high mortality rate but the mechanisms of its tumorigenesis are still unclear. Recently, it was proved that circRNA with a closed continuous loop structure is more stable than linear RNA, and its regulated competing endogenous RNA (ceRNA) network participates in many cancer-related biological processes. Mao W et al. (1) reported that overexpressed hsa_circ_0068871 could promote the progression of bladder cancer via miR-181a-5p/FGFR3. Furthermore, ceRNA is also important in immune infiltration remodeling which affects the progression and prognosis of patients. For example, overexpressed circUHRF1 upregulated the expression of TIM-3 via degradation of miR-449c-5p to inhibit NK cell function, which drove resistance to anti-PD1 immunotherapy (2). However, circRNA-mediated ceRNA network is complicated and more potential regulatory mechanisms remain to be researched.

Therefore, this study aims to construct a circRNA-mediated competing endogenous RNA (ceRNA)

network in the development of BLCA by comparing the expression profile of circRNA, miRNA and mRNA between BLCA tissues and normal bladder tissues, and investigate the ceRNA-related immune infiltration.

Methods and results

Comparing the expression profile in BLCA tissues vs normal bladder tissues, a total of 107 differentially expressed (DE) circRNAs from GSE92675-GPL19978 (Fig. 1A), 141 DE miRNAs from TCGA-BLCA (Fig. 1B), and 214 consistently up-regulated and down-regulated DE mRNAs from TCGA-BLCA as well as GSE7476-GPL570 were identified with the criteria of False discovery rate (FDR) < 0.05 and $|\log FC| \geq 2$ (Fig. 1 C-F). Enrichment analysis showed that multiple immune-related pathways such as TNF- α /NF- κ B, IL2/STAT5 signaling pathway and IFN- γ response, and multiple DNA replication-related biological processes were aberrant in the development of BLCA (Fig. 2 A, B).

DE circRNA-DE miRNA pairs were then

Key words: circRNA; ceRNA; bladder urothelial carcinoma; tumorigenesis; immune infiltration

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established based on CircBase and Cancer-Specific-CircRNA Database (CSCD), which provided information on predicted circRNA-regulated miRNAs, and established DE miRNA-DE mRNA pairs based on miRDB, miRTarBase and TargetScan database which provided information on predicted miRNA-regulated mRNAs, and eventually constructed the DE circRNA-

DE miRNA-DE mRNA network. The original ceRNA network consisted of 56 DE circRNAs, 32 DE miRNAs and 116 DE mRNAs.

In the original ceRNA network, among these circRNAs, six were previously found to be cancer-related (Table I), including circUBE2D2 (3), circCSPP1 (4), circVDAC3 (5), circRANGAP1 (6)

Table I. The characteristics of 6 circRNAs in the ceRNA network

circRNAs	Regulation	Type	Chromosome	Stand	Gene symbol
hsa_circ_0032821	Up-regulation	exonic	chr14	-	CEP128
hsa_circ_0005442	Up-regulation	exonic	chr2	+	CRIM1
hsa_circ_0001806	Up-regulation	exonic	chr8	+	CSPP1
hsa_circ_0004503	Up-regulation	exonic	chr5	+	UBE2D2
hsa_circ_0001235	Up-regulation	exonic	chr22	-	RANGAP1
hsa_circ_0005927	Down-regulation	exonic	chr8	+	VDAC3

Chromosome information is from CircBase database.

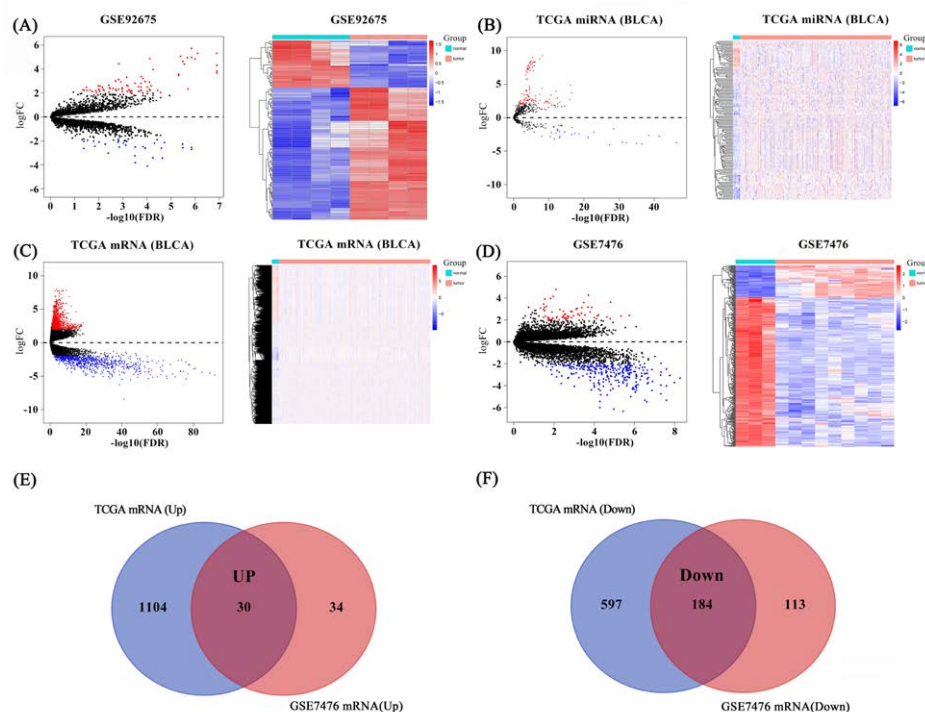


Fig. 1. Differentially expressed (DE) circRNAs, miRNAs and mRNAs in bladder urothelial carcinoma (BLCA). The identification of (A) DE circRNAs in GSE92675, (B) DE miRNAs in TCGA-BLCA, (C) DE mRNAs in TCGA-BLCA and (D) DE mRNAs in GSE7476 between BLCA tissues and normal tissues are performed and shown in volcano plot and heatmap. (E-F) The up-regulated and down-regulated mRNAs are respectively intersected in TCGA-BLCA and GSE7476 datasets.

circCEP128 (7, 8) and circCRIM1 (9). Eight of their regulated DEmRNAs in the ceRNA network were located in top10 ranking scores calculated by MNC algorithm in the protein-protein interaction (PPI) network which was constructed based on DEmRNAs through STRING database (Fig. 3A). Notably, we further validated the expression of these eight selected DEmRNAs in the GEPIA database, and it was found that all of them were consistently differentially expressed (Fig. 3B). In this way, a ceRNA network was eventually segregated from the original ceRNA network, which was made up of six circRNAs and

their regulated five miRNAs (miR-216a-5p, miR-139-5p, miR-205-5p, miR-145-5p and miR-195-5p) and eight mRNAs (EGR3, FOS, JUN, CYR61, CDK1, KLF4, NR4A2 and CTGF) (Fig. 3C).

To characterize the role of the ceRNA network in an immune microenvironment, we calculated the correlation between these eight hub mRNAs and the infiltration of 29 kinds of immune cells based on the TCGA-BLCA mRNA expression profile by the ssGSEA algorithm, and found many of them in the ceRNA network were closely associated with immune infiltration in BLCA microenvironment

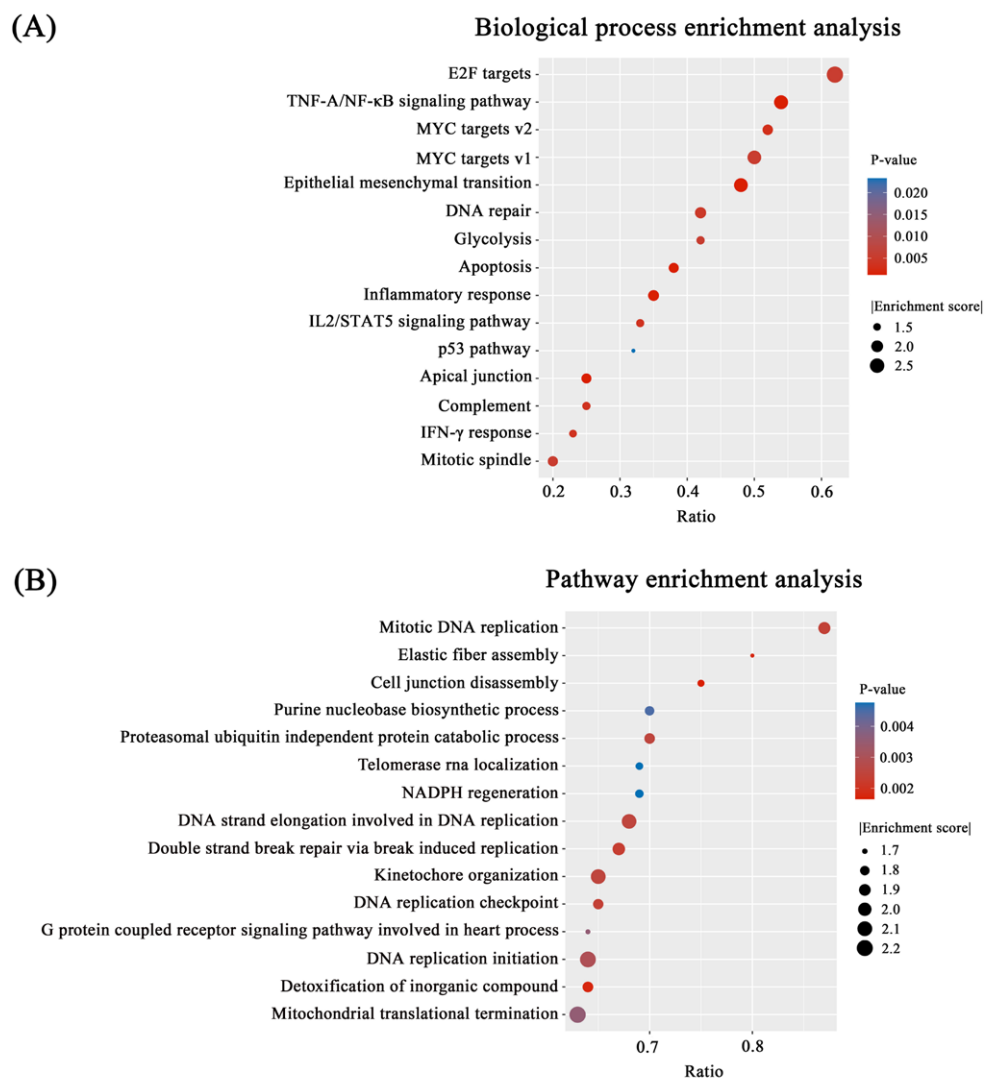


Fig. 2. Enrichment analysis pertaining to the development of BLCA. Enrichment results of (A) biological processes and (B) pathways. Colors, ranging from blue to red, indicate the significance of the results. The size of the dot indicates absolute value of normalized enrichment score. $P < 0.05$ is statistically significant.

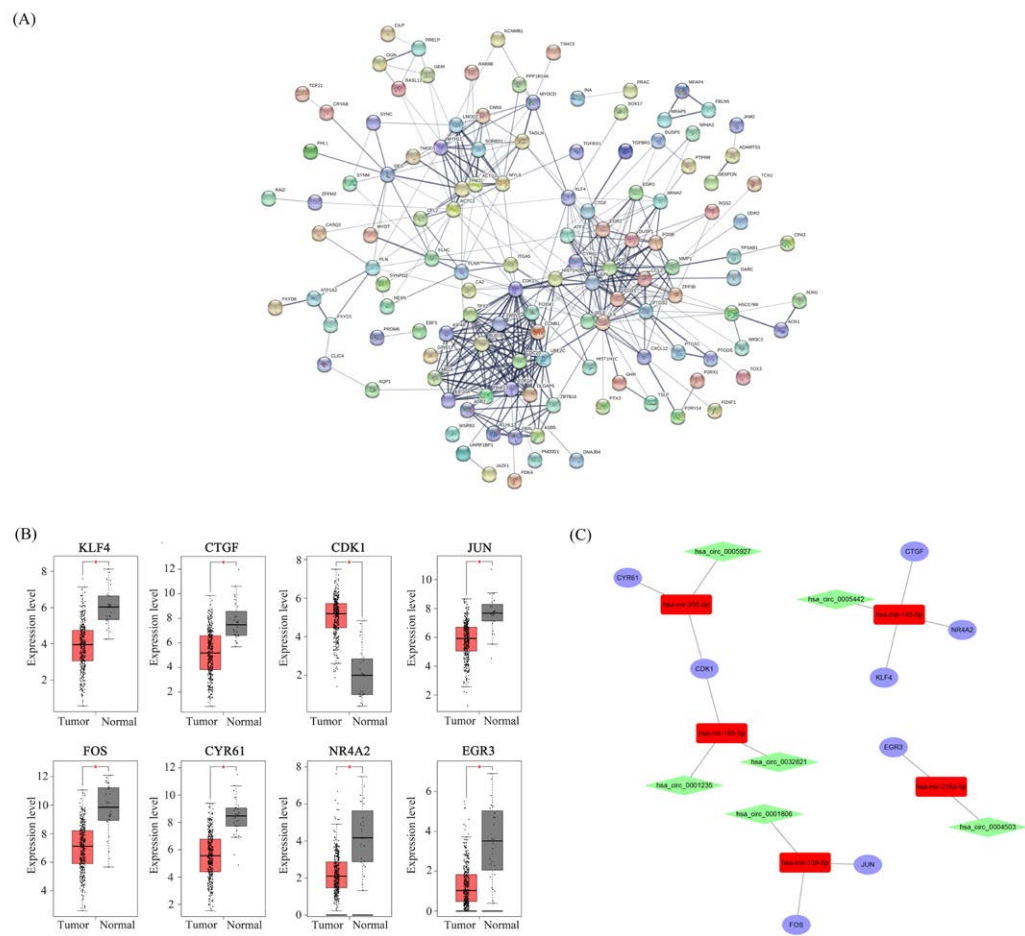


Fig. 3. The construction of ceRNA network in BLCA tumorigenesis. *A*) Protein-protein interaction (PPI) network is composed of 213 nodes and 420 edges. The nodes and edges indicate proteins and interactions, respectively. The thicknesses of edges indicate the degree of relationship among nodes. *B*) The validation of transcription of 8 mRNAs in the Gene Expression Profiling Interactive Analysis (GEPIA) database. *C*) The ceRNA network is composed of 6 circRNAs and their regulated 5 miRNAs as well as 8 mRNAs.

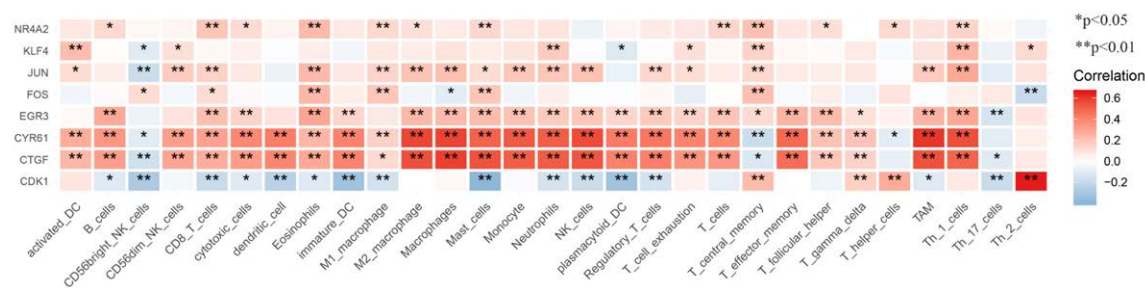


Fig. 4. The correlation between hub genes and immune infiltration. The heatmap shows the correlation between 8 mRNAs in the ceRNA network (EGR3, CTR61, JUN, FOS, CDK1, KLF4, NR4A2 and CTGF) and 29 immune cells in BLCA microenvironment. The blue and red colors indicate negative and positive correlations, respectively. * $P < 0.05$, ** $P < 0.01$.

(Fig. 4). For example, high expression of CDK1 was positively correlated with Th2 cell ($P<0.001$, $\text{cor}=0.651$), but negatively correlated with the infiltration of CD8⁺ T cell ($P<0.001$, $\text{cor}=-0.193$) and M1 macrophage ($P<0.05$, $\text{cor}=-0.156$). Down-regulated FOS and CTGF caused a decreased infiltration of M1 macrophage ($P<0.001$, $\text{cor}=0.183$; $P<0.05$, $\text{cor}=0.124$) and CD8⁺ T cell ($P<0.05$, $\text{cor}=0.115$; $P<0.001$, $\text{cor}=0.269$), while FOS could meanwhile boost the infiltration of Th2 cell ($P<0.001$, $\text{cor}=-0.194$). These genes might create a suppressive immune microenvironment *in situ*, fragile anti-tumor ability and further immune escape.

DISCUSSION

In the constructed BLCA tumorigenesis-related ceRNA network, these circRNAs are previously reported in cancers, but we exclusively focused on circCEP128 and its regulated downstream RNAs, which has been proved to be important in BLCA. Overexpressed circCEP128 could accelerate cell viability and mobility of bladder cancer by sponging miR-145-5p/MYD88 and miR-515-5p/SDC1 (7, 8).

Our results showed that circCEP128 might function through a different mechanism by promoting the expression of CDK1 after sponging miR-195-5p. High expression of miR-195-5p could inhibit the proliferation of BLCA via Cdc42/STAT3 (10). Moreover, overexpressed CDK1 regulated by LncRNA PVT1/miR-31 could boost growth, migration and invasion of BLCA (11). These results provide support for the role of circCEP128/miR-195-5p/CDK1 in BLCA tumorigenesis.

Additionally, we found CDK1 was highly close to immune infiltration, and it was predicted to inhibit the infiltration of CD8⁺ T cell and M1 macrophage, but promote the infiltration of Th2 cell. Consistently, CDK1 inhibitor could overcome IFN- γ -mediated adaptive immune resistance, and convert an immunologically 'cold' tumour into a 'hot' one (12). However, we should acknowledge that there are some limitations. The analysis of circRNA, miRNA and mRNA was carried out with different samples; the results are based on bioinformatics analysis and deserve to be further verified in follow-up studies.

In summary, the ceRNA network might play an important role in BLCA tumorigenesis, especially for circCEP128/miR-195-5p/CDK1, which might additionally induce an immunosuppressive microenvironment *in situ* by the recruitment of Th2 cell and inhibition of M1 macrophage and CD8⁺ T cell.

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LETTER TO THE EDITOR

**Umbilical cord blood platelet-rich plasma injections for epicondylitis treatment:
A prospective clinical study**

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To the Editor,

Epicondylitis has an incidence of 3% in the population. Epicondylitis seems to affect women more frequently than men and it is most common at the age of 35-50 years. Repetitive manual labor, handling of loads > 20 kg or heavy tools > 1 kg, working postures with upper limbs stretched forward, repetition of torsion-rotation movements, the use of a computer for more than 20 hours per week are significant risk factors (1). The histo-pathological pattern is characterized by a mucoid degenerative process, complicated by an inflammatory condition associated with neo-vascularization and fibroblastic hyperplasia, which are responsible for tendinosis. The main causes of epicondylitis are micro-traumatic muscle overload and partial lesions of the extensor radialis brevis tendon of the carpus. According to recent literature on the treatment of epicondylitis, Authors identified the physical therapy as first option for the success of treatment, whilst for refractory cases platelet-rich plasma (PRP) infiltration or surgical treatment

represent valid therapeutic options (2).

Shock waves (SWs) play a significant role in the treatment of epicondylitis. According to the literature, SWs are responsible for cellular responses and modelling growth factor release, including vascular endothelial growth factor and nitric oxide (NO). Due to revascularization, and modulation of inflammation, Extracorporeal Shockwave Therapy (ESWT) is applied for the treatment of epicondylitis (3). Recently, PRP infiltration has been investigated as an adjuvant therapy in the treatment of epicondylitis. The activation of platelets due to the release of the alpha granule content is able to promote the proliferation of tenocytes and to control the inflammatory process. Although many studies have examined the good results of autologous PRP application for epicondylitis treatment (4), further investigations are needed to assess the effects obtained by PRP from umbilical cord blood (CB-PRP) for treating other diseases such as skin ulcers. The aim of the study is to compare the results of

Key word: shock waves; PRP; umbilical cord; epicondylitis

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two different treatment options for lateral elbow epicondylitis: CB-PRP infiltration vs ESWT.

MATERIALS AND METHODS

This is a prospective randomized single-blind study with an evidence level of 1B. The study was approved by the local Ethics Committee (No. 6007 on 13.11.2019) and written consent was obtained from each patient. Thirty patients were enrolled from our Orthopedic Unit and randomized into two groups. Fifteen subjects received CB-PRP infiltration (group A) while the other fifteen subjects were treated with ESWT (group B). A randomization method (<http://www.randomization.com>) was applied to assign the subjects to one of the two groups. The following were used as inclusion criteria: age > 18 years; persistent pain for at least 6 months before recruitment; finger extensor tendon tendinosis confirmed by ultrasound and/or MRI exam; persistent pain and functional limitation; subjects non-responders to rest-ice-drug assumption and physical therapies for at least three months between previous treatments and recruitment. Exclusion criteria were: drug treatment (NSAIDs, analgesics, steroids or anticoagulants) at least 4 weeks before the start of the study; presence of cardiac pacemaker; previous surgery or fractures of the affected elbow or upper limb; the presence of systemic diseases (diabetes mellitus, rheumatoid arthritis, autoimmune diseases, tumor etc.); cervical radiculopathy; epilepsy; congenital or acquired deformities of the upper limbs; coagulopathy; severe unstable heart disease; pregnancy or breastfeeding.

Sample size

The aim of this study is to test the equality of VAS score for two groups: CB-PRP infiltration vs ESWT. In order to test this hypothesis, a significance level (α) was set at 0.050 and a 2-tailed test was used. To detect difference between the CB-PRP group and ESWT group the desired sample size is 22 patients, with 11 subjects in each group. Due to this sample size and assuming both a mean difference of 1.5 point (corresponding to the smallest effect that would be important to detect) between CB-PRP group and ESWT group and a common standard deviation of 1.3 within-group, the study had to have power of 82% to reach a statistically significant result. In consideration of the risk of drop-out, the number of recruited subjects improved to 15 subjects per group.

The subjects of group A were treated with a single injection of 2 ml CB-PRP along with 1 ml of 2% lidocaine; the injection was performed with a direct lateral approach on average 5 mm distal to the lateral epicondyle under ultrasound guidance, using a 22 G needle. The subjects of group B received ESWT that was applied using a shockwave device (Minilith, Storz®) equipped with in-line ultrasound-guidance Aloka SSD 900 (Aloka Co., Ltd. Tokyo, Japan). The subjects were placed in a sitting position, with the elbow flexed and pronated respectively to 90° and 45°; the insertion of epicondylitis tendon was identified by ultrasound guidance. The protocol included three sessions, one per week, giving 1,600 impulses per session at an energy flux density (EDF) between 0.01 and 0.10 mJ/mm² which was consistent with each patient's pain tolerance level. Repetition frequency of shockwave pulses was 4 Hz. This protocol was set up in accordance with previous studies (4, 5). All subjects enrolled were instructed to perform the same self-administered stretching and strengthening exercises for the extensor muscles of the fingers. The protocol included six stretching exercises and two muscle strengthening exercises to be performed for 10 repetitions, 5 times per day for 24 weeks.

The assessment times were: at the time of recruitment (T0), three months after recruitment (T1) and six months after recruitment (T2). For each subject, the epidemiological data (age, weight, BMI, gender, smoking habit), clinical data (pain onset, previous treatments, instrumental examinations, co-morbidities, etc.) were recorded at recruitment (T0). VAS and Quick DASH score were administered at each follow-ups, while the Roles and Maudsley Score was also evaluated at T1 and T2.

The primary endpoint was to analyze the efficacy of two treatments in terms of pain reduction in the study sample by VAS scale. The score ranges from 0 (no pain) to 10 (worst). The secondary endpoint was to evaluate the functional recovery of the arm using Quick DASH score ranging from 0% (no disability) to 100% (maximum disability). Furthermore, the Roles and Maudsley (R&M) Scale was adopted for the evaluation of the therapeutic outcome and perception of improvement at different follow-ups. This scale ranges from 1 to 4, with score of 1 indicating full functional recovery and 4 persistent discomfort.

A prospective randomized single-blind clinical study was conducted. The compiled forms were put into a database using Office Excel database and analyzed

with Stata MP16 software (IBM®, Armonk, NY, USA). Continuous variables were expressed in mean±standard deviation and range; categorical variables were expressed as proportions. The normality of continuous variables was assessed using the Skewness and Kurtosis test. Continuous variables were compared between groups using the Student's *t*-test for independent samples and between times and groups using the ANOVA test for repeated measurements; categorical variables were evaluated for detection times and groups using the *chi*-squared test and Fisher's exact test.

A multivariate linear regression model for each outcome was used to evaluate the association between VAS and Quick DASH scores comparing T2 and T0 and treatment types (group A *vs* group B), gender (males *vs* females), age (years), BMI and smoking habits (yes/no). The correlation coefficients were calculated, with the indication of 95% confidence interval (95% CI). For each test a value of 2-sided *p*-value <0.05 was considered significant.

RESULTS

The study group was made up of thirty subjects affected by epicondylitis, of whom 15 (50%) belonged to the CB-PRP group (group A) and the

remaining 15 (50.0%) to the ESWT group (group B). The mean age of the group was 51.6±8.4 years (range: 38-73), height 1.69±0.09 m (range: 1.47-1.81 m), weight 68.7±10.3 kg (range: 50-85 kg), BMI 24.0±1.7 kg/m² (20.8-27.4 kg /m²); 66.7% were male, 20% smokers. There were no statistically significant differences between the two groups at baseline. In the B group, during the first session, the energy released was 0.07±0.03 mJ/mm² (range: 0.01-0.09 mJ/mm²), during the second session the average value of energy released was 0.07±0.02 mJ/mm² (range: 0.02-0.08 mJ/mm²) and in the third session was 0.07±0.01 mJ/mm² (range: 0.04-0.08 mJ/mm²). Table I describes the mean values of the VAS scale, Quick DASH and Roles and Maudsley scores by detection times and group.

ANOVA analysis for repeated measures showed a statistically significant relationship in the comparison between the detection times (*p*=0.000) and groups (*p*=0.011) for VAS score. A statistically significant difference emerged with the comparison of Quick-DASH and R&M scores by detections times (*p*=0.000), while no statistically significant differences were found between the scores and times and groups (*p*> 0.05).

Table I. Mean±SD (range) of continuous variables divided by group and detection time

	T0	T1	T2
VAS			
Group A	7.5±1.0 (5-9)	4.2±2.4 (0-6)	2.8±1.7 (1-7)
Group B	6.0±1.8 (3-8)	2.7±2.7 (0-7)	1.2±2.1 (0-7)
Total	6.7±1.6 (5-9)	4.5±2.6 (0-8)	2.0±2.1 (0-7)
Quick-DASH			
Group A	65.4±11.9 (43.2-86.4)	21.1±15.1 (2.3-47.0)	7.7±7.8 (2.3-32.0)
Group B	55.9±28.3 (18.2-97.0)	19.5±17.1 (1.0-61.0)	9.1±16.7 (0-59)
Total	60.6±21.9 (18.2-97.0)	20.3±15.9 (1.0-61.0)	8.4±12.9 (0-59)
Roles and Maudsley Score			
Group A		2.1±0.7 (1-3)	2.3±0.5 (1-2)
Group B		1.7±1.0 (1-4)	1.3±0.8 (1-4)
Total		1.9±0.9 (1-4)	1.3±0.7 (1-4)

The multivariate analysis showed a statistically significant relationship between the VAS and smoking habit at T2 and T0 (coef.=2.6; 95% CI=0.4-4.8), while there were no further associations between the outcomes and the remaining determinants in analysis ($p>0.05$; table II). The multivariate analysis did not show statistically significant relationship between the Quick-DASH scores at T2 and T0 and the remaining determinants in analysis ($p>0.05$; Table III).

DISCUSSION

In this study, it was verified that both treatments used (umbilical cord PRP infiltration and ESWT) allowed to reach clinical and functional recovery in patients affected by epicondylitis. It has been found that the clinical improvement obtained by using PRP from umbilical cord proved to be comparable to shock waves.

Shock waves are recognized by the National Institute for Clinical Excellence (NICE) and the US Food and Drug Administration approved the use of SWs in the treatment of epicondylitis (6). This physical therapy improves and speeds up pain

reduction, modulates grip strength and functional recovery in patients with epicondylitis compared to other physical therapies used alone (5). This biological stimulation induces new vessel formation and increases the release of numerous growth factors (7); the application of SWs is able to modify the membrane permeability and to prevent the transmission of painful stimuli. This causes a direct suppressive effect on nociceptors and activation of the gate control mechanism.

Interest in regenerative medicine and biotechnologies has recently emerged. The treatment with PRP, plasma enriched with platelets and therefore with growth factors, could play a key role for the effectiveness of treatment. In fact, in degenerative tendinopathy, PRP is able to induce a regenerative effect, promoting correct differentiation and preventing abnormal differentiation of tenocyte precursors (8). As far as the epicondylitis treatment is concerned, the application of PRP is able to guarantee the relief of pain, with persistent results in the long term (9).

Umbilical cord blood (CB), obtained by venous sampling from the parental donation of umbilical

Table II. Analysis of VAS difference determinants between T2 and T0 in a multivariate linear regression model

Determinants	Coef.	95%CI	p-value
Group (A vs B)	0.1	-1.6-1.9	0.903
Gender (males vs females)	1.4	-1.0-3.7	0.233
Age (years)	-0.1	-0.1-0.1	0.551
BMI	-0.2	-0.9-0.4	0.478
Smoke (yes/no)	2.6	0.4-4.8	0.021

Table III. Analysis of Quick-DASH difference determinants between T2 and T0 in a multivariate linear regression model

Determinants	Coef.	95%CI	p-value
Group (A vs B)	14.2	-6.1-34.4	0.162
Gender (males vs females)	18.6	-8.9-45.8	0.171
Age (years)	-0.1	-1.3-1.2	0.896
BMI	-2.0	-9.8-5.7	0.591
Smoke (yes/no)	16.6	-8.8-42.1	0.189

cord vessels of term newborns, represents an important source of hematopoietic stem cells and progenitor cells, already used for patients affected by leukemia, lymphoma, thalassemia, immunodeficiency and metabolic disorders. US and Europe set up umbilical cord banks in the early 90s (10), which count about 600,000 units of CB that are maintained and cryopreserved for a long time; 80% of this material contains less than 1.5×10^9 nucleated cells and is therefore effectively excluded from banks for hematopoietic transplantation purposes. This material has become available for the infiltrative treatment in cases of orthopedic pathologies, such as tendinopathies. The use of umbilical cord PRP has to overcome some limitations of autologous PRP. On the one hand, the collection of autologous PRP can be difficult (e.g., due to disability) or clinically inappropriate (e.g., in the presence of thrombocytopenia) for some categories of patients; on the other hand, the CB-PRP contains cellular mediators which are responsible for the NK and T cell suppression, due to the fetus-maternal tolerance mechanism (11). Furthermore, the CB-PRP contains a higher concentrations of angiogenic, trophic and healing factors and lower concentrations of pro-inflammatory mediators than autologous PRP (12). Therefore, the use of CB-PRP could allow to enhance the effects of the autologous PRP: i) release of growth factors which are able to improve the stem cell differentiation towards tenoblast-like cells (13); ii) increase IL-17 that is responsible for the inflammatory effect due to neutrophils, and for the anti-nociceptive effect due to reduction of axon excitability (14).

The results of this study support the use of CB-PRP for the treatment of epicondylitis. These results are comparable to those obtained with shockwave treatment. The indication of CB-PRP infiltration should take into consideration the organizational and economic commitment and invasiveness of procedure; for this reason, this therapy should be reserved for patients who are not candidates for shockwave treatment (e.g., cardiac PM carriers, epileptics) and in whom it is not possible to use the autologous PRP (e.g., platelet deficiencies). Further investigations are needed to verify the effect of a

combined therapy (SW and infiltration of CB-PRP) for the treatment of epicondylitis, hypothesizing that the two therapies could have a synergistic effect; in fact, these treatments are able to induce a regenerative stimulus for tendon healing. This study has some limitations. Firstly, the study protocol provided a single CB-PRP infiltration, hence further studies are necessary to verify the advisability to increase the number of PRP infiltrations. Secondly, the source of the PRP is heterogeneous and could affect the results. Thirdly, a placebo group was not included in this study. Fourthly, a longer term follow-up may have been useful. The strong point includes the fact that this is the first study to investigate the clinical effects of CB-PRP for the treatment of tendinopathy comparing the results of a CB-PRP group with the results of a control group treated with SW therapy.

In conclusion, treatment with umbilical cord PRP infiltration may represent an appropriate therapy for tendinopathies and for epicondylitis. It has been found that PRP from umbilical cord allows to provide biological stimulus targeting pathogenetic aspects of tendon pathology. Further investigations are needed to define a model that may consider the phase of pathology, and is able to identify the proper patient enrollment, timing of application and number of infiltrations.

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LETTER TO THE EDITOR

Age-related changes in expression of autophagy genes in human large intestine

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To the Editor,

Studies conducted over the last 10 years show that autophagy is directly involved in cellular aging (1). In most organism and tissue studied to date, the activity of autophagy decreases with age. However, any genetic or pharmacological therapies stimulating autophagy are conducive to longevity. Aging tissues exhibit changes in the transcriptional activity of the genes involved in the regulation of autophagy, such as Beclin1, LC3, Atg5 and Atg7. However, the expression of AMPK and SIRT1 (sirtuin1) decreases with age (2). In turn, the overexpression of specific autophagy-related genes may contribute to longevity in some types of tissues (3). The results of those experiments indicate that impaired autophagy contributes to shorter life span in individuals or to the development of various age-related diseases. However, when autophagy is reinforced and there is overexpression of autophagy-related genes in the same mice, some of them live longer (4). In most tissues of an aging organism, autophagy is inhibited with age (5). Damaged and defective organelles accumulate in tissues, leading to the development of various age-related diseases and to the deterioration of organ functioning. Therefore, learning more about the mechanisms that contribute to countering adverse effects of aging is clinically significant (6).

The aim of this research is to assess the transcriptional activity of selected genes (*LAMP2*, *PINK1*, *BECN1*, *FOXO1*) involved in the process of autophagy depending on age. The selected genes are the most important regulators of autophagy involved in the three types of autophagy. *PINK1* is involved in mitophagy, *LAMP2* in chaperone-mediated autophagy (CMA), *BECN1* and *FOXO1* in macrophagy. In conclusion, our goal was to select the genes involved in each type of autophagy.

MATERIALS AND METHODS

Patients

For the analysis, specimens of the large intestine were utilized. Each selected sample was confirmed to be histopathologically healthy. They were collected from 100 patients during routine colonoscopies and categorized into 5 age groups:

group A - age 40-49 years (8 men, 9 women) – average age: 43.5 years

group B - age 50-59 years (8 men, 9 women) – average age: 56.3 years

group C - age 60-69 years (15 men, 14 women) – average age: 66.7 years

group D - age 70-79 years (12 men, 14 women) – average age: 74.7 years

Key words: aging; autophagy; intestinal microenvironment; *BECN1*, *FOXO1*, *LAMP2*, *PINK1* genes

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group E - age 80 years and up (4 men, 7 women) – average age: 83 years

The current study was conducted in accordance with the Declaration of Helsinki. The project was approved by the Bioethics Committee of the Medical University of Silesia in Katowice, Poland - decision No. KNW/0022/KB1/28/18 of April 17, 2018. The participants were informed in detail about the study and gave their written consent. Participation in the study was voluntary.

Methods

After the homogenisation of biological material using Polytron homogeniser (Kinematyka, AG, Switzerland), the RNA was fully isolated with the reactant TRIzol® (Invitrogen Life Technologies, California, USA) according to the manufacturer's instructions.

The validation of the matrix experiment was executed according to the methods of qPCR and RT-qPCR (real-time quantitative PCR). The number of *mRNA* copies of the analyzed genes was determined using the Engine OPTICON™ sequence detector (MJ Research) and Quanti Tect™ SYBRGreen RT-PCR Kit (QIAGEN) reagents according to the manufacturer's instructions. Reverse transcription (RT) reaction and quantitative PCR were performed in one reaction mixture using the reagent kit. The reaction mixture consisted of: 2×QuantiTect SYBRGreen RT-PCR Master Mix, forward and reverse primers 0.5M each, RNA template and pyrogen-free water. The reaction was performed using sequence-specific primer pairs for each test gene (Sigma-Aldrich, St. Louis, MO, USA):

Primer sequences 5' → 3'

- For BECN1: CAGTATCAGAGAGAATACAGTG (starter forward) to TGGAAGGTTGCATTAAAGAC (starter reverse),
- For LAMP2: AACAAAGAGCAGACTGTTTC (starter forward) to CAGCTGTAGAATACTTTCCTTG (starter reverse),
- For PINK1: TGTAACGACGGCCAGT (starter forward) to CAGGAAACAGCTATGACC (starter reverse),
- For FOXO1: GTCAAGACAACGACACATAG (starter forward) to AAATAAGGGAGTTGGTG

The samples tested amounted to 10 µl. The results were normalized to be the β -actin and GADHP control. The standard curves were generated based on the above

results and in turn, were used as the basis for calculating the number of *mRNA* copies for the studied genes. The results of the analysis of transcriptional activity are presented as the *mRNA* copy number per 1 µg of the total RNA.

Statistical analysis

The statistical significance of the observed differences between the *mRNA* concentration profiles was assessed based on methods of descriptive statistics and the results of the one-way ANOVA test. A comparative analysis of the results was obtained by the RT-qPCR method which was performed in the Statistica 12.5 program (StatSoft, Tulsa, OK, USA). For each analyzed parameter, the most significant elements of descriptive statistics were generated: the average, median, minimum and maximum value, standard deviation, as well as upper (75%) and lower (25%) quartiles. The hypothesis regarding the compliance of the distribution of a given parameter with the normal distribution was verified by the Shapiro-Wilk test, which showed that the obtained results did not follow the assumptions of normal distribution.

RESULTS

The transcriptional activities of genes involved in the autophagy process (*LAMP2*, *BECN1*, *PINK1*, *FOXO1*) in the large intestine samples taken from different patients aged 40 years to 80+ years were compared. The results were normalized to be the β -actin and GADHP control. When comparing the number of *mRNA* copies in the genes studied within a section of the large intestine, no statistically significant differences were found (one-way ANOVA), assuming that the *p* value was < 0.05.

Table I illustrates the summary of descriptive statistical values and the ANOVA test of gene expression levels for each of the genes studied in sections of the large intestine samples for each of the five age groups. The validation of the experiment with the use of the RT-qPCR technique to assess *mRNA* levels of each gene in individual age groups is shown in Fig. 1. The highest number of *LAMP2 mRNA* copies/µg total RNA was found in the subjects up to 49 years of age. *LAMP2* is most active in younger people, which means that chaperone-mediated autophagy decreases with age. *PINK1* is

also most active in younger people. Therefore, in the group of those aged 50-59 years its activity declines. Analysis of other genes in the autophagy process showed the highest number of copies of *BECN1 mRNA* in groups C and D (people in the groups aged 60-69 and 70-79 years, respectively). For *FOXO1 mRNA*, the highest number of copies were shown in

colon samples of subjects aged 70-79 years. In very old age (age 80+) *mRNA* levels for each of the genes was very low.

DISCUSSION

The results indicate that *LAMP2* shows the

Table I. Messenger RNA levels for genes in intestinal tissue collected from subjects in 5 different age groups

Age	Avg	Me	Q1	Q3	SD	Test ANOVA
BECN1						
40-49	1.9x10 ⁴	7.9x10 ³	3.9x10 ³	5.1x10 ³	2.6x10 ⁴	p=0.87
50-59	1.3x10 ⁴	1.1x10 ⁴	1.3x10 ²	1.9x10 ⁴	1.6x10 ⁴	
60-69	1x10 ⁷	1.1x10 ⁴	3.5x10 ³	1.8x10 ⁴	8.6x10 ⁷	
70-79	1.2x10 ⁷	1.1x10 ⁴	7.7x10 ³	2.5x10 ⁴	6.0x10 ⁷	
>80	1.4x10 ⁴	1.2x10 ⁴	4.4x10 ³	2.3x10 ⁴	9.5x10 ³	
LAMP2						
40-49	1.4x10 ⁷	4.0x10 ⁴	1.3x10 ⁴	7.2x10 ⁴	4.1x10 ⁷	p=0.21
50-59	3.9x10 ⁴	4.9x10 ⁴	2.1x10 ⁴	5.1x10 ⁴	1.6x10 ⁴	
60-69	3.7x10 ⁴	4.4x10 ⁴	1.6x10 ⁴	5.2x10 ⁴	2.1x10 ⁴	
70-79	4.0x10 ⁶	2.7x10 ⁴	1.2x10 ⁴	5.0x10 ⁴	2.1x10 ⁷	
>80	2.1x10 ⁴	1.5x10 ⁴	8.x10 ³	3.4x10 ⁴	1.7x10 ⁴	
PINK1						
40-49	6.7x10 ³	2.1x10 ³	2.9x10 ²	4.1x10 ³	8,879x10 ⁹	p=0.26
50-59	1.2x10 ⁵	71,76z	0,16	1.1x10 ⁴	3,342x10 ⁴	
60-69	3.3x10 ³	11,78	0,33	9.3x10 ²	8,245x10 ⁹	
70-79	1.5x10 ³	4,99	0,02	5.4x10 ²	7,905x10 ⁴	
>80	2.5x10 ²	1,67	6.8x10 ⁻³	1,67	1,809x10 ⁵	
FOXO1						
40-49	3.0x10 ³	2.6x10 ²	6,40	5.2x10 ³	5.2x10 ³	p=0.56
50-59	2.1x10 ⁴	1.3x10 ³	0,95	4.7x10 ⁴	3.0x10 ⁴	
60-69	1.5x10 ⁴	2.0x10 ³	3,45	1.8x10 ⁴	2.7x10 ⁴	
70-79	1.3x10 ⁷	1.5x10 ²	0,89	1.5x10 ⁴	4.7x10 ⁷	
>80	200	1,39	0,92	1,85	4.4x10 ²	

Avg: arithmetic average; Me: median; Q1: lower quartile; Q3: upper quartile, SD: standard deviation

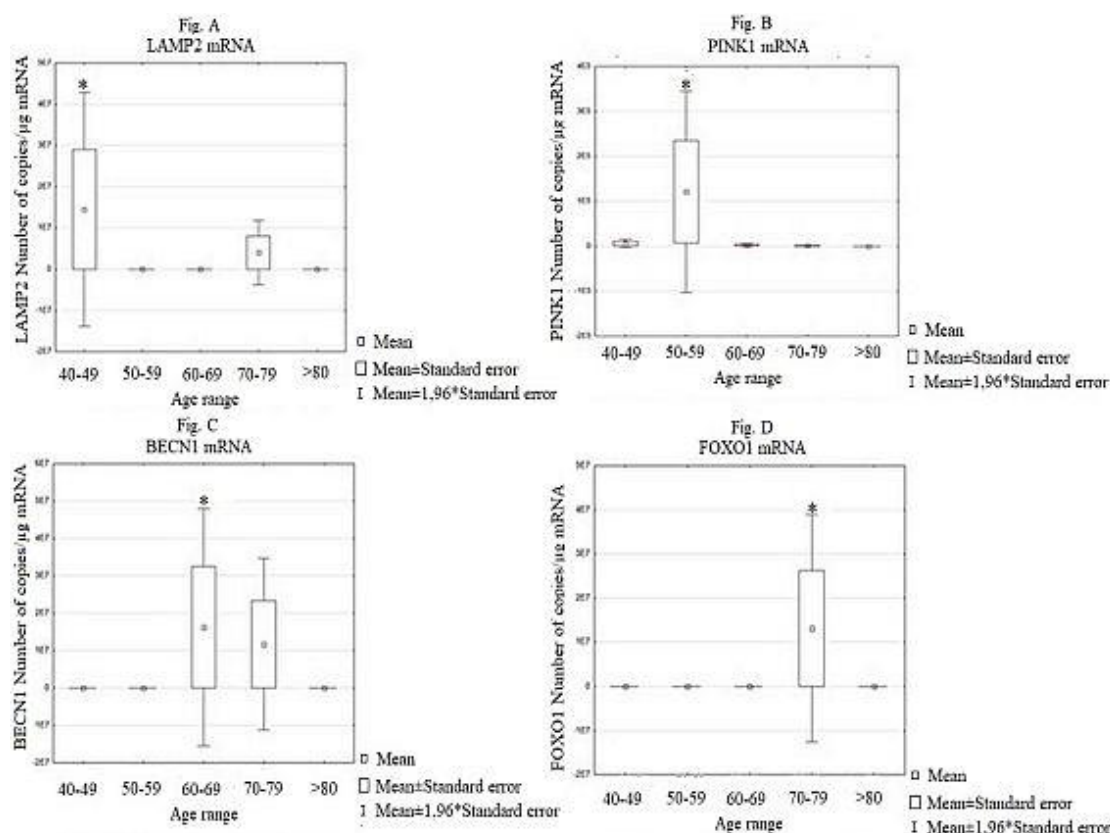


Fig. 1. A-D). Changes in the expression of *LAMP2*, *PINK1*, *BECN1* and *FOXO1* genes depending on age. *highest number of copies of each gene expression levels in individual age groups

highest activity in patients up to 49 years of age, which is associated with a decrease in CMA with age. This may be related to changes in the lipids of lysosomal membranes, which change the dynamics and weaken the stability of *LAMP2* in older individuals (7). Undesirable changes in the lipid composition intensify the degradation of *LAMP2* in the lysosome lumen, thanks to which translocation of substrates to the lysosome is significantly impaired in the elderly. *PINK1* is the most active in the group of people aged 50-59. It is involved in degradation of damaged mitochondria which may contribute to the development of age-related pathologies (8). Mitochondrial dysfunction is recognized as a hallmark of ageing and is also associated with apoptosis, ageing, genome instability, inflammation and metabolic disorders (9). The highest expression of *BECN1* was observed in the age group from 60 to 79, which means removing damaged organelles to prevent the development of age-related diseases

(10). In younger people, the expression of *BECN1* is lower. The increase in *FOXO1* activity was observed in patients aged 70-79 years. This transcription factor plays a role in cellular ageing and cell homeostasis by regulating genes involved in lipid and glucose metabolism. It has been shown that increased activity of *FOXO1* prolongs the viability of *C. elegans*, and *Drosophila* in mice, and is observed in elderly people (11). Very low transcription activity of analyzed genes after 80 years of age is also important. This groundbreaking research suggests that stimulation of autophagy may be an act as an anti-ageing mechanism (12). Our results show an increase in the activity of *BECN1* and *FOXO1* involved in autophagy and probably the role of macrophages in the large intestine cells of older compared to younger patients. It is possible that investigating the interplay between autophagy, the intestinal microenvironment and aging may provide novel therapeutic approaches to maintain intestinal health.

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LETTER TO THE EDITOR

Efficacy of ultrasound-guided intra-articular injection with hyaluronic acid for the management of hip osteoarthritis patientsB. Zucchi¹, F. Agostini², M. Paoloni², A. Bernetti², M. Mangone² and S. Zucchi³

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To the Editor,

Osteoarthritis (OA) of the hip is a disease responsible for severe patient disability, both in terms of pain and functional limitation and for the consequent reduction in the patient's quality of life. In a prominent US-based population study, prevalence of symptomatic hip OA was reported at 9.2% among adults aged 45 years and older, with 27% showing radiologic signs of disease; prevalence was slightly higher among women (1). The etiopathogenesis is multifactorial although the main risk factors for its development are represented by age and obesity (1). The treatment of OA must consider various factors such as the pain intensity, patient disability, comorbidities, multiple drug therapies, BMI, patient functional activity and, finally, the presence of joint malformations (2). In accordance with the Osteoarthritis Research Society International (OARSI) Guidelines and Recommendations for OA management, lower limb muscle strengthening programs and aerobic activity programs to reduce body weight are used as the first therapeutic approach, associated with a nutrition education program for the patient (3). Physical therapy fails to reduce pain in some cases, so it is necessary to switch to pharmacological treatment, which involves the use of paracetamol as first choice for

mild-to-moderate pain, a non-steroidal anti-inflammatory drug (NSAID) is added or substituted in patients who respond inadequately to paracetamol (4, 5). In recent years, intra-articular infiltrative therapy of HA has become increasingly popular, and is considered as the best option for non-operative therapy for OA (6, 7). The use of this procedure in hip osteoarthritis makes the debate still open as there is still no clear evidence to understand how HA can have an action both in improving painful symptoms and the articular and radiographic morphology of a pathological hip. The aim of the present study was to investigate efficacy of ultrasound-guided intra-articular infiltrative treatment of HA in a cohort of patients with different grades, according to Kellgren-Lawrence (KL) Classification, of hip osteoarthritis (8).

MATERIALS AND METHODS

In this retrospective cohort study, we analyzed hip osteoarthritis patients who underwent ultrasound-guided intra-articular infiltrative treatment of HA in Viterbo Hospital (Viterbo, Lazio, Italy) in the period between February 2014 and February 2017. The study was conducted in accordance with the Declaration of Helsinki and in accordance with the guidelines

Key words: hip osteoarthritis; rehabilitation; pain; hyaluronic acid; infiltration

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of Good Clinical Practice (GCP). All patients gave their informed consent, and the study protocol was approved by the Viterbo Hospital Ethics Committee.

Patients

Patients were enrolled during an orthopedic visit for hip pain. After examination and X-ray, indications were given to perform ultrasound-guided intra-articular infiltrative therapy with HA. Before performing any study procedure, a clinical investigator, medical expert in infiltrative therapy, had explained the nature, aim and technical procedures, and each patient was asked to give written consent to participate. Therefore, patients aged 30 to 80 years, with painful osteoarthritis of the hip with a degree of osteoarthritis 1 to 4 according to the Kellgren-Lawrence (KL) (8) radiographic classification, resistant to pain medication and physical rehabilitation therapies were included in the study. Patients who did not want to undergo hip replacement surgery were also included although the surgical indication was relevant.

The exclusion criteria for the study were: when one or more clinically significant conditions could have interfered in the treatment and/or evaluation of the study; patients who had undergone previous surgery involving spine and lower limbs; patients with ongoing infections or on antibiotic treatment; patients under chronic corticosteroids therapies; patients under anticoagulant therapy, patients with cognitive impairment/dementia; patients who had an allergic reaction to an injection of HA; patients involved in other trials.

Procedure

Each infiltrative procedure was performed on the patient in a supine position with the hip to be treated in internal rotation of 15° - 20°. A 3.5 MHz Convex probe (ASTRO, Hitachi-Esaote, Genova, Italy) was used with a sterile biopsy guide applied. A sterile field was prepared, and sterile gloves were used. The probe was aligned with the axis of the femoral neck which also included the acetabulum and the femoral head. After abundant skin disinfection, the ultrasound examination was carried out using an anterior parasagittal approach, lateral to the femoral vessels, which

can be highlighted with the color doppler. Intra-articular injection was performed by inserting a 20 G (0.9 x 90 mm) spinal needle into the biopsy guide. Then, using real-time biopsy ultrasound guidance software, the needle was introduced into the anterior recess of the capsule, at the level of the femoral head. In all infiltrations, we used medium-high molecular weight HA (1200-1500 Kilo Dalton, 2ml/40mg); its presence within the joint is highlighted by real-time monitoring (direct visualization of viscous liquid) also using the power-doppler ultrasound image (flow signals in the intra-articular recess). In all cases, there was a direct visualization of the introduction of the needle into the joint cavity and of the injection of the liquid inside it. The duration of the execution of the injection technique varied between 5 and 7 minutes. No local or general anesthesia was required. No precautions are necessary either before or after the procedure. The patient could return home independently. Each patient's weight and height were recorded with an electronic column scale (Seca). The treatment efficacy assessment was performed by administering the Visual analogue scale (VAS) (8) for pain assessment to each patient, at baseline and at each follow-up; the Harris Hip Score (HHS) (9) was used to evaluate the functionality in daily activities, the presence of structured deformities and the joint Range of Motion (RoM) before the start of therapy and at the end of treatment. Any change in the use of anti-inflammatory/anti-pain medications was recorded as well as whether the patient had undergone hip replacement surgery or was planning the surgery. A total of three infiltrations were performed: T0 - first infiltration (baseline), T1 - 30 days from the first infiltration and T2 - 3 months after the second infiltration

Statistical analysis

When we considered all patients as a single group (independently of KL) the following analysis was performed: for the analysis of the results, a Friedmann Anova statistical test was performed for repeated measurements over time to evaluate the difference between the Vas values calculated at T0, T1, T2 and T3 for all patients regardless of the degree of osteoarthritis. The Mann-Withney test was

performed to evaluate the differences in the HHS values calculated at T0 and T3. When the group was stratified according to the degree of osteoarthritis assessed with the KL scale into 4 groups (KL1, KL2, KL3, KL4) the following analysis was performed: a Friedman Anova for repeated measurements was used for the evaluation over time of the VAS in each group. the time variations in HHS (Δ HHS) were also compared between the four groups using the Kruskal-Wallis test for independent samples with subsequent post-hoc analysis. The data relating to VAS and HHS are reported in terms of median and range of variation (Min-Max).

RESULTS

Two hundred patients (66 males, 134 females) were included in the study. All characteristics of the included patients are shown in Table I. All the comparisons made between the T1, T2, T3 follow-ups demonstrated a statistically significant decrease in pain. The analysis showed a statistically significant reduction in the value of VAS from Pre to T1, from

T1 to T2 and from T2 to T3 (Table II), as well as in the value of HHS from T0 to T3 (Table II). The results of the statistical analysis for the temporal variations of the VAS in the 4 groups showed that for groups KL2, KL3 and KL4, there was a statistically significant evaluation for all comparisons performed at T0, T1, T2 and T3. Only for the KL1 group the VAS was not statistically significant in respect to the comparisons made between T1-T2 and T2 and T3, but the difference T0-T1, T0-T2 and T2-T3 was significant (Table III). The results of the statistical analysis for the evaluation of the temporal variation of the HHS between T0 and T3 showed a statistically significant difference between G1-G2, G1-G3 and G1-G4. The other comparisons were not significant (Table IV).

VAS

VAS value was statistically significantly different for 8 months. For each group post hoc analysis is shown in Table II. Medians of VAS scores were statistically significantly different between the KL groups (Table III).

Table I. *Characteristics of patients included in the study*

Patients (n)	200 (66M, 134F)
Age (Mean $\pm$ standard deviation)	60.2 $\pm$ 12.6
Weight (Mean $\pm$ standard deviation)	69.16 $\pm$ 10.4
Height (Mean $\pm$ standard deviation)	1.70 $\pm$ 0.009
KL1 (n)	33
KL2 (n)	99
KL3 (n)	48
KL4 (n)	30
VAS (Mean $\pm$ standard deviation)	8.5 $\pm$ 1.25
HHS (Mean $\pm$ standard deviation)	62.23 $\pm$ 13.6

KL: Kellgren-Lawrence; VAS: Visual analogue scale; HHS: Harris Hip Score

HHS

No differences were found between delta HHS; $\chi^2 = 1.51$ p 0.68. There were significant differences between pre-HHS therapy and post-HHS therapy independently of the KL group. The results are shown in Table III.

DISCUSSION

The results show that the VAS improved for each time point but with a different trend in the different groups. In the first group (KL1), patients with very severe pre-treatment pain, experienced a decrease in pain, up to the total absence of the same, after intra-articular infiltrations. Then the pain was reduced until it disappeared for patients who had mild osteoarthritis. In the second and third groups (KL2 and KL3), the pain had a decreasing temporal trend, it improved significantly until the first infiltration, but there was no significant difference between the different infiltrations, i.e. the patients

could not perceive a temporal improvement between infiltrations although they improved after treatment. In the group with grade 4 osteoarthritis (KL4), patients had severe pain with severe functional limitation that had subsided after the first infiltration, subsequently recurring with similar intensity, forcing the patient to re-perform the treatment to relieve the symptom. However, considering the results obtained for HHS, it can be noted that in the KL1 group, the HHS values slightly improved, by virtue of the still high functionality of these patients in relation to their mild degree of arthrosis. In the KL2 group, on the other hand, there was a greater improvement in functionality, and the KL3 and KL4 groups improved more than the KL1 group. Therefore, function improved more in all groups with progressively worsening osteoarthritis than in the KL1 group where function did not improve but only because the impairment of function in these patients was mild. In terms of functionality, the KL4 group was the one that significantly improved the most, despite all the

Table II. Evaluation of all groups of the repeated measures over time of the Visual analogue scale (VAS) and the Harris Hip Score (HHS); Median (min-max).

	T0	T1	T2	T3	p - value	POST HOC					
						T0-T1	T1-T2	T2-T3	T0-T2	T0-T3	T1-T3
VAS	8 (6-10)	5 (0-9)	3 (0-8)	1(0-7)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
HHS	67.5 (36-89)	-	-	83 (41-98)	<0.001	-	-	-	-	<0.001	-

Table III. Evaluation of repeated measures over time divided by degree of Kellgren-Lawrence (KL); Median (min-max).

KL						POST HOC					
Group	T0	T1	T2	T3	p -value	T0-T1	T1-T2	T2-T3	T0-T2	T0-T3	T1-T3
KL 1	8 (6-10)	3 (0-7)	0 (0-4)	0 (0-3)	<0.001	0.003	0.18	1	<0.001	<0.001	<0.001
KL 2	8 (6-10)	5 (0-8)	3 (0-6)	0 (0-6)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
KL 3	9 (6-10)	5 (0-7)	4 (0-7)	2 (0-6)	<0.001	<0.001	0.012	0.004	<0.001	<0.001	<0.001
KL 4	10 (8-10)	7 (4-9)	6 (3-8)	5 (1-7)	<0.001	0.012	0.012	0.345	<0.001	<0.001	<0.001

Table IV. Comparisons between the deltas of the Harris Hip Score (HHS) between the Kellgren-Lawrence (KL) groups; Median (min-max).

	KL1	KL2	KL3	KL4	KL1-KL2	KL1-KL3	KL1-KL4	KL2-KL3	KL2-KL4	KL3-KL4
DELTA HHS	9 (3-36)	16 (4-54)	14 (4-30)	18.5 (8.5-35.6)	0.02	0.03	0.014	0.463	1	0.992

differences of the KL2 and KL3 groups compared to the KL1 group. Patients with severe osteoarthritis (KL4) were considered with a surgical indication of hip replacement, but for those who did not want to undergo the operation, it was possible to perform an ultrasound-guided infiltrative procedure, resulting in an improvement in the quality of life, but not a disappearance of pain.

The limitations of our study are represented by the absence of a control group. We found an improvement in the quality of life in terms of pain reduction in all groups in the different degrees of osteoarthritis and an improvement in hip function in the highest degrees of osteoarthritis. Future studies with a larger patient sample and longer follow-up are needed to better highlight the benefits of this treatment.

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LETTER TO THE EDITOR

Targeted regulation of BBOX1-AS1 on miR-361-3p and its effect on the biological function of non-small cell lung cancer cellYF. Lian¹, XM. Jiang¹, LH. Sui¹ and XJ. Shi^{2*}¹Department of Oncology, Weihai Central Hospital, Weihai, Shandong, China; ²Department of Respiratory and Critical Care, Weihai Central Hospital, Weihai, Shandong, China*Received March 19, 2021 – Accepted June 11, 2021*

To the Editor,

Non-small cell lung cancer (NSCLC) accounts for the majority of lung cancers, and has a high morbidity and mortality rate (1). Although the clinical treatment of NSCLC has been developing rapidly in recent years, the 5-year survival rate is still very low (2). Targeted drugs and immunotherapy are the most important therapeutic strategies for NSCLC (3). The research direction of NSCLC is to detect molecular markers related to NSCLC progression or prognosis, develop targeted drugs and establish individualized treatment methods (4). Therefore, it is necessary to find new prognostic markers for NSCLC to improve the accuracy of survival prediction and targeted drug development.

As a class of non-coding RNA molecules with a length of 200 nt-100 kb, long non-coding RNAs (lncRNA) are commonly transcribed in eukaryotic cells and affect the transcriptional translation process of protein-coding genes through mechanisms such as transcriptional and post-transcriptional regulation and epigenetic modification (5). The abnormal expression of lncRNA is closely related to human diseases and plays an important regulatory role in tumor genesis, growth, invasion, metastasis, recurrence, and drug resistance (6).

BBOX1-AS1 is a new lncRNA, which was found

to be up-regulated in NSCLC by using the Ensembl database in a recent study (7). However, the prognostic significance of BBOX1-AS1 on NSCLC and its effect on cellular function has not been demonstrated. In the present study, the important role of BBOX1-AS1 in the development and prognosis of NSCLC was explored.

MATERIALS AND METHODS

Patients and tissues collection

One hundred and thirty-five pairs of tumor tissue samples and adjacent normal tissue samples from NSCLC patients treated in Weihai Central Hospital from 2011 to 2015 were collected, and clinical data were obtained through case histories. Patients enrolled in this study met the following inclusion criteria: patients with a pathologic diagnosis of NSCLC; no other treatment was performed before the surgery, such as chemotherapy or radiotherapy; none patient had the mental disease, autoimmune disease, complications with other cancers. The obtained tissue samples were immediately stored in liquid nitrogen until use. All the patients signed written informed consent before surgery and the tissues were made anonymous. This study was approved by the Ethics Committee of Weihai Central Hospital, and five-year survival information of patients was collected by telephone or outpatient follow-up.

Key words: lncRNA BBOX1-AS1; NSCLC; miR-361-3p; cellular function

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Cell culture and transient transfection

Human normal lung cell lines 16HBE and four NSCLC cell lines (A549, HCC827, H1299, and NCI-H650) were obtained from the Institute of Cell Biology, Chinese Academy of Sciences, Shanghai (Shanghai, China). All cells were cultured routinely with RPMI1640 medium containing 10% fetal bovine serum (FBS; Gibco, NY, USA) and 1% penicillin-streptomycin (37°C, 5% CO₂), and the solution was changed once every other day. The experiment was carried out when the degree of cell fusion reached 70~80%.

Small interfering RNA (si-BBOX1-AS1) was used for silencing of BBOX1-AS1 and si-NC was the small interfering negative control. The si-BBOX1-AS1, si-NC, miR-361-3p mimics and miR-361-3p inhibitors were obtained from Genepharma Co. (Shanghai, China). Lipofectamine 2000 kit (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized to transfect si-BBOX1-AS1 or si-NC into A549 and H1299 cells following the protocol description.

RNA extraction and quantitative real-time PCR

Cells of each group were collected and total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). RNA reverse transcription reversed total RNA into cDNA using the Superscript III transcriptase (Invitrogen, Grand Island, NY), and the qRT-PCR assay was performed according to the instructions of the SYBR® Premix Ex Taq™ kit (Takara Bio, Inc.) at a Thermal Cycler Dice™ Real-Time System III (Takara Bio Inc.). GAPDH was used as the internal reference for BBOX1-AS1, while U6 for miR-361-3p. The 2^{-ΔΔCt} method was used to calculate the relative expression. Each sample had 3 replicates.

Cell proliferation assay

Transfected cells in each group were seeded into 96-well plates, and 10 μL CCK-8 solution was added at 0, 24, 48, and 72 h after incubation, respectively. The absorbance at 450 nm was detected by constant temperature culture. The proliferation of cells was evaluated with untreated cells as a reference.

Cell migration and invasion assays

Cells in each group were resuspended in a serum-free medium. For the cell migration assay, 200 μL cell suspension (1 × 10⁵ cells) was taken inoculation in the

upper chamber of the Transwell chamber, and 700 μL medium with 10% FBS was added in the lower chambers. The cells were incubated for 24 h under the condition of 37°C and 5% CO₂. Then the chambers were taken out, the upper cells were wiped gently with cotton swabs after removal of nutrient solution. After washing with PBS, the migrated cells were fixed using 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 10 min. After PBS washing again, the number of cells was observed under a microscope. For the cell invasion assays, RPMI-1640 medium was added at a ratio of 1:5 to dilute Matrigel, then spread in the upper chamber. After drying at room temperature, the cell invasion experiment was carried out consistent with the steps of the cell migration assay. Finally, the invasive cells were stained with crystal violet and the number of cells was counted in five random fields under a microscope (repeated three times).

Targeted prediction and identification of dual-luciferase reporting systems

TargetScan database showed that the 3'-UTR of BBOX1-AS1 had miR-361-3p binding sites. The 3'UTR luciferase expression vectors WT-BBOX1-AS1 and Mut-BBOX1-AS1 were constructed for the wild-type and mutated gene targets BBOX1-AS1. A549 cells in the growth phase were inoculated into 24-well plates, which exhibited the highest BBOX-AS1 expression levels. When the cells grew to 80% fusion, WT- BBOX1-AS1 or Mut-BBOX1-AS1 groups were cotransfected with miR-361-3p mimic, miR-361-3p inhibitor, or negative control (NC) by Lipofectamine 2000 (Invitrogen), respectively. According to the instructions, the dual-luciferase reporter gene assay kit (GM-040502A, Qcbio Science and Technologies) was used for the double-luciferase reporter assay.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM, Aemonk, NY, USA) and GraphPad 5.0 software (La Jolla, CA, USA). Measurement data were expressed as mean ± standard deviation, Students' *t*-test was performed for comparison between two groups, and one-way analysis of variance (ANOVA) was used for comparison among multiple groups. Kaplan-Meier curve and multivariate Cox regression assay were used to explore the prognostic significance of BBOX1-AS1. A value of *P* < 0.05 indicated a statistically significant difference.

RESULTS

Expression of BBOX1-AS1 in LSCLC tissues and cell lines

BBOX1-AS1 expression was detected in 135 pairs of NSCLC and paracancerous normal lung tissues. The expression of BBOX1-AS1 in lung cancer tissues was significantly higher than that in paracancerous normal tissues ($P < 0.001$, Fig. 1A). For the relative expression level of BBOX1-AS1 in NSCLC cell lines (A549, HCC827, H1299, and NCI-H650) and human normal lung cell lines 16HBE, the qRT - PCR results showed that the expression of BBOX1-AS1 in NSCLC cell lines was notably increased compared with that in 16HBE ($P < 0.001$, Fig. 1B). It was noteworthy

that BBOX1-AS1 expression in A549 and H1299 cell lines were relatively higher than the other two cell lines, which were chosen for subsequent studies.

Relationship between BBOX1-AS1 expression and clinical data of patients with NSCLC

NSCLC patients were divided into a high expression group ($n = 68$) and a low expression group ($n = 67$) according to the median expression of BBOX1-AS1 (1.563) in tissues. The incidence of positive lymph node metastasis ($P = 0.001$) and high TNM stage ($P = 0.001$) in the high expression group was significantly higher than in the low expression group (Table 1). There were no significant differences in age, gender, tumor size, tumor number, smoking

Table 1. Correlation of the lnc BBOX1-AS1 expression with clinical characteristics in NSCLC

Parameters	Cases (n = 135)	lnc BBOX1-AS1 expression		P-value
		Low (n = 67)	High (n = 68)	
Age				0.538
< 50	60	28	32	
≥ 50	75	39	36	
Gender				0.425
Male	78	41	37	
Female	57	26	31	
Tumor size				0.070
≤ 5 cm	70	40	30	
> 5 cm	65	27	38	
Tumor number				0.668
solitary	69	33	36	
multiple	66	34	32	
Smoking status				0.262
Non-smoker	56	31	25	
Smoker	79	36	43	
Differentiation				0.265
Well, Moderate	62	34	28	
Poor	73	33	40	
Lymph node metastasis				0.001
Negative	84	51	33	
Positive	51	16	35	
TNM stage				0.001
I,II	89	53	36	
III, IV	46	14	32	

history, and degree of pathological differentiation between the two groups ($P > 0.05$, Table I).

Upregulation of BBOX1-AS1 is associated with poor prognosis in NSCLC patients

Kaplan-Meier curves were used to determine the difference in overall survival rate between the two groups of patients with NSCLC. The results showed that the overall survival rate of the high expression group was significantly lower than that of the low expression group ($P = 0.036$, Fig. 1C). Multivariate

Cox regression analysis showed that BBOX1-AS1 (HR = 2.067, $P = 0.035$), lymph node metastasis (HR = 1.985, $P = 0.040$) and TNM stage (HR = 1.989, $P = 0.046$) were independent prognostic factors for overall survival in NSCLC patients (Table II).

Effect of BBOX1-AS1 on cell proliferation, migration, and invasion

According to the expression of BBOX1-AS1 in the above cell lines, we selected A549 and H1299 cell lines with relatively high expression levels to construct

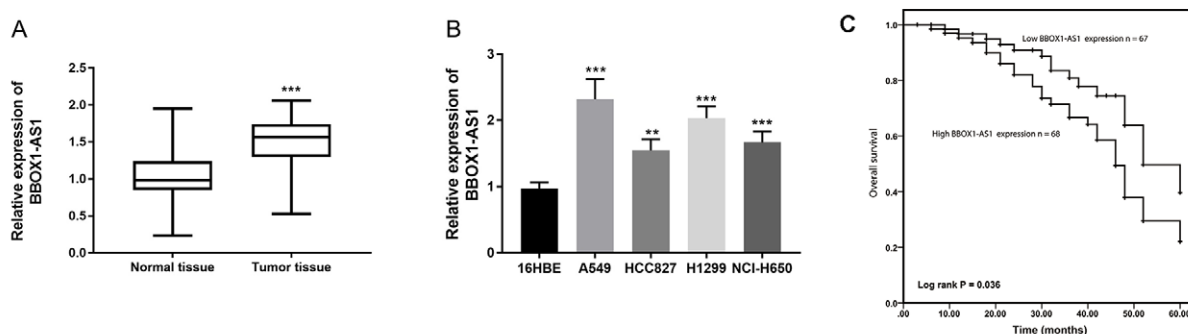


Fig. 1. Relative BBOX1-AS1 expression in NSCLC tissues and cell lines. **A)** The qRT-PCR analysis detected the expression level of BBOX1-AS1 in the NSCLC tissues and the corresponding normal tissues. **B)** qRT-PCR analysis of BBOX1-AS1 in NSCLC cell lines and human normal lung cell lines. *** $P < 0.001$, ** $P < 0.01$. **C)** Kaplan-Meier curves for NSCLC patients with high and low levels of BBOX1-AS1 (log-rank test: $P = 0.036$).

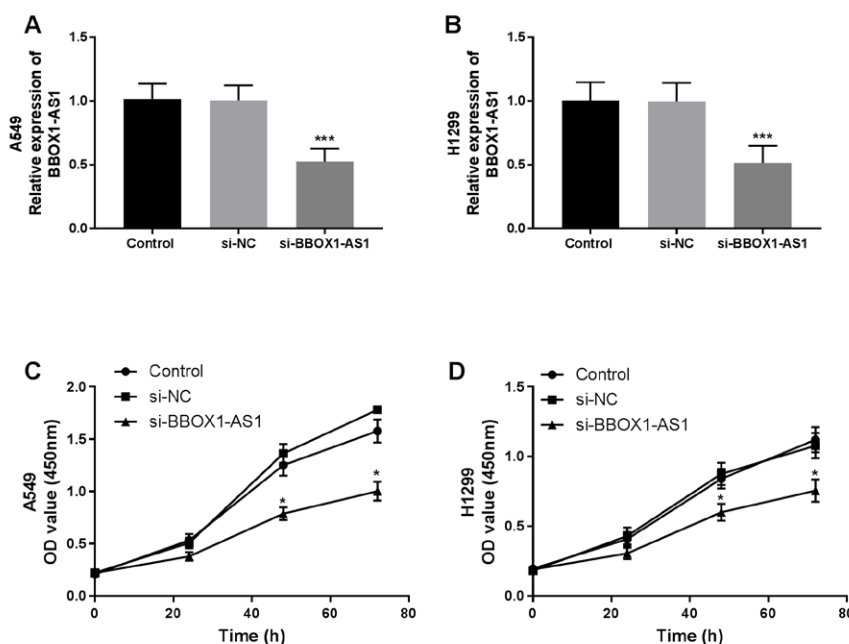


Fig. 2. Effects of BBOX1-AS1 expression levels on cell proliferation, migration and invasion abilities in H1299 and A549 cells. **A, B)** the expression level of BBOX1-AS1 was verified by qRT-PCR after transient transfection with si-NC/si-BBOX1-AS1. **C, D)** The CCK-8 assay was performed to examine cell proliferation. *** $P < 0.001$, * $P < 0.05$.

knockdown cell models to study the role of BBOX1-AS1 in the biological function. The expression of BBOX1-AS1 was confirmed after transfection of si-BBOX1-AS1 or si-NC in A549 and H1299 cells. The expression levels of BBOX1-AS1 in the si-BBOX1-AS1 group were significantly decreased, compared with the control groups in A549 and H1299 cell lines ($P < 0.001$, Fig. 2 A, B). CCK-8 results showed that the proliferation activities of A549 and H1299 cells in the si-BBOX1-AS1 group were decreased at 48 and 72 h after transfection ($P < 0.05$), while the difference of proliferation activity between the control group and si-NC group was not statistically significant (Fig. 2 C, D).

For migration and invasion, Transwell assays were used. The number of migration and invasion with A549 and H1299 cells in the si-BBOX1-AS1 group was markedly decreased, compared with the control group (Fig. 3 A, D) ($P < 0.001$).

LncRNA BBOX1-AS1 targets miR-361-3p

The presence of binding sites between BBOX1-AS1 and miR-361-3p were predicted through the TargetScan database, as shown in Fig. 4A. The luciferase reporter assay results showed that WT-BBOX1-AS1 luciferase activity of the miR-361-3p mimic group was significantly decreased, while it was increased by miR-361-3p inhibitor, compared with the control group in the A549 cell line ($P < 0.001$, Fig. 4B). However, there was no significant difference in MUT-BBOX1-AS1 luciferase activity of the A549 cell line (Fig. 4B). Moreover, BBOX1-AS1 was proved to be significantly negatively correlated with miR-361-3p ($r = -0.63$, $P < 0.0001$) (Fig. 4C). The relative expression level of miR-361-3p in NSCLC tissues was lower than that in normal tissues ($P < 0.001$) (Fig. 4D). The qRT-PCR results showed that the expression level of miR-361-

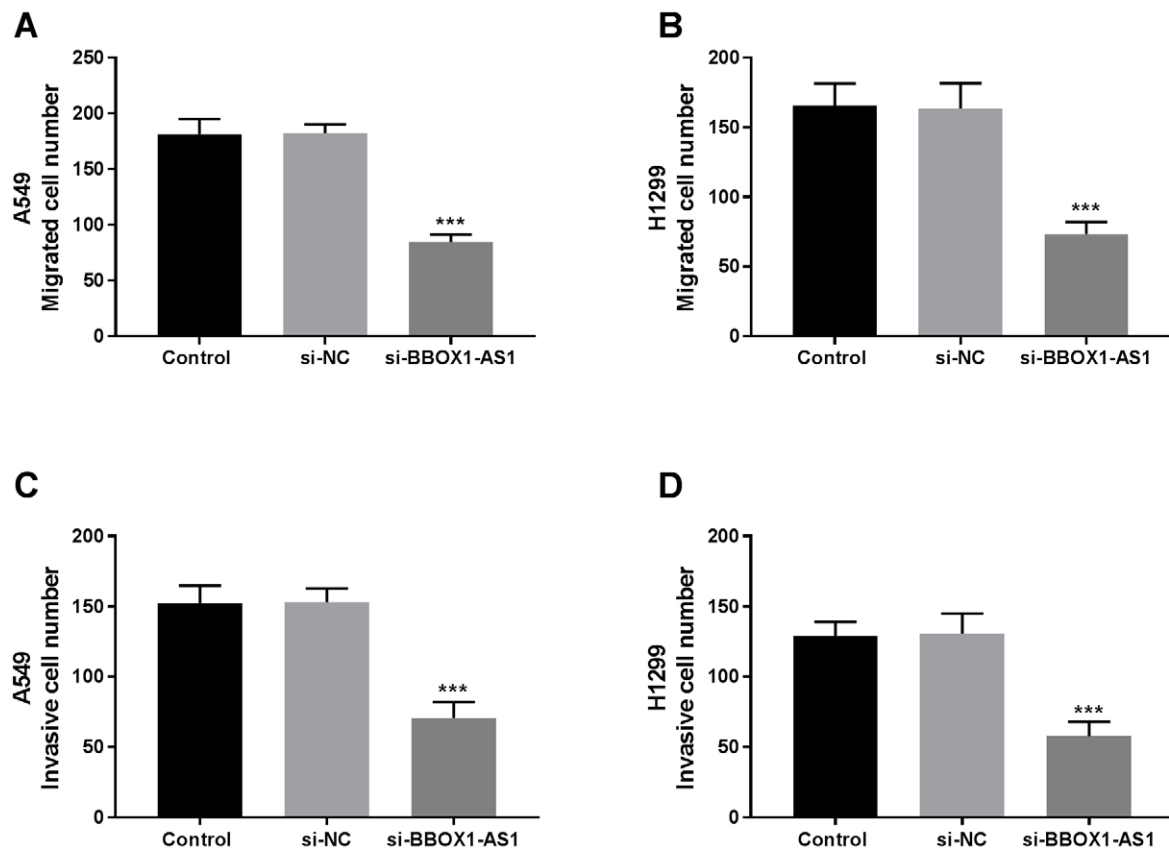


Fig. 3. The migration and invasive abilities of H1299 and A549 cells were assessed with Transwell assay. **A)** Cell migration and **(B)** invasion abilities. *** $P < 0.001$.

3p in the si-BBOX1-AS1 group was significantly increased compared with that in the control group.

DISCUSSION

In this study, we firstly observed that BBOX1-AS1 was highly expressed in NSCLC tissues and cell lines, and the prognosis of patients with high expression of BBOX1-AS1 was poorer than patients with low expression. It is suggested that BBOX1-AS1 may be involved in the development of NSCLC. BBOX1-AS1 was also reported to be upregulated in other tumors, such as colon cancer (8) and gastric cancer (9).

Cell function results revealed that BBOX1-AS1 could promote cell proliferation, migration, and invasion. Similar results have been found in

colorectal cancer cell lines; BBOX1-AS1 promoted CRC progression by sponging miR-361-3p and up-regulating SH2B1(10). LncRNA BBOX1-AS1 promoted the process of cervical cancer by upregulating HOXC6 expression via miR-361-3p and HuR (11). Thus, it is hypothesised that BBOX1-AS1 may play an oncogenic role in the occurrence and development of NSCLC. In addition, an interesting finding was that the target genes of BBOX1-AS1 seem to uniformly point to miR-361-3p. Based on the above phenomena, an online prediction confirmed that BBOX1-AS1 could bind to the miR-361-3p sequence. And this study further proved that BBOX1-AS1 had a targeting relationship and a significant negative correlation with miR-361-3p. One study indicated that miR-361-3p played a suppressor role by inhibiting the proliferation,

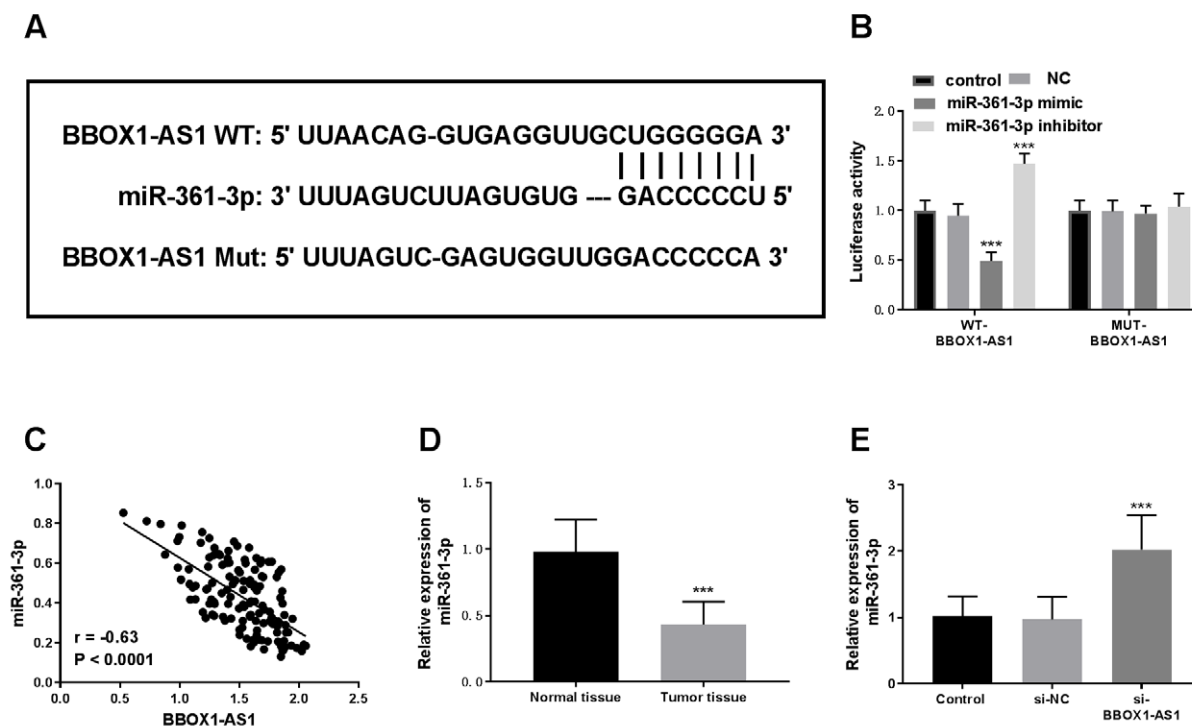


Fig. 4. miR-361-3p acted as the target of BBOX1-AS1 using A549 cells. **A)** The binding sites between BBOX1-AS1 and miR-361-3p. **B)** The interaction between miR-361-3p and BBOX1-AS1 was confirmed in A549 cells co-transfected with WT- BBOX1-AS1 or MUT-BBOX1-AS1 by luciferase reporter assay. **C)** Correlation analysis between BBOX1-AS1 and miR-361-3p. $r = -0.630$, $P < 0.0001$. **D)** qRT-PCR analysis detected the expression level of miR-361-3p in the NSCLC tissues and the corresponding normal tissues. **E)** qRT-PCR was used to determine the expression of miR-361-3p, and the groups were divided into control, NC, si-NC and si-BBOX1-AS1. *** $P < 0.001$.

Table II. Multivariate Cox analysis of clinical characteristics in relation to overall survival

Characteristics	Multivariate analysis		
	HR	95% CI	P-value
Lnc BBOX1-AS1	2.067	1.052 - 4.061	0.035
Age	0.583	0.288 - 1.179	0.133
Gender	0.663	0.348 - 1.264	0.212
Tumor size	1.970	0.941 - 4.125	0.072
Tumor number	0.732	0.380 - 1.410	0.352
Smoking status	0.566	0.269 - 1.188	0.133
Differentiation	1.520	0.765 - 3.022	0.232
Lymph node metastasis	1.985	1.031 - 3.822	0.040
TNM stage	1.989	1.012 - 3.907	0.046

migration, and invasion of NSCLC by directly targeting SH2B1 (12). All the conclusions proved that miR-361-3p may be a target of BBOX1-AS1, and both of them jointly affect tumor progression.

In conclusion, BBOX1-AS1 was highly expressed in NSCLC tissues and cell lines, and plays a promoting role in NSCLC, which is closely related to the proliferation, migration, and invasion of NSCLC. The interaction between BBOX1-AS1 and miR-361-3p provides theoretical support for targeted NSCLC therapy.

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LETTER TO THE EDITOR

A case of very early onset inflammatory bowel disease and relative treatment

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To the Editor,

Inflammatory bowel disease (IBD) is an autoimmune disease characterized by chronic non-specific inflammation of the intestines; the major examples include Crohn's disease (CD) and ulcerative colitis (UC) (1). In recent years, the IBD incidence rate in China has continuously increased. The pathogenesis of IBD is attributed to genetic factors, living habits, and environmental factors; however, specific pathogenesis for this disease is still unclear. It has been proposed that genetic and immune factors are closely associated with changes in cytokine levels in IBD patients (2, 3). In the colon tissue of patients with UC, the levels of pro-inflammatory factors are significantly increased, while the levels of anti-inflammatory factors are significantly decreased. This imbalance of pro-inflammatory factors/anti-inflammatory factors causes excessive immunity of the body reaction, which is an important factor in the onset of UC. Very early onset IBD (VEO-IBD), which is categorized as IBD before 6 years of age, is genetically and phenotypically different from IBD in adults or older children (4). VEO-IBD accounts for 4-10% of children's IBD, and genetic defects can often be detected. Among the single-gene mutations that can cause IBD-like phenotypes, loss-of-function mutations of IL-10 and IL10R are the most common (5).

In this study, we report the case of a 12-day-old male newborn with IBD, which is strongly associated with

the IL-10 receptor gene. Our case study demonstrates that VEO-IBD is serious early in life, and timely and correct treatment of the disease is critical. In addition, it is difficult to achieve clinical remission.

Case presentation

The patient was a 12-day-old male, and presented clinical symptoms such as intermittent fever, salivating for 6 days, and rash. He was admitted to the hospital at 21:22 on January 13, 2019; the patient is G1P1, with a gestational age of 41+3 weeks, was birthed by cesarean section, and had a body weight of 4 kg. The umbilical cord, placenta, and amniotic fluid were normal, with no premature rupture of the amniotic membrane. The patient has a history of resuscitations.

Six days previously, the child was suspected to have a fever after being in contact with a family member who had an upper respiratory tract infection; the highest recorded temperature was 39.2°C, which was accompanied by reduced food intake, poor mental response, and foaming at the mouth. At the same time, a red rash had developed on the skin all over the body, especially on the neck and limbs. From January 9th until January 13th he was admitted to the Second People's Hospital of Mengcheng County with "neonatal pneumonia and sepsis", and was given supportive treatment with imipenem antibiotic

Key words: VEO-IBD; IL-10RA; ulcerative colitis; intermittent fever

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and gamma globulin. Following hospital admission, the child still had intermittent fever, hence, his family was referred to Anhui Children's Hospital for further medical treatment. The emergency department recorded the case as "neonatal pneumonia, neonatal sepsis", and admitted the patient to the neonatology department. During the course of the disease, the child was in poor spirits accompanied by screaming, and displayed poor feeding and diarrhea. However, there were no vomiting, convulsions, or abnormalities in urine or feces.

Physical examination

The child showed poor mental response and was irritable. There was bruising around the mouth, with red rash spread all over the skin, mainly on the neck, limbs and buttocks, and a soft fontanel (frontal suture). There were also 3–4 white ulcers on the soft palate; the largest was approximately 1×1 cm. Pupils were normal, and light reflection was present. Nasal congestion, shortness of breath, and salivating was observed in the child; there was no nasal fan and three concave signs; breathing was R75 times/divided, with thick breath and phlegm sounds in both lungs. Heart rate was 180 beats/min; the patient

had strong heart beats with regular rhythm and no murmurs. The abdomen was flat and soft, with no obvious enlargement of liver, spleen, or intestines; no masses were observed. Physical examination showed that hearing, muscle tension in the limbs, and reflexes were all normal. Aside from shortness of breath, neonatal pneumonia, septicemia infection, and perianal abscess were also present. Swelling of the skin in the 12 o'clock direction was observed at the site of the perianal calculus; no obvious redness was found, however, there was a purulent discharge from the perianal area. Lastly, anti-streptococcal hemolysin "0" was found to be 38.4 Iu/mL; both white blood cells and C-reactive protein (CRP) levels were elevated.

Auxiliary examination

Cerebrospinal fluid testing and biochemistry index analyses were performed in the Mengcheng County Second Hospital: the cerebrospinal fluid was colorless and transparent. A negative result was obtained from the PANS test. Nucleated cell number was $3 (10^6/L)$, cerebrospinal fluid glucose was 3.25 (mmol/L), cerebrospinal fluid chloride was 103.1 (mmol/L), and cerebrospinal fluid lactate



Fig. 1. Electronic gastroscopy. The esophageal mucosa is smooth, the vascular network is clear, and there are no ulcers, erosions, polyps, or varicose veins. Cardia is normal. The gastric fundus mucosa is normal. Stomach mucus is clear and of medium amount. The gastric mucosa has no congestion and edema, and the folds are arranged regularly. The stomach angle is curved and symmetrical, and the mucous membrane is smooth. Swelling of the gastric antrum mucosa. The pylorus is round and opens and closes well. The duodenal cavity has no deformation, congestion, edema, and ulcers, and the descending segment and horizontal mucosal folds and mucosa are normal. After the end of the gastroscopy, the endoscopy was followed through the anus, and it was successfully inserted into the ileocecal area. Multiple round dimples and small ulcers were seen in the mucosa of the descending colon and sigmoid colon. There was no active bleeding or abnormalities.

dehydrogenase was 26 (U/L). Head CT showed a small amount of subarachnoid hemorrhage; admission blood gas analysis showed: pH = 7.423, PO₂ = 57.7 mmHg, PCO₂ = 43.6 mmHg, HCO₃⁻ = 27.9 mmol/L, BE = 2.87 mmol/L, SO₂ = 95.6%; Na⁺, K⁺, and Ca₂⁺ were 132.8, 3.78, and 1.153 mmol/L, respectively; Hb = 18.2 g/dL, BS = 5.5 mmol/L.

The stool was found to be mucous pus-like and bloody. The number of white blood cells in the stool was more than 100/HP, and red blood cells was 15–20 /per HP; the stool was also positive for pus ball and occult blood. Further examination using a microscope indicated that epithelial cells, starch granules, fat globules, fungal hyphae, fungal spores, parasite eggs, and parasites were not present in the stool.

Immunological index testing

Allergic reactions to wheat, tomato, shrimp, rice pork, mushroom, milk, egg, crab, corn, cod, soybean, chicken, and beef were found to be negative. Immunological antibody test results excluded toxoplasma gondii, rubella virus, cytomegalovirus, and herpes simplex virus infections. G-lipopolysaccharide was < 5 pg/ml, which ruled out bacterial infections; anti-double-stranded DNA

antibody, anti-nucleosome antibody, anti-SmD1 antibody, anti-DNP antibody, anti-histone antibody, anti-riboprotein antibody, anti-Sjogren's syndrome A/Ro60 antibody and Anti-Sjogren's syndrome A/Ro52 antibody, anti-Sjogren's syndrome antibody B, anti-scleroderma antibody, anti-centromere protein B antibody, and anti-J0-1 antibody were all found to be negative. In addition, procalcitonin and interleukin 6 levels were 0.963 ng/mL and 597.00 pg/mL, respectively, which suggested that serum pro-inflammatory factors were significantly upregulated. Analysis of anticoagulant blood samples showed that both the ratio and number of neutrophils were increased; and the ratio of lymphocytes was decreased. Additionally, serum amyloid A and C-reactive protein levels were in the ranges of 87.81–156.5 mg/L and 34.2–61.7. mg/L, respectively, which indicated onset of inflammation. Red blood cell and hemoglobin levels were measured to be 3.03–3.36 (reference value 5.2–6.4 10¹²/L) and 93.0–107 g/l (reference value 170–200 g/L), respectively, which suggested that the patient was anemic.

The proportion of T-cell subsets and CD64 positive cells were measured by flow cytometry. The proportion of CD3⁺ cells was 81.3%, which was increased compared to the reference value (61.7%–

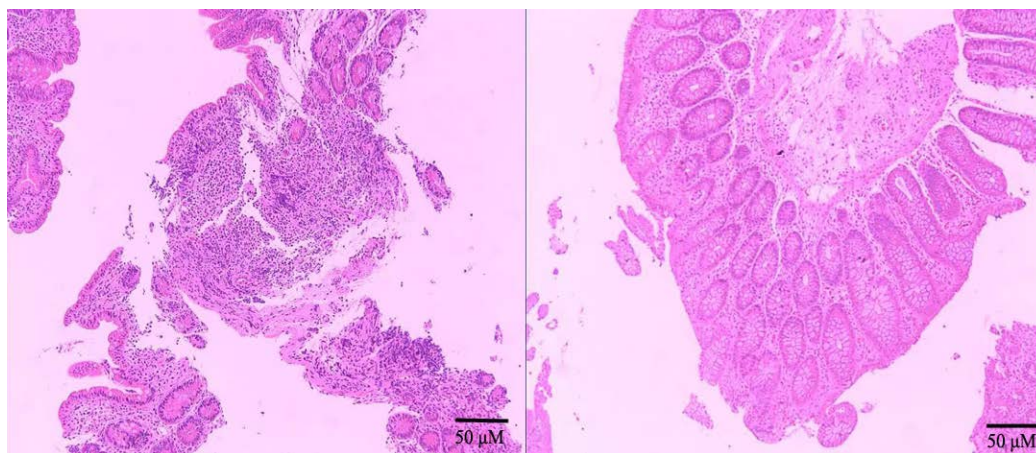


Fig. 2. Pathological diagnosis report. From the descending part of the duodenum, two small gray tissues, 35 cm from the anal margin, one small gray tissue, 20 cm from the anal margin, and three small gray tissues, each with a diameter of 0.05 cm, were taken for pathological analysis. Intestinal mucosal epithelium and glands have regular structures, a small amount of lymphoplasmic cell infiltration in the lamina propria, and focal superficial erosion.

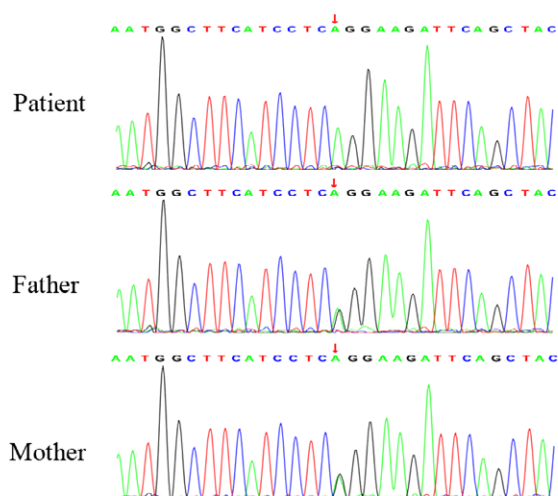


Fig. 3. Genetic testing. The chromosome location of *IL-10RA* is chr11:117864009. The variant proband is homozygous, and his parents are heterozygous.

77%). The proportion of CD3⁺ CD4⁺ cells was 66%, which was also increased compared to the reference value (25.8%–41.6%); the proportion of CD3⁺ CD8⁺ double positive cells was 14.6%, which was decreased compared to the reference value (18.1%–29.6%); the ratio of CD3⁺ CD4⁺/CD3⁺ CD8⁺ cells was 4.52, which was significantly increased compared to the reference value (0.9–1.9); the neutrophil CD64 index was 4.18, which was also increased compared to the reference value (0–3.5). Total IgE level was 45.7 IU/mL, which was higher than the normal value, indicating that the patient may also have an allergic response.

Electronic gastroscopy showed multiple round dimples and small ulcers in the mucosa of the descending colon and sigmoid colon (Fig. 1). Tissues were taken from the descending part of the duodenum, 35 cm from the anal margin. One tissue sample was taken 20 cm from the anal margin, and three additional tissue samples were taken for pathological analysis. Results showed that the intestinal mucosal epithelium and the gland structure were normal; however, a small amount of lymphoplasmic cell infiltration was observed in the lamina propria, and focal superficial erosion was found (Fig. 2).

Genetic testing and clinical characteristic

The chromosome location of *IL-10RA* is chr11:117864009. The patient's genetic variant is homozygous, while his parents' are heterozygous. This confirms that the disease is an autosomal recessive (AR) disease (Fig. 3). This disease was found to be associated with inflammatory bowel disease type 28, another autosomal recessive disease (OMIM: 613148). Some shared clinical features between the two diseases include pyoderma, hematochezia, enterocolitis, and perianal abscess. Additionally, color doppler ultrasound diagnostic analysis of body surface and superficial masses showed that a 1.3×0.8×1.2 cm heterogeneous echo area can be seen in the subcutaneous soft tissue, at 12 o'clock of the anus. It was close to the anus, and had a clear boundary and regular shape; it was partly liquefactive, and demonstrated poor sound transmission. There was no obvious blood flow color signal in the CDFI display, and spot-like red blood flow signals were observed around the area. These findings suggested that a prompt perianal abscess may be present.

During treatment, 0.3 mL acetaminophen, 1.5 mg furosemide, intravenous injection of 100 mL 10% GS, 6% AA, 2 mL 10% NaCl, 1 mL 10% KCl, 0.25g Vitamin C was given; the patient was also intravenously injected with 2.5 g Human immunoglobulin (PH4); 22 mg fluconazole, mesalazine, and potassium permanganate was administered, and a warm water bath was given for 5–10 min. In addition, he was fed with formula milk to increase the intake of certain nutrients (such as iron, calcium, and magnesium) as well as fat-soluble vitamin supplements. This was also supplemented with other fluids and electrolytes to compensate for nutrient loss caused by diarrhea. Despite these treatments, sustained relief could not be achieved due to systemic inflammation, and the patient was transferred to the hospital for further treatments. Due to severe malnutrition, poor wound healing, and sepsis, he died of severe infection at the age of eight months. There was no follow-up, and no autopsy was performed. Written informed consent to report the case was obtained from his parents.

DISCUSSION

We report the case of a newborn with IBD symptoms at 12 days of age, which is a typical case

of VEO-IBD (6). We believe that the incidence of this disease will increase, and therefore, disease onset in these very young patients need to be taken seriously (7). VEO-IBD can often result from genetic defects. Among the single-gene mutations that can cause IBD-like phenotypes, loss-of-function mutations of IL-10 and IL-10R are the most common (8). IL-10R is composed of 2 subunits: IL-10RA and IL-10RB. The most common gene defect of VEO-IBD in China is the IL-10RA mutation (9). Most VEO-IBD children with IL-10RA gene dysfunction display severe colonic diseases; since the colon contains the most abundant bacteria in the digestive tract, it is possible that intestinal flora may play an important role in the development of intestinal inflammation in children with IL-10RA gene dysfunction.

Our gene sequencing results showed that this patient was homozygous recessive for the mutated IL-10RA variant, and the associated disease was inflammatory bowel disease type 28. This has important significance in the clinical diagnosis of inflammatory bowel disease. VEO-IBD is very serious, its prognosis is severe and life-threatening; the disease requires active therapeutic intervention. An important factor responsible may be genetic susceptibility to IBD in early infancy. In conclusion, VEO-IBD is a disease that seriously threatens the health of children, and the clinical treatment effect is poor. For growing VEO-IBD, early diagnosis and active treatment are required. In addition, VEO-IBD may be genetically susceptible, and IL-10R genetic testing may be used as an indicator of early diagnosis of the disease.

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LETTER TO THE EDITOR

Protective role of lncRNA H19 against LPS-induced inflammatory injury in WI-38 cells by upregulating miR-454-3p in neonatal pneumonia

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To the Editor,

The greatest risk of death from pneumonia in childhood is during the neonatal period. Neonatal pneumonia (NP) is frequent in premature and very low birth weight infants (1). At least one-third of the annual 10.8 million child deaths worldwide occur in the first 28 days of life. Strong inflammatory response is closely related with NP progression, thus anti-inflammatory treatment plays a key role in this disease (2). A number of long non-coding RNAs (lncRNAs) have been reported to have crucial functions in diverse processes and diseases, including inflammatory response. lncRNA H19 (H19) is a developmentally regulated RNA, the expression of which is retained only in the adult heart and skeletal muscle. The re-expression of H19 in other tissues is closely related to inflammatory disorders (3). Notably, in the study of abnormal expression of lncRNAs in pulmonary tuberculosis, lncRNA H19 upregulation was found in patients with pneumonia (4). However, the expression and function of H19 in NP are unknown.

Recent studies have demonstrated that lncRNAs act as competing endogenous RNA (ceRNAs) of microRNAs (miRNAs) in various biological processes. This study found the binding site of miR-454-3p on the fragment of H19. MiR-454-

3p is an inflammation-related miRNA, and has the function of alleviating LPS-induced lung injury (5). Therefore, this study aimed to analyze the expression level of the H19/miR-454-3p axis in NP. An in vitro model of pneumonia was established by stimulating human lung fibroblasts WI-38 with LPS to explore the regulatory role of the H19/miR-454-3p axis in LPS-induced inflammatory injury of WI-38 cells.

MATERIALS AND METHODS

Patients and sample collection

This study included 15 neonates with NP and 10 healthy control neonates, who were monitored at Weifang People's Hospital in 2017. Inclusion criteria were neonates less than 28 days of age with clinical manifestations of NP. The diagnosis of pneumonia was made by history, clinical findings, laboratory findings and chest X-ray. The neonates exhibited symptoms of infection, such as fever, poor lactation, and poor mental response. Clinical signs included respiratory distress (respiratory frequency >60 times/min), thoracic depression, moaning, cyanosis, smearing, cough, expectoration. Lung signs were thickened breathing sounds or dry or wet rales. Chest X-ray suggestive of pneumonia includes nodular or coarse patchy infiltrate, diffuse haziness or granularity, air bronchogram and lobar or segmental consolidation. Blood samples were collected

Key words: neonatal pneumonia; H19; miR-454-3p; inflammation; cell viability; LPS-induced

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from the patients and healthy controls on admission, and were centrifuged to separate serum for further use. A signed written informed consent was obtained from parents of each child and the experimental procedures were in accordance with the guideline of the Ethics Committee of Weifang People's Hospital.

Cell culture and stimulation

Human lung fibroblasts WI-38 were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA). Cells were induced with 10 ng/mL lipopolysaccharide (LPS) (Sigma, Louis, MO, USA) for 6 h to construct an *in vitro* model of pneumonia, and maintained in a 5% CO₂ atmosphere at 37°C.

Cell transfection

Cell transfection was used in this study to achieve the *in vitro* regulation of H19 and miR-454-3p. The siRNAs-H19 (si-HI9), si-NC, nucleocapsid protein (NC) mimic, miR-454-3p mimic, NC inhibitor, and miR-454-3p inhibitor were synthesized from GenePharma (Shanghai, China) and were transfected into WI-38 cells using Lipofectamine 3000 (Invitrogen, CA, USA). The cells transfected with only transfection reagents were used as mock. After 48 h of transfection, the cells could be used for the further analyses.

RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was obtained from serum samples and cells by TRIzol reagent (Invitrogen, Life Technologies,

Paisley, UK), then the RNA was reversely transcribed into single-stranded cDNA with PrimeScript reverse transcriptase reagent kit (TaKaRa, Shiga, Japan) following the manufacturer's guidelines. The expression of H19 and miR-454-3p were measured by qRT-PCR, which was carried out using a SYBR green I Master Mix kit (Invitrogen, Carlsbad, CA, USA) on a 7500 Real-Time PCR System (Applied Biosystems, USA). U6 was used as an endogenous control for miR-454-3p, and GAPDH was used as an endogenous control for H19. The final expression value was calculated using the 2^{-ΔΔC_t} method.

Cell viability assay

The Cell Counting Kit 8 (CCK-8) assay was used to analyze cell proliferation rate. Cells were seeded into 96-well plates (5 × 10³ cells/well). Then, 10 μL of CCK-8 reagent (Beyotime, Shanghai, China) was added to each well. The optical density at 450 nm was analyzed using a microplate reader.

Evaluation of pro-inflammatory cytokine levels

The levels of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β), in the WI-38 were measured with the enzyme-linked immunosorbent assay (ELISA) kit (R&D System, Abingdon, UK) according to the manufacturer's protocols.

Bioinformatics prediction and dual-luciferase reporter assay

Prediction of binding sites of H19 and miR-454-3p was performed using the bioinformatics prediction platform DIANA-LncBaseV2 (<http://carolina.>

Table I. Baseline characteristic comparison between pneumonia and healthy neonates

Feature	Healthy (n=10)	NP (n=15)	P value
Age (days)	16.10±2.76	16.60±2.72	0.659
Gender (female/male)	4/6	5/10	0.734
Birth weight (kg)	3.51±0.17	3.36±0.18	0.063
Gestational age (weeks)	39.70±0.95	39.06±0.88	0.102
Delivery methods (vaginal/C-section)	8/2	10/5	0.476

imis.athenainnovation. gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted) (6) and used a luciferase reporter assay to confirm the interaction between H19 and miR-454-3p. The wild type (WT) and mutant type (MUT) of H19 sequences, that contained the binding site of miR-454-3p, were combined into the luciferase reporter vector pGL3 (Promega, Madison, WI, USA). The combined vectors were then co-transfected into WI-38 with miR-454-3p mimic, mimic NC using Lipofectamine 3000 (Invitrogen). At 48 h post-transfection, the luciferase activity changes were analyzed by dual luciferase reporter assay (Promega).

Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD) and analyzed at SPSS 21.0 (SPSS, Inc, Chicago, Ill, USA) and GraphPad 7.0 (GraphPad Software, Inc, USA). Differences between groups were analyzed with Student's *t*-test or analysis of variance and Tukey post hoc test. Pearson correlation analysis was used for the expressions of H19 and miR-454-3p. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Baseline characteristic comparison between pneumonia and healthy neonates

Student's *t*-test and *chi*-squared test were used to compare the basic data of the two groups of newborns. The results showed that there were no

differences in age ($P = 0.659$), gender ($P = 0.734$), birth weight ($P = 0.063$), gestational age ($P = 0.102$), and delivery methods ($P = 0.476$) between the two groups (Table I).

Expression of H19 and miR-454-3p in NP newborns

To further understand the role of H19 and miR-454-3p in NP, its expression in NP serum was quantified by qRT-PCR. We observed that compared with healthy controls, NP patients had upregulated serum H19 ($P < 0.001$ Fig. 1A) and downregulated serum miR-454-3p ($P < 0.001$ Figure 1B), and serum H19 and miR-454-3p levels were significantly negatively correlated in NP patients ($r = -0.649$ $P = 0.009$ Fig.1C).

Expression of the H19/miR-454-3p axis in in-vitro pneumonia model

According to the DIANA-LncBaseV2, we found binding sites for H19 and miR-454-3p (Fig. 2A). The results of the luciferase reporter assay showed that the luciferase activity of the H19-WT was promoted by miR-454-3p inhibitor but suppressed by miR-454-3p mimic (all $P < 0.01$, Fig. 2B). However, there was no significant luciferase activity change in the MUT group ($P > 0.05$, Fig. 2C). Construction of pneumonia cell model was then performed by LPS treatment in WI-38. The expression of H19 was significantly increased in the pneumonia cell model compared with normal cells, and downregulation of

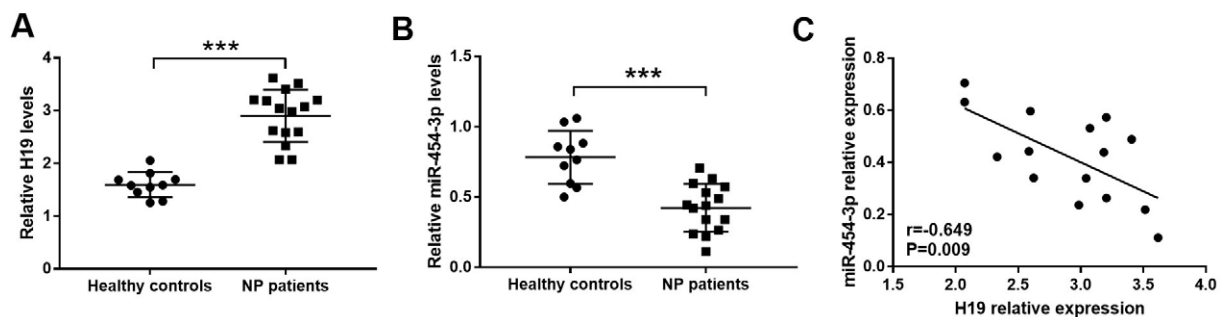


Fig. 1. Expression of H19 and miR-454-3p in pneumonia neonates. **A)** H19 levels were higher in patients with NP than that in healthy controls. **B)** The expression of miR-454-3p was downregulated in NP serum sample. **C)** Serum H19 and miR-454-3p levels were significantly negatively correlated in ISR patients. *** $P < 0.001$.

miR-454-3p levels was observed in pneumonia cell models (all $P < 0.001$, Fig. 2 D, E). The results were consistent with those in serum of NP.

Inhibition of H19 alleviates LPS-induced inflammatory damage in WI-38 cells

The expression level of H19 was significant inhibited by si-H19 ($P < 0.001$, Fig. 3A). We induced cell injury with LPS at a concentration of 10 $\mu\text{g/mL}$, and results of CCK-8 assay demonstrated that cell viability was obviously decreased, but si-H19 could reverse this phenomenon (all $P < 0.001$, Fig. 3B). Further result showed that LPS evidently increased the secretion of pro-inflammatory cytokines (IL-6, IL1 β and TNF- α), which were then suppressed when H19 was downregulated (all $P < 0.001$, Fig.

3C). Taken together, these results suggested that H19 knockdown might play a protective effect on LPS-induced inflammatory injury.

Overexpression of miR-454-3p alleviates LPS-induced inflammatory damage in WI-38 cells

MiR-454-3p mimic significantly upregulated the level of miR-454-3p in WI-38 cells ($P < 0.001$, Fig. 3D). A CCK-8 assay showed that LPS decreased cell viability by nearly 50%. Furthermore, the upregulation of miR-454-3p also alleviated LPS-induced cell viability decline (all $P < 0.001$, Fig. 3E). LPS increased the expression of IL-6, IL-8 and TNF- α , meaning a severe inflammatory response induced by LPS, while the upregulation of miR-454-3p effectively decreased these inflammatory

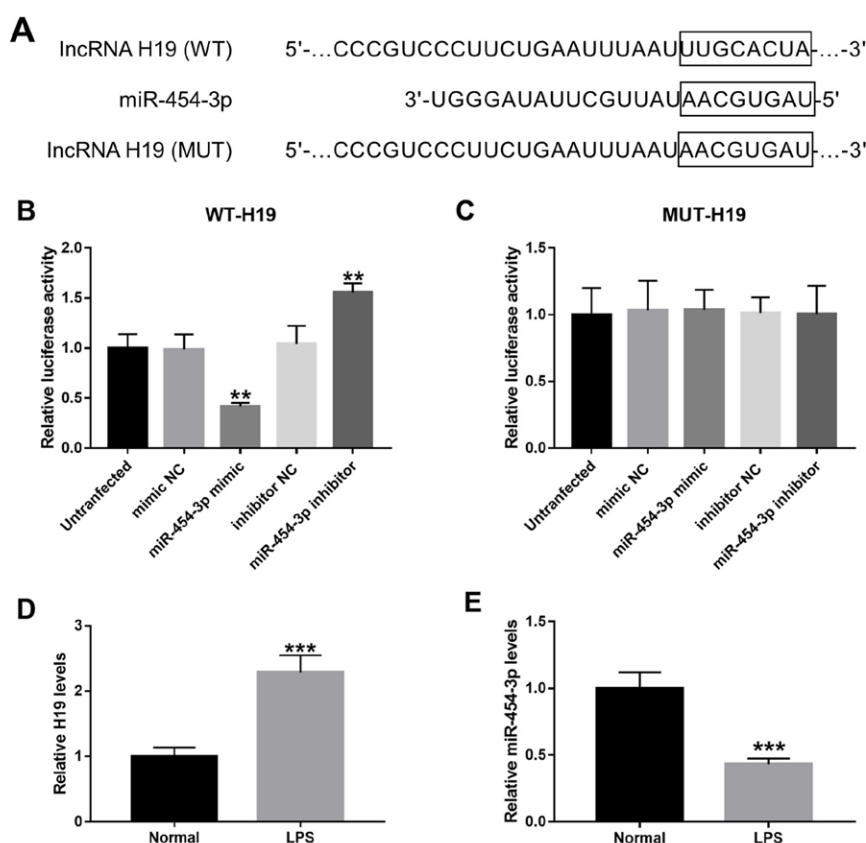


Fig. 2. Expression of the H19/miR-454-3p axis in in vitro pneumonia model **A)** The predicted target sequence in the miR-454-3p for H19 binding. **B, C)** In WI-38 cell lines, the luciferase activity of the H19-WT was decreased by miR-454-3p overexpression but enhanced by its low expression and no significant changes in the relative luciferase activity were observed in the MUT group. **D)** The expression of H19 was upregulated in LPS-induced WI-38 cells. **E)** MiR-454-3p levels were lower in LPS-induced WI-38 cells than that in normal cells. ** $P < 0.01$ *** $P < 0.001$.

cytokines (all $P < 0.001$, Fig. 3F). The experimental results suggest that miR-454-3p overexpression might also have a protective effect on LPS-induced inflammatory injury.

MiR-454-3p mediates the protective effects of H19 reduction in in vitro pneumonia model

To explore the regulatory relationship between H19 and miR-454-3p, the expression of H19 and miR-454-3p were co-regulated in the cell model. Si-H19 could significantly enhance the level of miR-454-3p in the cell model. Furthermore, in the cells co-transfected with si-H19 and miR-454-3p inhibitor, the increased miR-454-3p expression induced by H19 knockout was significantly decreased (all $P < 0.01$, Fig. 4A). Downregulated H19 improved cell viability, which was decreased by LPS, while downregulated miR-454-3p could significantly reverse the effect made by H19 (all $P < 0.001$, Fig. 4B). The detection of relative levels of

inflammatory cytokines showed that the knockdown H19 decreased the elevated production of IL-6, IL-8 and TNF- α induced by LPS, and downregulated miR-454-3p suppressed the effect caused by H19 (all $P < 0.001$, Fig. 4C).

DISCUSSION

Pneumonia is a major cause of neonatal morbidity and mortality around the world, especially in developing countries. NP is associated with inflammatory stimulation from microorganisms. LncRNA H19 is located on chromosome 11 in humans. Fang et al. found that H19 acted as a ceRNA in regulating miR-874 and restoring LPS dysregulated inflammatory responses and myocardial dysfunction (7). In the present study, H19 was upregulated in NP patients and LPS-induced WI-38 cells. Further investigation demonstrated that inhibition of H19 significantly improved WI-38 cell viability and inflammation that was affected by LPS.

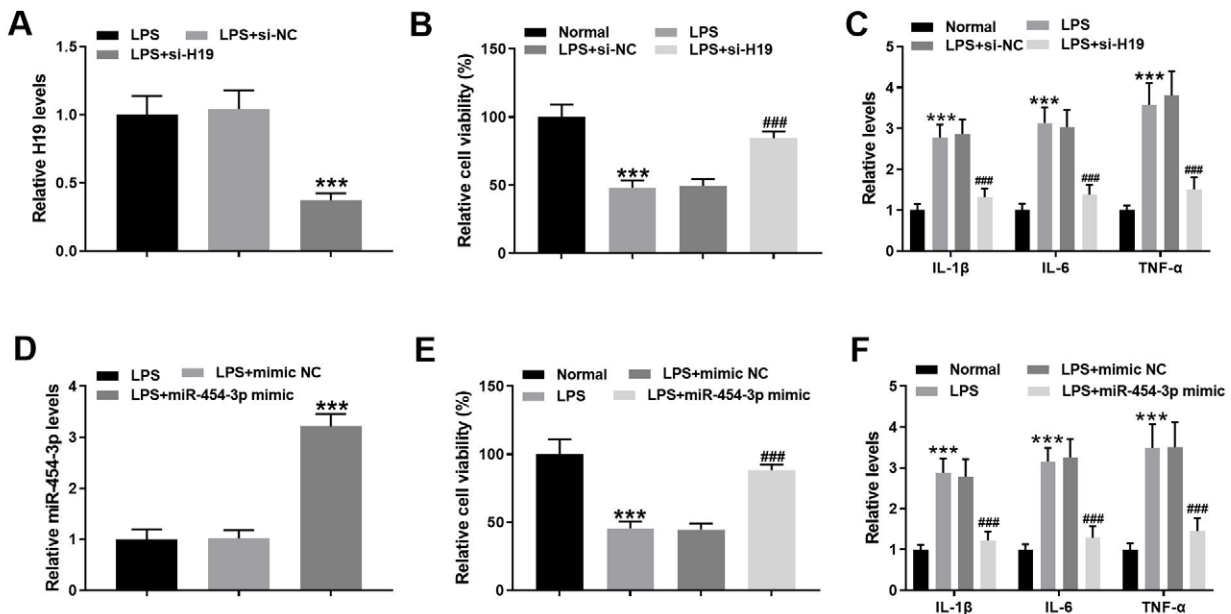


Fig. 3. Effects of H19 and miR-454-3p on LPS-induced inflammatory damage in WI-38 cells. **A)** Expression of H19 was downregulated following transfection with a si-H19 in LPS-induced WI-38 cells. **B)** LPS-induced decrease in cell viability, and knockdown of H19 can reverse this phenomenon. **C)** Downregulated H19 can reduce the expression level of inflammatory factors in LPS-induced WI-38. **D)** The expression of miR-454-3p was upregulated by miR-454-3p mimic in LPS-induced WI-38 cells. **E)** LPS decreased cell viability, upregulation of miR-454-3p alleviated LPS-induced cell viability decline. **F)** Overexpression of miR-454-3p can reduce the expression level of inflammatory factors. *** $P < 0.001$ compared to Normal; ### $P < 0.001$ compared to LPS.

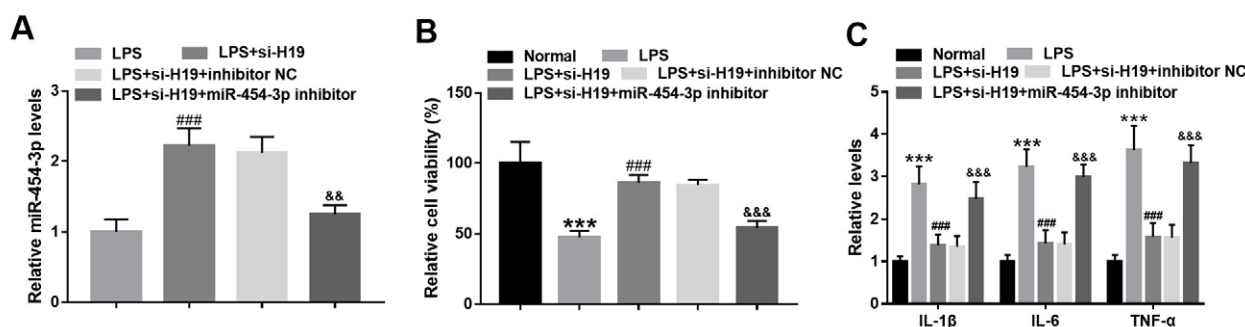


Fig. 4. MiR-454-3p mediates the protective effects of H19 reduction in in vitro pneumonia model. **A)** Expression of miR-454-3p was upregulated following transfection with si-H19, and downregulated following transfection with miR-454-3p inhibitor in LPS-induced WI-38 cells. **B)** Knockout of H19 increased cell viability of LPS-induced WI-38, but miR-454-3p inhibitor can abolish the promoting effect. **C)** The inhibition of inflammatory factor expression level by downregulation of H19 could be reversed by the down-regulation of mir-454-3p in LPS-induced WI-38 cells. ** $P < 0.01$, *** $P < 0.001$ compared to Normal; ## $P < 0.01$, ### $P < 0.001$ compared to LPS; &&& $P < 0.001$ compared to LPS+si-H19.

Therefore, H19 may play a regulatory role in the progression of NP.

Recent research has shown that ceRNA has been hypothetically proposed to explain a new regulatory mechanism of lncRNA through sponging miRNAs in many diseases (8). MiR-454-3p have been shown to be associated with the development of a variety of diseases, such as glioblastoma (9), and lung cancer (10). In this study, miR-454-3p was determined as a target of H19. miR-454-3p expression was decreased in NP cells, and was negatively correlated with H19. Additionally, the inhibition of miR-454-3p partially reversed the effects of H19 knockout on LPS-induced WI-38 cell viability and inflammation. Thus, we considered that H19 might be involved in the progression of NP by directly regulating miR-454-3p.

In conclusion, our study demonstrated that H19 was significantly upregulated, and miR-454-3p was downregulated in NP newborns and the LPS-induced WI-38 cell model. The knockdown of H19 could improve cell viability and inflammation in LPS-induced WI-38 cells by sponging miR-454-3p. Our findings implied that the H19/miR-454-3p axis might provide new therapeutic targets for NP.

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LETTER TO THE EDITOR

Levodropropizine in children: over thirty years of clinical experienceG. Ciprandi¹, A. Licari², M.A. Tosca³ and G.L. Marseglia²

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To the Editor,

Under normal conditions cough is a protective reflex with two main purposes: to clear excessive secretions and mucous from the large airways, and to prevent foreign material from entering the lower airways. The cough reflex is triggered by stimulation of receptors on vagal afferent nerves ending in the larynx, trachea and bronchi. These stimuli trigger sensory nerve fibres in the respiratory tract, including C-fibres and mechanically sensitive A δ -fibres, and reach the neural cells normally dedicated to the generation of the breathing pattern. However, cough can sometimes become excessive and potentially harmful to the airway mucosa, in fact, it is one of the most common reasons why individuals seek a medical consultation in primary health.

Acute cough lasts less than four weeks. An acute cough is commonly associated with upper respiratory tract infection (URTI), which is usually of viral origin so that it is called post-viral cough. Consistently, acute cough due to URTI is the most common reason for primary care worldwide. It is generally self-resolving, but may be very bothersome for a child, but overall, for the family.

Chronic cough (lasting more than 8 weeks) can be caused by several lung disorders such as asthma, chronic obstructive pulmonary disease (COPD) and lung cancer, or extra-pulmonary disorders such

as gastro-esophageal reflux disease. In children, differently from adults, the etiology of coughing is often related to viral URTI, prolonged bacterial bronchitis and asthma. As a result, antitussive treatments are commonly used in clinical practice. However, cough control is a challenge as there is a need to identify an antitussive medication that has to be effective and above all safe in children.

There are two classes of antitussive agents available for the treatment of cough in children: central action cough suppressants (e.g. codeine, dextromethorphan and cloperastin), and peripheral action antitussive agents (such as levodropropizine). The efficacy and safety of most over-the-counter (OTC) symptomatic antitussive drugs has recently been questioned, because centrally acting antitussive drugs have shown poor tolerability related to side effects on the central nervous system.

In the last five years, several studies and surveys, carried out by Regulatory Authorities and Scientific Societies, reported that cough negatively impacts quality of life, but for most patients their cough medications have limited or no effectiveness. Thus, the safety of such drugs, particularly when they are being administered to paediatric populations and patients with respiratory disorders, is very important.

There is therefore a clear need for safe and effective drugs for cough treatment, especially in

Key words: children; cough; levodropropizine; respiratory infections

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children. Levodropropizine (LDP) is a non-opioid peripheral antitussive drug that is therefore devoid of effects on the central nervous system. LDP is indicated for adults and children over 2 years of age for the symptomatic therapy of cough (1).

The current paper presents the literature concerning LDP treatment in children with cough, analyzing its efficacy and tolerability profile and its use in clinical practice in the paediatric population,

Levodropropizine

LDP (S(-)-3-(4-phenyl-piperazin-1-yl)-propane-1,2-diol) is the levorotatory isomer of dropropizine, a piperazine derivative. LDP is rapidly absorbed from the gastrointestinal tract, reaching peak plasma concentrations between 40 and 60 minutes after oral administration, and is also rapidly distributed throughout the body (1). In addition, it has a plasma half-life of approximately 1.5 to 3 hours and has linear pharmacokinetics over a concentration range of 30, 60 and 90 mg in healthy volunteers (1). This enantiomer has an antitussive effect comparable to dropropizine racemic drug, but with the advantage of causing fewer adverse effects, mainly related to the central nervous system (12). Its peripheral antitussive action is due to the inhibition of vagal C fiber activation. Several studies on animal models and on humans support the thesis of the lack of centralized action. Gunella and colleagues conducted a controlled study *versus* placebo to evaluate the effects of 60 mg levodropropizine t.i.d. for four days on the respiratory center in 40 hypercapnic and hypoxiemic patients. Findings in the two treatment groups were similar, confirming the absence of effect at the central level of levodropropizine (1).

Recently, a single-blind crossover study was carried out on patients with chronic cough of different origin to investigate the effect of levodropropizine (60 mg), or dihydrocodeine (15 mg), or placebo on the respiratory response to a standard CO₂ re-breathing testing. The results showed that, unlike dihydrocodeine, both levodropropizine and placebo did not significantly affect respiratory response to hypercapnia, supporting the peripheral action of levodropropizine (2).

Since the launch, more than 30 years ago, the

antitussive activity of LDP has been extensively studied and the drug has been proven to be effective and safe in patients of all ages (3,4). In particular for pediatric age, many clinical studies, including meta-analysis, have confirmed the antitussive efficacy and tolerability of LDP for the treatment of acute and chronic cough in children (5, 6).

The evidence in children

The research included published studies cited in PubMed (the search included the term "levodropropizine in children"). In 1989, three studies investigated the efficacy of LDP in children with ages ranging between the first month of life and 12 years, who were suffering from several respiratory diseases, including acute bronchitis, asthma, bronchopneumonia, tracheitis. The efficacy was assessed in terms of reducing the intensity and frequency of coughing after treatment and tolerability as absence of adverse events.

In the first published study, Fiocchi and colleagues enrolled 70 children (mean age 4.5 years, 31 males) and administered LDP oral drop formulation at 2 mg/Kg/day for five days. LDP was effective in 69/70 children and cough scores significantly ($p < 0.01$) diminished. In no case was there any early interruption of treatment, and somnolence was reported in 3 (4.28%) children (7). The second study, carried out by Cogo and colleagues, was a multicenter study with 172 children aged between 1 and 12 years, out of a total sample of 1,304 patients (mean age 4.6 years), who had acute cough due to upper tract respiratory infection. 3-6 mg/kg t.i.d. of LDP oral drops were administered for 7 days. The effectiveness of the treatment was judged by doctors to be good in 93% of cases, having found a reduction or absence of the cough symptom, while tolerability was considered good in 98% of cases. Patients and parents of the children reported that the improvement in coughing occurred during the first 3-4 days of therapy. The last of the three studies, was another multicenter study only, focused on a wide range of pediatric respiratory diseases. A total of 180 children, age between 5 months and 12 years, were enrolled and treated with 1-2 oral drops/kg die of LDP for 7 days. The clinical judgment on

the efficacy of LDP was good in 94% of cases, and tolerability was good with only 3% of cases judged unsatisfactory, probably due to the simultaneous presence of antibiotic therapy which makes it difficult to attribute responsibility.

Two comparative double-blind, randomized clinical trials evaluated antitussive efficacy and tolerability of levodropropizine *versus* dropropizina and dextromethorphan in children. Banderali and colleagues compared LDP (2mg/kg) *vs* dropropizine (1mg/kg) 3 times a day for 3 days in 258 children (aged between 2 and 14 years) with dry cough. The results showed that both treatments were significantly ($p < 0.001$) effective in reducing cough, but LDP was better tolerated. Sleepiness was found to be twice as frequent in the dropropizin group compared to the LDP group (10.3% *vs* 5.3%) (8). Instead, Kim Doon Soo and colleagues, compared LDP syrup *vs* dextromethorphan, one of the major central antitussive drugs, in 77 pediatric patients (aged between 2 and 3 years) with non-recurrent or slightly recurrent coughs accompanied by acute chronic bronchitis. The severity and frequency of cough were more significantly reduced after 2-3 days in the LDP group than in the dextromethorphan group ($p = 0.003$). In conclusion, LDP has a more favourable antitussive efficacy and benefit profile than dextromethorphan.

De Blasio and colleagues reviewed cough management from a practical point of view. The review identified that among the drugs used for the symptomatic treatment of cough, peripheral-acting antitussive drugs, such as LDP, showed higher effectiveness in relieving cough, mainly in childhood. Moreover, all these studies provided evidence that LDP was more tolerated than central antitussive drugs (9). Consequently, the same authors analyzed the efficacy of LDP in pediatric cough, highlighting the concept that the cough morbidity extends to parents, other family members, caregivers, and teachers. The most common medications used to relieve acute cough in children have clinically relevant sedative effects because they are narcotics or first-generation antihistamines. On the contrary, LDP, being a peripheral antitussive agent, is substantially devoid of adverse effects on

the central nervous system (10).

Furthermore, these authors carried out a pediatric observational study evaluating children with acute cough associated with URTI. This study investigated cough epidemiology and its impact on the quality of sleeping and children's daily activities, but also on their parents. The results reported that cough significantly affected sleep. A total of 433 children (mean age 6.1 years) were enrolled and divided into 3 treatment groups: 101 children were treated with LDP, 60 with central antitussive agents (cloperastine or codeine), and 80 without any active drug for six days. The results showed a cough resolution was significantly higher with levodropropizine than with central antitussives (47% *vs* 28% respectively, $p = 0.0012$) or no therapy, independent of antibiotic use or concomitant illnesses (11).

Zanasi and colleagues provided a meta-analysis of LDP in the treatment of cough in adults and children. This meta-analysis evaluated seven clinical studies, including 1,178 patients, to compare LDP with control treatments (codeine, cloperastine, dextromethorphan). The analysis showed that LDP was an effective antitussive ($p = 0.0015$) drug in both pediatric and adult populations, as it induced a more significantly reduced cough intensity and frequency, as well as nocturnal awakenings in comparison with central antitussive drugs (6).

The same authors carried out a prospective observational study to evaluate 330 children with acute post-viral cough. The treatment lasted six days. Children were subdivided in four groups: the first group (123 children) was treated with antitussive medications, including central antitussive agents (44 children) and peripheral ones (79 children). The second group was treated with antibiotics alone (89 children). The third group took antitussive agents combined with antibiotics. The last group was without active treatment (80 children). Severity, frequency, and type of cough were assessed at baseline and the end of the study. Antitussive drugs were more effective than antibiotics. Moreover, considering peripheral antitussives, the resolution of cough was significantly higher with antitussives than with antibiotics ($p < 0.01$). Therefore, the authors concluded that antibiotics are not indicated

to treat acute cough due to URTI, whereas peripheral antitussive drugs should be the treatment option.

Recently, an international group of experts specialized in cough management met to discuss and increase their knowledge on the cough mechanism and the activity of LDP as a peripherally acting antitussive drug (1). The authors analyzed the studies in the last thirty years conducted on adults and children with a cough. They concluded that LDP trials showed a documented efficacy in cough control, associated with no evident central depressant activity.

Development of new cough treatments still remains a challenge, as even the latest available pharmacological approaches are still unsafe and ineffective. Therefore, the authors suggest that, at the present time, LDP is an important therapy for the treatment of different types of cough, especially in children, thanks to its very good efficacy and safety profile (1).

CONCLUSION

Cough is one of the most frequent symptoms and is consequently the most common reason for a medical visit. Cough is a very bothersome symptom and significantly affects the quality of life of both the child and the parents (12). Therefore, even though the acute post-viral cough is self-resolving, there is a need to relieve it. The majority of antitussive medications act on the central nervous system and consequently have relevant side effects. In this regard, LDP acting peripherally represents an important therapeutic option. LDP exerts its activity on the sensory fibers, namely the C-fibers, involved in the genesis of cough. In animal models, it was demonstrated that levodropropizine could act on C-fibers. Clinical studies on humans confirmed its peripheral action.

In conclusion, all these results support the favorable benefit/risk profile of LDP in the management of cough. At present, LDP seems to be an antitussive drug of first choice that acts on peripheral mechanisms of the cough reflex and is devoid of central adverse events, contrary to the common centrally-acting antitussives. The clinical experience highlights that LDP is effective and safe in children with cough.

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LETTER TO THE EDITOR

Overexpression of miR-150-5p alleviates lipopolysaccharide-induced A549 cell injury in type II alveolar epithelial cells by inhibiting the FSTL1 signaling pathwayC-F. Shi^{1*}, Z-H. Wang^{1*}, J-C. Li^{2*} and M-W. Liu²

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To the Editor,

At present, acute lung injury (ALI) caused by sepsis has poor prognosis, and the mortality rate can be as high as 70% (1). Therefore, the discovery of new therapeutic targets and effective drug treatments may reduce the mortality of ALI induced by sepsis follicle inhibition protein 1 (follistatin-like protein 1, FSTL1 - also known as follistatin-related protein) which is involved in the occurrence and development of a variety of inflammatory diseases (2, 3).

Different miRNAs involved in the inflammatory response play a promotive or inhibitive role towards inflammation (4). In monocyte/macrophage differentiation, miR-223, miR-15a, and miR-16 commonly regulate IKK α . Their expressions are reduced when three IKK α show increased phosphorylation, activating nuclear factor (NF)- κ B(5, 6). In peripheral blood mononuclear cells of patients with type 1 diabetes, the overexpression of miR-885-3p can inhibit the inflammatory response of mononuclear cells by targeting the activity of Toll-like receptor 4/NF- κ B signaling pathway (7). However, the mechanism by which miRNAs regulate inflammation is still unclear.

As a major regulator of negative regulation

of the development and differentiation of diverse immune cells, miR-150-5p is widely involved in the regulation of inflammatory responses induced by various causes (8), however, the exact mechanism is unclear. In this study, we predicted that FSTL1 is the regulatory target of miR-150-5p by using TargetScan software. We hypothesized that miR-150-5p plays an important role in the occurrence and progression of endotoxin-induced ALI and further explored whether baicalein can increase miR-induced lung injury.

MATERIALS AND METHODS

Culture and grouping of type II alveolar epithelial A549 cells

The 1640 culture medium containing 10% fetal bovine serum was used to culture A549 cells until 70% A549 cell confluence at 37°C in a 5% CO₂ incubator, achieving 1 × 10⁴/ml per orifice plate density in the culture dish. The cells were subjected to 0.25% trypsin digestion and cultivated for 7 days in 1:2 batches. The cells were inoculated in 25 cm² culture flask at a density of 1×10⁵ cells/mL. When the fusion reached about 60%, cell growths were synchronized for 24 h, and cell grouping was performed. Different concentrations of lipopolysaccharide (LPS) (0.5, 1.0, 1.5, and 2.0 mg/ L) were added and used for culture for 24 h to

Key words: A549 cells inflammatory response; miR-150-5p; FSTL1; ERK1/2

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observe the effect of LPS on the activity and proliferation of A549 cells. The cells were divided into the following groups to observe the effect of miR-150-5p on endotoxin-induced A549 cell injury: normal control, LPS-induced, LPS+miR-150-5p mimic, LPS+miR-150-5p inhibitor, LPS+miR-150-5p mimic control, and LPS+miR-150-5p inhibitor control groups. The cells were cultured for 24 h, and the indicators were measured afterward.

CCK-8 assay for determination of A549 cell viability

CCK-8 kit (CCK-8, Qihai Bio) was used to determine the proliferation viability of cells in different treatment groups. The A549 cells were injected in 96-well plates and fused until 70% confluence. Then, the medium was changed to serum-free medium. At different conditions, 10 μ L CCK-8 solution was added to each well, and the plates were incubated in the dark for 2–4 h. The absorbance was measured at 450 nm using a microplate meter (A450 nm).

A549 cell transfection

An appropriate amount of A549 cells in logarithmic growth phase were inoculated into a six-well plate. When the cells adhered to the wall overnight and grew to 60% confluence, miR-150-5p mimic, miR-150-5p inhibitor, mimic control, and inhibitor control were transfected into A549 cells following the manufacturer's instructions for Lipofectamine 2000 transfection reagent. The transfection concentration was 50 nmol/L, and medium was replaced with a fresh one after transfection for 6 h. The cell culture was continued for 24 h. Then, the cells were collected for real-time polymerase chain reaction (RT-PCR) and Western blot detection.

Survival rate of A549 cells

The liquid in each well of the 96-well plate was sucked and discarded. Dulbecco's Modified Eagle Medium (DMEM) was added with 270 μ L, 5 g/L MTT working solution was added with 30 μ L and evenly mixed, and the culture medium was incubated at 37°C for 4 h. The culture medium in the wells was discarded, and 270 μ L dimethyl sulfoxide was added to each well. The solution was fully dissolved after 10 min of oscillation. The optical density (OD) of each well was measured by using a microplate analyzer at 490 nm wavelength. With the OD of the normal group as the control, the survival rate of cells in each well (%) = the OD value of each well / the mean OD value of the normal group $\times$ 100.

Target gene prediction

The miR-Base (<http://www.mirbase.org/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdc-Berlin.DE/>) target gene prediction software predicted whether the target gene of miR-150-5p is FSTL1. Wild-type (WT) and mutant (MUT) FSTL1 luciferase gene vectors were constructed by conventional methods. These plasmids were mixed with miR-150-5p mimics, miR-150-5p inhibitor, mimic control, and inhibitor control and transfected into A549 cells. Cells with WT + miR-150-5p mimics, MUT + miR-150-5p, WT + negative control, and MUT + negative control were counted. After storage at 37°C and incubation in 5% CO₂ incubator for 48 h, a double luciferase reporter gene kit was used to detect double luciferase activity. At the same time, the level of FSTL1 in A549 cells overexpressing miR-150-5p was detected by RT-PCR and Western blot.

Quantitative RT-PCR analysis

A549 cells in the treatment group were collected, and cellular RNA was extracted by the total RNA extraction kit and reverse transcribed into cDNA in accordance with the instructions for PrimeScript™ RT Reagent Kit. The expressions of FSTL1 mRNA and miR-150-5p were detected by quantitative RT-PCR. The reaction system was as follows: SYBR Premix Ex Taq™ II 10 μ L, 2 μ L PCR primers, DNA template 2 μ L, DEPC water 6 μ L, and 20 μ L total reaction system. Each sample was provided with three parallel duplicate holes. The reaction system was completed on a PCR apparatus. To detect the level of miR-150-5p, we used the TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA, USA) and measured the expression level of miR-150-5p using the 2^{- $\Delta\Delta$ CT} method. The reaction conditions were as follows: 95°C pre-denaturation for 30 s, 95°C reaction for 5 s, and 60°C annealing for 30 for 40 cycles. The primer sequences were as follows: miR-150-5p: F-5'-GCGGGCTAATACTGCCTGG-3', R-5'-ATCCAGTCCAGGGTCCGAAA-3'; U6: F-5'-CTCGCTTCGGCAGCAC-3', R-5'-AACGCTTCACGAATTTGCGT-3'; FSTL1 mRNA: F-5'-TTATGATGGGCAGGCAAAGAA-3', R-5'-ACTGCCTTTAGAGAACCCAGCC-3'; β -actin: F-5'-ATGGTGAAGGTCGGTGTG-3', R-5'-AACTTGCCGTGGGTAGAG-3'. U6 was obtained as the internal reference of miR-150-5p; β -actin was used as the internal reference for the expression of FSTL1, ERK1, and

JNK1 genes. After the reaction, the circulating threshold (Ct) of each gene was calculated and compared with the internal reference gene U6 to determine the expression level.

Western blotting analysis

The cell lysate, protease inhibitor, and phosphatase inhibitor were mixed with cell lysate buffer at the ratio of 8:1:1.150 μ L. Radioimmunoprecipitation assay cell lysis buffer was added to each well, and the plates were placed in an ice bath for 30 min lysis to extract the total protein of each group. Protein purity was determined by BCA protein quantitative kit after 15 min centrifugation at 4°C. The 50 μ g protein extract was separated by 10% SDS-PAGE electrophoresis, added with 5% skim milk, and incubated for 1.5 h. The corresponding NF- κ B65, P-I κ B- α , I κ B- α , β -actin, FSTL1, and ERK1/2 primary antibodies 1:1 000 (Abcam Company, United Kingdom) were added at 4°C overnight. HRP-labeled goat anti-rabbit IgG (Wuhan Google Biological Co., Ltd, Wuhan, China) was added the next day after washing the membrane with Tris buffered saline with Tween® 20 (TBST) thrice (volume dilution ratio: 1:3000), and the reaction time was 1 h at room temperature. The film was washed thrice with TBST, with each wash lasting for

10 min, and enhanced chemiluminescence solution was added for color development. The gray values of the strips were analyzed by Image Lab software, and β -actin was used as the internal reference.

ELISA analysis

The logarithmic growth-phase cells were induced to absorb heavy suspension inoculation in six-well plates. After the adherent cells absorbed the old culture medium, the cells and cell culture supernatant from the treatment group were collected after 24 h. Cell inflammatory factors, including prostaglandin E₂ (PGE₂), IL-6, IL-1 β , and TNF- α , were detected with ELISA kits (Wuhan Huamei Biological Co., Ltd., Wuhan, China) with the operation steps strictly performed in accordance with the instructions.

Statistical analysis

GraphPad Prism 6 was used for statistical analysis. The experimental data were expressed as mean $\pm$ standard deviation. One-way analysis of variance was used for comparison between groups, and least significant difference method was used for comparison of significance between groups. $P < 0.05$ indicated statistically significant difference.

Table I . LPS affects the inflammatory cytokines, viability and survival rate of type II alveolar epithelial A549 cells

Group	IL-6 (ng/L)	IL-1 β (ng/L)	TNF- α (ng/L)	Viability	Survival
Control	0.48 $\pm$ 0.19	0.19 $\pm$ 0.07	0.37 $\pm$ 0.14	0.81 $\pm$ 0.25	0.69 $\pm$ 0.23
LPS(0.5mg/L)	0.57 $\pm$ 0.23	0.41 $\pm$ 0.13*	0.48 $\pm$ 0.17	0.73 $\pm$ 0.21	0.61 $\pm$ 0.21
LPS(1.0mg/L)	1.34 $\pm$ 0.52*	0.67 $\pm$ 0.26*	0.83 $\pm$ 0.25*	0.56 $\pm$ 0.16*	0.47 $\pm$ 0.17*
LPS(1.5mg/L)	1.68 $\pm$ 0.61*	0.79 $\pm$ 0.29*	1.04 $\pm$ 0.36*	0.41 $\pm$ 0.14*	0.36 $\pm$ 0.14*
LPS(2.0mg/L)	1.93 $\pm$ 0.69*	0.93 $\pm$ 0.38*	1.38 $\pm$ 0.42*	0.35 $\pm$ 0.12*	0.31 $\pm$ 0.12*

Alveolar epithelial A549 cells were incubated with various concentrations of LPS (0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h. The levels of inflammatory cytokines (IL-6, IL-1 β , and TNF- α) in A549 cells were measured by ELISA analysis. CCK-8 was used to measure the viability of A549 cells, and MTT assay was performed to measure their proliferation. The levels of inflammatory cytokines, viability and survival rate were expressed as mean $\pm$ standard error of the mean (SEM). * $P < 0.05$ compared with the control group.

RESULTS

Effect of LPS on inflammation, viability and survival rate in type II alveolar epithelial A549 cells

Alveolar epithelial A549 cells were incubated with various concentrations of LPS (0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h to observe the effect of LPS on their inflammation, viability and survival

rate. IL-6, IL-1 β , and TNF- α in A549 cells were determined by ELISA, CCK-8 was used to measure A549 cell viability, and MTT was used to measure A549 cell proliferation (Table I). LPS increased the levels of IL-6, IL-1 β , and TNF- α in A549 cells, and reduced the viability and survival of A549 cells in a concentration-dependent manner.

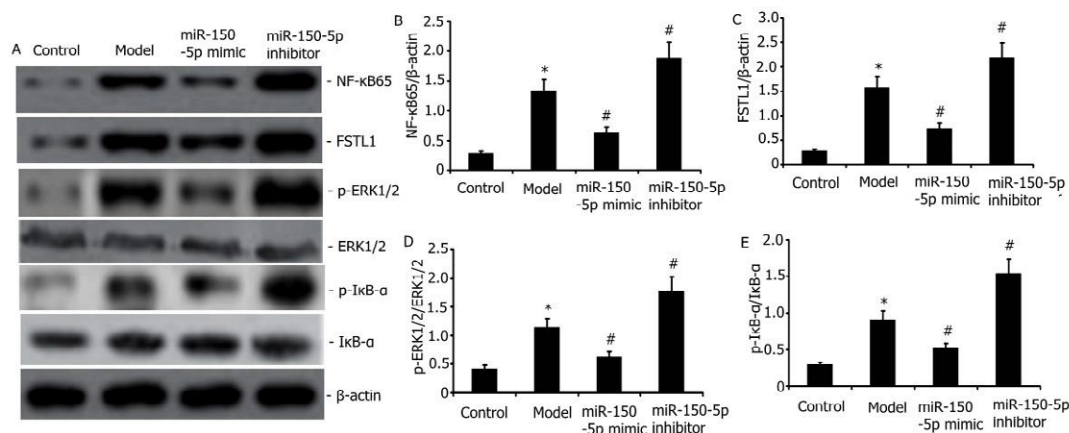


Fig. 1. Effect of miR-150-5p overexpression on the FSTL1 and ERK signaling pathway in type II alveolar epithelial A549 cell inflammation induced by LPS. A549 cells were transfected with miR-150-5p mimics and miR-150-5p inhibitors for 24 h and then cultured with 1 mg/L LPS for 24 h. The protein expressions of FSTL1, P-ERK1/2, NF- κ B65, and P-I κ B- α were detected by Western blot. The level of protein expression in each group was expressed as mean $\pm$ SEM. * P < 0.05 compared with the control group. # P < 0.05 compared with the model group. A) Typical protein bands of FSTL1, p-ERK1/2, NF- κ B65, and p-I κ B- α were analyzed by Western blot. B–E) quantitative analysis of the expression levels of various proteins.

Table II. Effect of miR-150-5p over-expression on the inflammatory cytokines, viability and survival rate of type II alveolar epithelial A549 cells of type II alveolar epithelial A549 cells induced by LPS

Group	IL-6 (ng/L)	IL-1 β (ng/L)	TNF- α (ng/L)	Survival	Viability
Control	0.46 $\pm$ 0.13	0.18 $\pm$ 0.06	0.38 $\pm$ 0.15	0.81 $\pm$ 0.25	0.69 $\pm$ 0.23
LPS	1.36 $\pm$ 0.47*	0.69 $\pm$ 0.25*	0.89 $\pm$ 0.32*	0.52 $\pm$ 0.17*	0.48 $\pm$ 0.16*
LPS+ mimic	0.69 $\pm$ 0.26#	0.43 $\pm$ 0.12#	0.52 $\pm$ 0.18#	0.67 $\pm$ 0.25#	0.61 $\pm$ 0.19#
LPS+inhibitor	2.17 $\pm$ 0.61#	1.49 $\pm$ 0.43#	1.93 $\pm$ 0.57#	0.34 $\pm$ 0.13#	0.26 $\pm$ 0.10#

A549 cells were transfected with miR-150-5p mimics and miR-150-5p inhibitors for 24 h and then cultured with 1 mg/L LPS for 24 h. The levels of inflammatory cytokines (IL-6, IL-1 β , and TNF- α) in A549 cells were measured by ELISA analysis. CCK-8 was used to measure the viability of A549 cells, and MTT assay was performed to measure their survival. The levels of inflammatory cytokines, viability and survival rate were expressed as mean $\pm$ SEM. * P < 0.05 compared with the control group. # P < 0.05 compared with model group.

Effect of LPS on miR-150-5p over expression on the inflammation, viability and survival rate of type II alveolar epithelial A549 cells induced by LPS

A549 cells were transfected with miR-150-5p mimics and miR-150-5p inhibitors for 24 h and then cultured with 1 mg/L LPS for 24 h. The levels of IL-6, IL-1 β , and TNF- α in A549 cells were measured by ELISA, and CCK-8 and MTT assays were used to measure A549 cell activity and survival rate, respectively. As shown in Table II, miR-150-5p simulants improved the activity and survival rate of A549 cells, and reduced the secretion of IL-6, IL-1 β , and TNF- α in A549 cells.

Mir-150-5p over-expression blocked FSTL1 and ERK signaling pathway of type II alveolar epithelial A549 cell inflammation induced by LPS

A549 cells were transfected with miR-150-5p mimics and miR-150-5p inhibitors for 24 h and then cultured with 1 mg/L LPS for 24 h. The protein expressions of FSTL1, p-ERK1/2, NF- κ B65, and p-I κ B- α were detected by Western blot. As shown

in Fig. 1, miR-150-5p mimics decreased the protein expressions of FSTL1, p-ERK1/2, NF- κ B65, and p-I κ B- α . MiR-150-5p inhibitor increased the expression of FSTL1, p-ERK1/2, NF- κ B65, and p-I κ B- α proteins.

FSTL1 is the regulatory target of miR-150-5p

In this study, FSTL1 and miR-150-5p played important roles in LPS-induced A549 cell inflammation. TargetScan was used to predict whether FSTL1 is the regulatory target of miR-150-5p to determine whether miR-150-5p can regulate LPS-induced A549 cell inflammation by regulating FSTL1. Analysis showed that FSTL1 mRNA and protein levels significantly decreased after miR-150-5p overexpression and significantly increased after miR-150-5p down-regulation (Fig. 2 A, B). WT and MUT FSTL1 were constructed for dual luciferase reporter gene detection to verify the interaction between miR-150-5p and FSTL1. miR-150-5p bound to the WT FSTL1 3'-UTR but not to the mutant FSTL1 3'-UTR (Fig. 2 E, F).

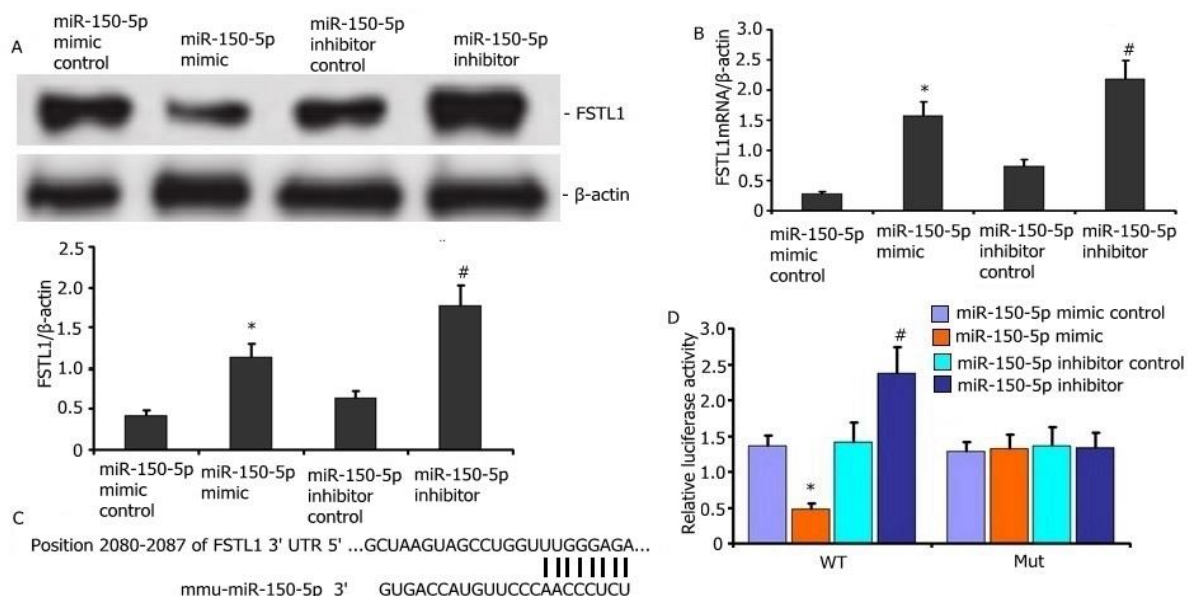


Fig. 2. FSTL1 is the downstream regulatory target of miR-150-5p. **A, B)** FSTL1 was reduced in LPS-induced A549 cells with miR-150-5p overexpression compared with the control group ($P < 0.05$). FSTL1 significantly increased in A549 cells with miR-150-5p down-regulation. **C, D)** After the overexpression of miR-150-5p, the mRNA level of FSTL1 in LPS-induced A549 cells decreased ($P < 0.05$). MiR-150-5p increased after upregulation ($P < 0.05$). **E)** TargetScan predicted FSTL1 as the regulatory target of miR-150-5p, and miR-150-5p bound to the 3'-UTR region of FSTL1. **F:** Dual luciferase reporter assay showed that miR-150-5p mimics bound to the 3'-UTR region of WT FSTL1 but not to MUT FSTL1 ($P < 0.05$).

DISCUSSION

A549 cells are widely used in the study of the injury-protective mechanism of alveolar epithelial cells (9). In this study, we observed that the administration of endotoxin to A549 cells can lead to the secretion of inflammatory mediators, such as IL-6, IL-8, TNF- α , and PGE2, with the increase in endotoxin dose. After baicalein treatment, A549 cells secreted IL-6, IL-8, TNF- α , and PGE2, whereas the levels of other inflammatory mediators decreased. The activity of A549 cells and cell proliferation also increased.

FSTL1 was transfected into U937 and COS-7 cells and stimulated by endotoxin or phloboxate, respectively, to produce a large number of pro-inflammatory factors, such as IL-1 β , IL-6, and TNF- α . FSTL1 can activate human peripheral blood macrophages to secrete a large number of inflammatory factors (10). Studies have also shown that FSTL1 can regulate cell and tissue inflammation by regulating the ERK/JNK pathway activity (11). In this study, we further observed that endotoxin induced A549 cells with increased expression of FSTL1 and increased the secretion of inflammatory mediators. LPS promoted ERK1/2 phosphorylation, leading to increased ERK pathway activity and A549 cell inflammation. Moreover, inhibition of the expression of FSTL1 reduced the ERK/JNK pathway activity and improved LPS-induced A549 cell inflammation.

In conclusion, the present study demonstrated that miR-150-5p plays an important role in the occurrence and development of endotoxin-induced ALI. The overexpression of miR-150-5p can regulate the expression of LPS-induced A549 cell inflammation by targeting FSTL1, reducing the decreased expression of ERK1/2 phosphorylation, inhibiting the activity of ERK pathway, and reducing the inflammatory response induced by endotoxin in A549 cells.

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LETTER TO THE EDITOR

Dimethyl fumarate attenuates lipopolysaccharide-induced acute lung injury by inhibiting inflammation and oxidative stressX.-F. Cui[#], P. Lin[#], J. Yu, L. Liu, Z.-Y. Wang and X.-J. Tang**EICU, The Second Hospital of Dalian Medical University, Dalian, China**Received March 23, 2021 – Accepted July 27, 2021*

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To the Editor,

Acute lung injury (ALI)-induced acute respiratory distress syndrome (ARDS) is a disease with high mortality in respiratory departments and intensive care units, and is characterized by excessive proteinaceous exudation and inflammatory mediators in the alveoli (1). Despite progress in the emergency treatment and intensive care of ALI, pharmacotherapy is presently ineffective in inhibiting the progression of the disease. Pathologically, disruption of alveolar structures and parenchyma cell death result in granulocyte infiltration and albumin excretion in the alveoli (2). Effective blocking of neutrophil infiltration attenuates tissue damage in injured lungs, indicating that negative modulation of granulocyte infiltration in ALI may mitigate disease progression. Inflammation acts as an adaptive response to injury (3, 4). However, an excess of leukocytes driven by inflammation can exacerbate disruption of the alveolar-capillary barrier, finally leading to the formation of alveolar dead spaces in the lung (5). In addition, unbalanced oxidation-reduction reactions caused by the dysfunctions of superoxide dismutase (SOD) and catalase (CAT) induce the generation of reactive oxygen species (ROS) in

cells, resulting in oxidative damage in lungs. Hence, the use of antioxidant drugs is considered as a promising treatment to protect lung tissues during ALI. Dimethyl fumarate (DF), a novel antioxidant drug, has been reported to exert antioxidant effects by activating Nrf2 (6). Furthermore, DF has shown anti-inflammatory properties by inhibiting the nuclear factor kappa B (NF- κ B) pathway *in vitro* (7). Here, we hypothesized that DF may alleviate inflammation and oxidative stress during ALI. In this study, the anti-inflammatory and antioxidant roles of DF were identified during ALI, suggesting that DF is a potential pharmacotherapeutic option for ALI.

MATERIALS AND METHODS

Animals and modeling

Adult male C57/B6J mice (20–22 g) were obtained from Dalian Medical University Animal Center (Dalian, China). This study was approved by the Animal Ethics Committee of Dalian Medical University Animal Center. The mice were randomly assigned to three different experimental groups: Sham (Sham), Sham+DF (Sham+DF), lipopolysaccharide (LPS)-induced ALI (ALI), and LPS-induced ALI+DF (ALI+DF). For

Key words: dimethyl fumarate; acute lung injury; inflammation; oxidative stress.

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ALI modeling, the mice were anesthetized using 1% pentobarbital sodium [50 mg/kg, intraperitoneally (I.P.)]. The trachea was then exposed and 25 µg of LPS diluted in 75 µL phosphate-buffered saline (PBS) was injected using a microsyringe; the control animals were instead injected exclusively with PBS. Immediately after and 12 h after LPS injection, DF 10 mg/kg, I.P., q.d. (Sigma-Aldrich, St. Louis, MO, USA) dissolved in dimethylsulfoxide was administered to the animals using oral gavage.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from lung tissue using TRIzol reagent (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. A qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) was used to conduct complementary deoxyribose nucleic acid (cDNA) synthesis. Quantitation of the RNAs was performed using the Perfecta SYBR Green Supermix (Quanta Biosciences). A melting curve was used to determine each RNA level. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. The relative mRNA expressions were quantitated using the $2^{-\Delta\Delta C_t}$ method. The RNA primers are listed in Table I.

Myeloperoxidase (MPO) level detection

Lung tissue was placed into 0.5% hexadecyltrimethylammonium bromide and homogenized.

After centrifugation, the supernatant was sonicated and mixed with tetramethylbenzidine and hydrogen peroxide. The MPO level was measured using a spectrophotometer at 650 nm.

SOD and CAT measurements

The level of SOD was measured spectrophotometrically at 480 nm using a SOD Assay Kit (KeyGen, Nanjing, China) according to the manufacturer's instruction. The CAT level was spectrophotometrically measured using a CAT Detection Kit (KeyGen) at 240 nm.

Malondialdehyde (MDA) and reactive oxygen species (ROS) measurements

MDA and ROS were measured using an MDA Assay Kit and an ROS Assay Kit, respectively, following the manufacturer's protocol (KeyGen).

Immunohistochemistry and hematoxylin & eosin (HE) staining

Lung tissue was fixed with 4% paraformaldehyde, and dehydration was conducted using different gradients of alcohol. The sample was then embedded in paraffin and cut into 5-µm sections using a rotary microtome. Hematoxylin and Eosin (H&E) staining was conducted using a staining kit (Beyotime). For immunohistochemistry (IHC), the sections were incubated with anti-iNOS 1:100 (Abcam, Cambridge, MA, USA) overnight at 4°C. A rabbit and mouse 3,3'-diaminobenzidine HRP Kit (Solarbio, Beijing, China) was used for IHC analysis following the manufacturer's

Table I. Primer sequences used by qRT-PCR

Oligo Name		Sequence (5' -----> 3')
Occludin (Mouse)	Forward	TGAAAGTCCACCTCCTTACAGA
	Reverse	CCGGATAAAAAGAGTACGCTGG
ZO-1 (Mouse)	Forward	GCTTTAGCGAACAGAAGGAGC
	Reverse	TTCATTTTTCCGAGACTTCACCA
Claudin 1 (Mouse)	Forward	GCCTTGATGGTAATTGGCATCC
	Reverse	GGCCACTAATGTCGCCAGAC
GAPDH (Mouse)	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	GGGGTCGTTGATGGCAACA

instructions. Nuclei were stained with H&E, then the images of sections were observed using a microscope.

Evans Blue assay

Mice were injected with 1% Evans Blue dye solution (Sigma-Aldrich) in normal saline via the caudal vein. After 60 min, the mice were sacrificed and their lung tissues were removed for analysis.

Wet/dry weight measurements of lung tissue

The lung tissue was removed following sacrifice of the animal, and the wet weights of the left lungs were determined. The wet lungs were then dried at 60°C for 24 h and weighed to determine the dry weight. The data were used to calculate the ratio of wet/dry weight.

Enzyme-linked immunosorbent assay (ELISA)

Mouse bronchoalveolar lavage fluids (BALFs) were collected at 24 h post-ALI, then centrifuged for 5 min. The supernatant was measured using an ELISA kit (MultiSciences, Hangzhou, China) according to the manufacturer's instructions. The absorbance of each well was measured at 450 nm using a spectrophotometer.

Statistical analysis

Data were analyzed and expressed as the mean $\pm$ SD (standard deviation). Differences between two groups were analyzed using Student's *t*-test. Comparison between multiple groups was conducted using one-way analysis of variance followed by a post-hoc test (least significant difference). Data were collected and analyzed using the Statistical Package for the Social Sciences (SPSS), version 21.0 (IBM, Armonk, NY, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

DF reduces neutrophil infiltration and inflammation in ALI mice

To verify whether DF inhibited neutrophil infiltration and inflammation, we examined the expression of MPO, a classical chemokine of neutrophils, following DF treatment of ALI mice. The results showed a remarkable increase of MPO expression in the ALI group, but a decrease in mice treated with DF (Fig. 1A). In addition, the

inflammatory level was determined by measuring the levels of inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and IL-6 in BALF as well as the expression of inducible nitric oxide synthase (iNOS) in lung tissues. ELISA showed that the levels of TNF- α , IL-1 β , and IL-6 in BALF significantly increased after LPS injection, but treatment with DF alleviated the expressions of these inflammatory cytokines (Fig. 1 B, D). Also, IHC showed an increased iNOS positive area after LPS stimulation, while DF administration decreased iNOS positive areas in the lung (Fig. 1E). Taken together, these results indicated that DF inhibited neutrophil infiltration and proinflammatory expression after treatment with ALI.

DF attenuates the level of oxidative stress by rescue of the SOD/CAT system in lungs

The levels of oxidative stress were then measured. Measurements of MDA and ROS showed a significant increase in LPS-injected mice, but DF treatment remarkably reduced their expressions in injured lung tissue (Fig. 2 A, B). To measure the antioxidant/peroxidase system during oxidative stress, we determined SOD and CAT levels with or without DF treatment in ALI-treated animals. The results showed that the SOD level significantly decreased and CAT expression remarkably increased in the ALI group, while administration of DF significantly rescued the SOD levels, but only minimally changed the expression of CAT (Fig. 2C, D). These results showed that treatment with DF inhibited oxidative stress by rescuing the levels of SOD.

Administration of DF prevents disruption of the lung alveolar-capillary barrier in ALI animals

The integrity of the alveolar-capillary barrier was evaluated after LPS induction in the lung. Tight junction markers were measured using qRT-PCR, which showed that RNA levels of claudin-1 (CLDN1), zonula occluden-1 (ZO-1), and occluding (OCLN) were significantly decreased after LPS injection; however, DF treatment increased the expressions of these tight junction markers in lungs, when compared with the lungs of untreated mice (Fig. 3 A-C). To analyze the integrity of the alveolar-

capillary barrier of lung tissue, Evans Blue assay was used to measure alveolar permeability, which showed that LPS significantly increased Evans Blue staining of lung tissues of mice, while DF treatment

reduced this staining (Fig. 3D). Overall, the results showed that treatment with DF protected the integrity of the alveolar-capillary barrier by preserving tight junctions in the lung during ALI.

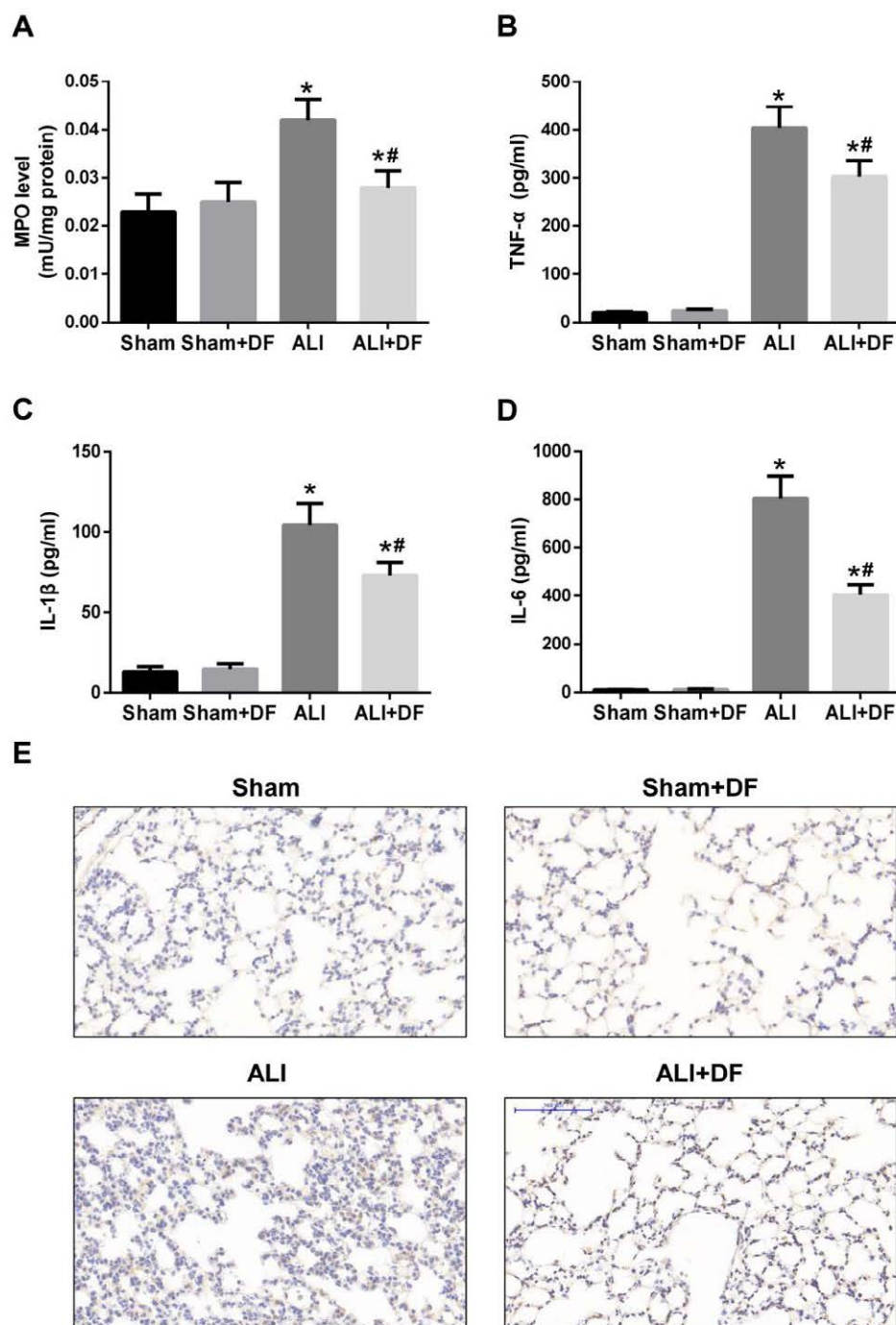


Fig. 1. DF reduces neutrophil infiltration and inflammation in ALI mice. **A)** Representative MPO level in Sham, Sham+DF, ALI and ALI+DF groups. **B-D)** Representative ELISA of TNF-α, IL-1β and IL-6 in Sham, Sham+DF, ALI and ALI+DF groups. **E)** Representative IHC staining of iNOS in Sham, Sham+DF, ALI and ALI+DF groups, (magnification: 100×). * denotes vs Sham group and # signifies vs ALI group with statistical significance. ALI: Acute lung injury; DF: Dimethyl fumarate.

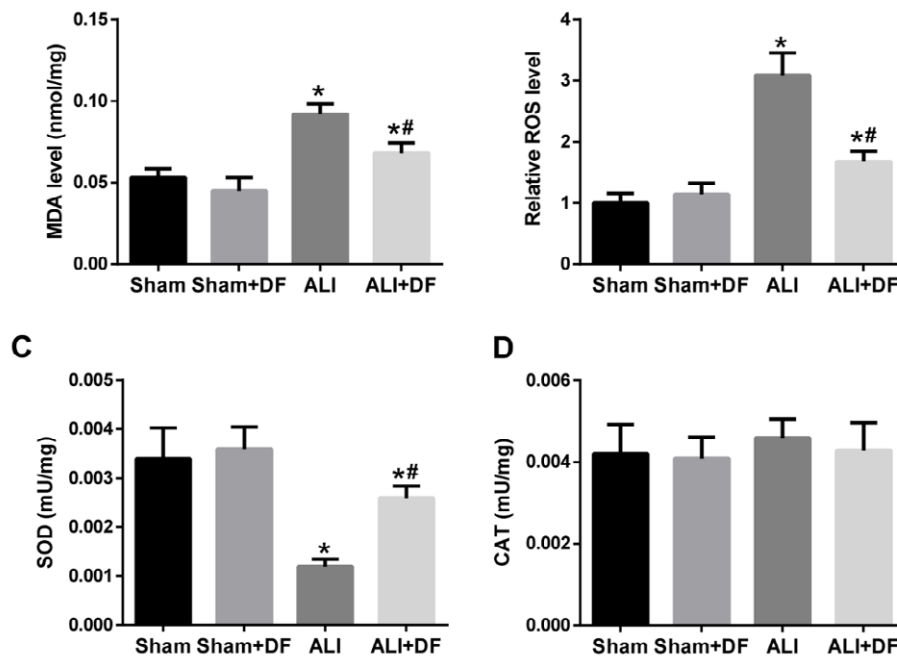


Fig. 2. DF attenuates the level of oxidative stress by rescue of SOD/CAT system in lung. **A)** Representative MDA levels in Sham, Sham+DF, ALI and ALI+DF groups. **B)** Representative ROS levels in Sham, Sham+DF, ALI and ALI+DF groups. **C)** Representative SOD levels in Sham, Sham+DF, ALI and ALI+DF groups. **D)** Representative CAT levels in Sham, Sham+DF, ALI and ALI+DF groups. * signifies vs Sham group and # denotes vs ALI group with statistical significance. ALI: Acute lung injury; DF: Dimethyl fumarate.

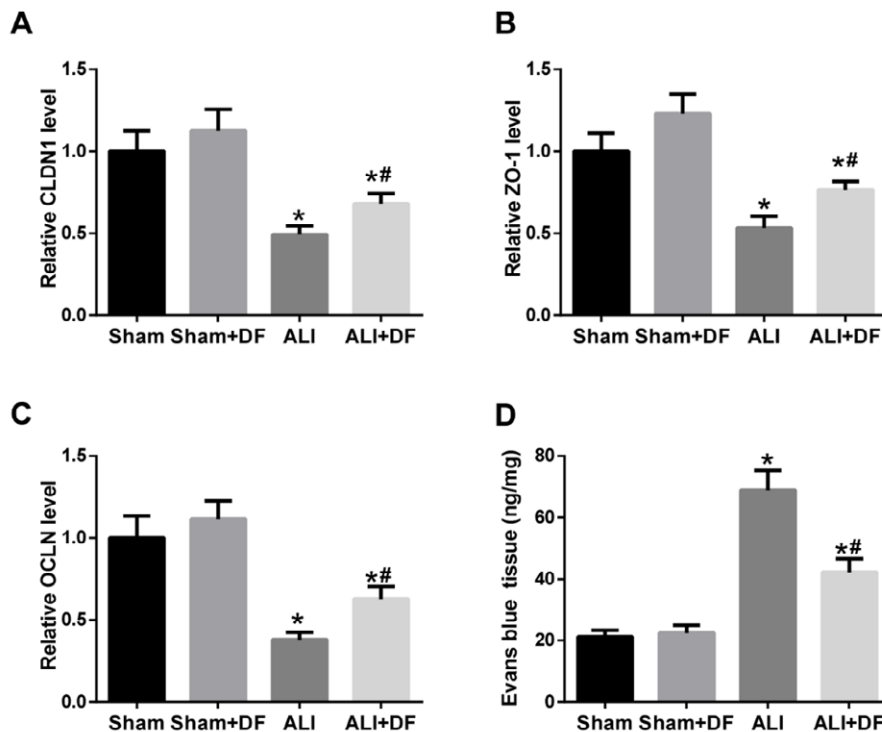


Fig. 3. Administration of DF prevents disruption of lung alveolar-capillary barrier in ALI. **A-C)** Representative RNA levels of CLDN1, ZO-1, and OCLN in Sham, Sham+DF, ALI and ALI+DF groups. **D)** Representative Evans blue tissue in Sham, Sham+DF, ALI and ALI+DF groups. * denotes vs Sham group, and # denotes vs ALI group with statistical significance. ALI: Acute lung injury; DF: Dimethyl fumarate.

DF treatment alleviates alveolar edema and maintains lung tissue integrity during ALI

The effects of DF treatment on alveolar edema and histological damage were then determined. The degree of edema was reflected by the lung wet/dry weight ratio. LPS-induced ALI significantly increased the wet/dry ratio of lung tissue, while DF decreased this ratio in ALI mice (Fig. 4A). Moreover, the histology of the lung was examined using HE staining, which showed thin alveolar walls and minority granulocytes in the Sham and Sham+DF groups, while LPS-treated lungs showed excessive macrophages and neutrophils in interstitial tissues and the alveoli, as well as an increased number of alveolar walls. However, these histological disorders were at least partially alleviated after treatment with DF (Fig. 4B). Taken together, the results indicated that DF treatment ameliorated lung edema and alleviated histological injuries during ALI.

DISCUSSION

The inflammatory response associated with neutrophil infiltration is a critical process aggravating tissue destruction after ALI. Rapid inflammatory cascades cause extensive lung injury, resulting in

ARDS, hypoxia, and even a high probability of respiratory failure. DF, an Nrf2 activator, has been shown to be a promising antioxidant in various neurological disorders. Our findings showed the inhibitory effects of DF on inflammation and oxidative stress in LPS-induced ALI, and further identified a negative effect of DF during neutrophil infiltration. Neutrophil infiltration is a typical biological event in acute inflammation, which increases the permeability of vessel walls. Our results showed that DF treatment decreased MPO expression in LPS-induced lung tissue, contributing to the inhibition of neutrophil infiltration. Importantly, a recent study reported that DF reduced neuroinflammation and apoptosis in the pentylenetetrazol kindling model in rats (8). The present study showed that DF decreased the expressions of classical inflammatory cytokines, including TNF- α , IL-1 β , and IL-6 and the level of iNOS in ALI mice. Acting as a specific antioxidant, DF conferred neuroprotection during intracerebral hemorrhage by activating the Nrf2/HO-1 pathway (9). In a similar manner, DF treatment of ALI mice inhibited expressions of MDA and ROS after LPS induction, which could be attributed to the effective rescue of decreased SOD expression. Moreover, LPS-induced ALI severely damaged tight junctions

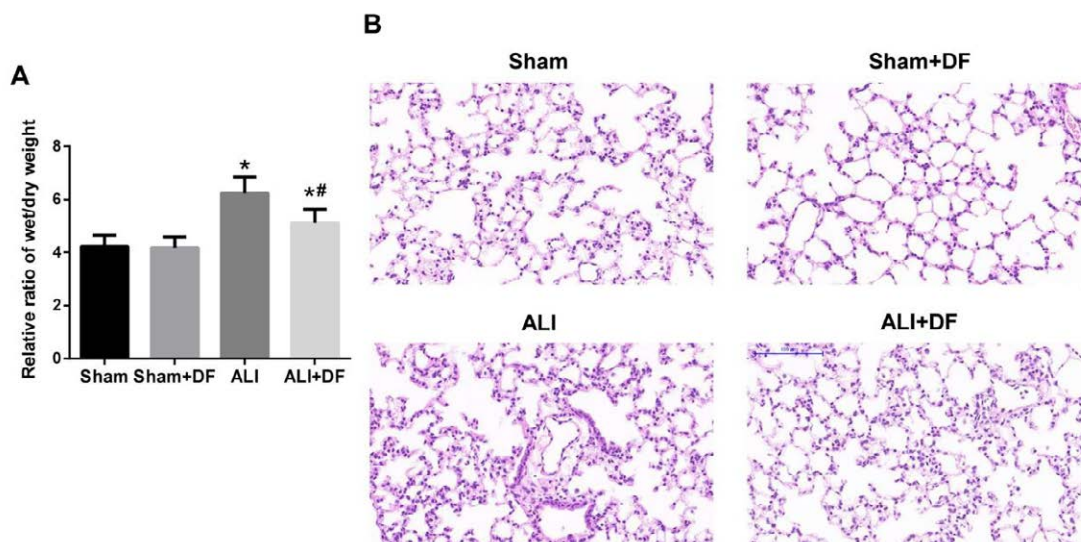


Fig. 4. DF treatment alleviates alveolar edema and maintains lung tissue integrity during ALI. **A)** Representative ratio of lung wet/dry weight in Sham, Sham+DF, ALI and ALI+DF group. **B)** Representative HE staining of lung tissue in Sham, Sham+DF, ALI and ALI+DF group, (magnification: 100 $\times$). * denotes vs Sham group and # denotes vs ALI group with statistical significance. ALI: Acute lung injury; DF: Dimethyl fumarate.

of endothelial cells and destroyed the alveolar-capillary barrier. Tight junction markers, including CLDN1, ZO-1, and OCLN, were reduced in ALI mice, while DF reversed these expressions. The Evans Blue positive lung tissues in DF-treated mice were decreased when compared with untreated mice, indicating that DF preserved the superior integrity of the alveolar-capillary barrier during ALI. Because of the above ameliorative effects, DF protected the alveolar-capillary barrier and mitigated alveolar edema in lung tissue, contributing to better histology of the lung. Previous studies suggested that DF therapy induced liver injury and diffuse dermatophytosis in multiple sclerosis patients (10, 11). The adverse reaction of DF concerning pharmaceutical metabolism and immunosuppression therefore requires clarification in further studies.

In summary, the present study showed that DF treatment reduced lung injury by inhibiting neutrophil infiltration, and alleviated oxidative stress by increasing SOD expression, resulting in decreased inflammatory mediators and oxidative products. DF therefore mitigated destruction of the alveolar-capillary barrier and decreased lung tissue edema, resulting in the protection of histological integrity during ALI.

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LETTER TO THE EDITOR

Individuals with temporomandibular disorders exhibit psychological impairment based on a case control nested studyA. Monaco¹, R. Cattaneo¹, L. De Simone¹, P. Smurra², F. Adib³, D. Pietropaoli¹ and E. Ortu¹¹MeSVA Department, Dental Unit, University of L'Aquila, L'Aquila, Italy; ²Private practice, Torino, Italy; ³Myotronics-Noromed, Inc., Kent, WA, USA*Received May 5, 2021 – Accepted July 28, 2021*

To the Editor,

Chronic Myofascial Temporomandibular disorders (CM TMD) are currently considered a multifactorial disorder characterized by complex pathogenesis. Central disturbance to the regulation of descending modulation systems of autonomous signals has been suggested as a possible etiology (1). Pain and arousal involve many systems controlling signal transmission between the central nervous system and the periphery, and have also been investigated as possible etiologies (2).

In these disorders, somatic signs and symptoms (muscular, articular, and fascial connective tissue) are related to a set of psychosocial variables, which can be verified through psychometric tests, and evaluated with a widely used, broad descriptive multiaxial classification (3). Although an evolution from descriptive classification to physio-pathogenetic-based characteristics of the disorder is preferred, it is difficult to construct a simple framework that improves the prognosis of the treatment and the best treatment based on the identified classification criteria.

Thus, it has recently been proposed to include many disorders in the bio-psycho-social model of TMD, especially those characterized by chronic pain. For chronic TMD, the development of areas

where there is integration between the body and brain justifies the pathogenesis of disorders characterized by chronic pain (4). Most of these disorders are known as Central Sensitization (CS) syndromes, which have high comorbidity, and include TMD. In this group of disorders, psychometric scales are used to assess psychological components and CS. Out of existing scales, the DASS42 (**Depression Anxiety Stress Scale 42**) and the CSI (Central Sensitization Index) are self-assessing and allow rapid analysis by operators (5-6).

This study aimed to investigate the distribution of CS characteristics in a group of chronic myofascial TMD, to associate the psychometric characteristics that emerged from DASS42 to CSI characteristics, and to evaluate their possible correlation with the specific clinical results. This method could help the dentist to make a differential diagnosis of TMDs.

MATERIALS AND METHODS

This study was carried out in accordance with the Declaration of Helsinki, and all subjects provided written informed consent. Twenty-eight subjects classified as chronic myofascial TMD, according to RDC/DC criteria, were progressively enrolled from January 2018 to January 2019, as part of a study group that was followed for one

Key words: temporomandibular disorders; depression anxiety stress scale; Central Sensitization Index; transcutaneous electrical nerve stimulation

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year. A control group, homogeneous with the TMD group for age and sex, was selected from subjects that attended the clinic for routine dental check-ups or for professional hygiene, during the same period. The criteria for inclusion in the control group were the absence of signs or symptoms in the temporomandibular area and diseases characterized by chronic pain (e.g., non-specific back pain, migraine and musculo-tensive headache, irritable bowel, and recurrent cystitis). All patients were advised not to take pain relievers for the 15 days prior to the study.

Both groups were given CSI and DASS42 scales, with the specification that the investigators did not know which group the collected data came from. The scales were compiled in a room in which the patient, who underwent the self-assessment test, was alone. The average compilation time for each scale was approximately 5 min. To promote anonymity, an alphanumeric abbreviation was applied to each subject, which was kept by a third operator, who also took care of the collection and numerical evaluation of the scales. The assessment and assignment of the numerical scores for both scales lasted about 3–4 min per subject (1 min for the CSI and 2–3 min for the DASS42).

The diagnosis was made by a clinician based on RDC/DC criteria. The same clinician subsequently passed the cases to the staff, and each subject entered the clinical routine. This routine involved the creation of a bite using the information provided by the neuro-myofascial technique for establishing the bite. The use of ULF TENS was discussed, and analyzed based on existing publications (7). The subjects were visited and checked each month, and again in January 2019. A clinical check-up was performed at the end of the treatment, and the clinical evaluation of the scale was compiled by assigning a score from -3 to +3.

DASS 42

DASS42 was used to quantify the values of depression, anxiety and stress for evaluation. It is typically used in studies evaluating the quality of life, characteristics associated with particular population samples, and health events (8). DASS42 was therefore used here to predict general and specific outcomes characterizing the psychological component of TMD (9). The principal purpose of DASS in a clinical setting is to clarify the locus of emotional disturbance. DASS is based on a dimensional, rather than categorical, conception of

psychological distress. The scores emphasize the degree to which a person is experiencing symptoms, rather than diagnostic cutoff points.

CSI

CSI has been proposed to evaluate the signs and symptoms of disorders characterized by CS. CSI is reliable and sensitive to the severity of CS, and the Italian version of this scale has been previously validated (10). CSI was correlated with pain in different pathologies, also in association with DASS42 (11). Combining these two scales for the same subjects provides a valid and quick way of obtaining information on the influence of the psychological state in disorders characterized by CS. Because both scales can be used to “predict” the outcome, they could be used simultaneously to detect a possible relationship between CSI and psychological characteristics (DASS42) in subjects affected by chronic myofascial TMD.

Statistical analysis

Due to the limited sample size, a more conservative non-parametric statistical approach was adopted in this study. Statistical differences between continuous variables were evaluated by Wilcoxon’s test. The risk of TMD was estimated by generalized linear logistic multivariate models. Specifically, TMD was used as the independent variable, while the dependent variables were CSI, depression, anxiety, stress, age and gender. The risk of TMD over the controls was estimated in terms of odds ratio (OR) and 95% confidence intervals (95% C.I.). The collinearity of the covariates was previously tested. P value significance was set at $p < 0.05$. The statistical analysis and graphs were carried out using R software (v.4.0.2).

RESULTS

Table I shows the results and the statistical significance of the comparison between the TMD group and control group. Analysis of the distribution and recruitment of control group subjects showed that the sample was statistically homogeneous in terms of age. All CSI and DASS variables showed significant differences in the two groups, with TMD patients having higher scores compared to control subjects. The only exception was the S component

of DASS which, although greater in the TMD group, did not show any significant values. The most significant values were represented by CSI (17.60 vs 38.67; $p = 2.5e-07$) and the sum of DASS and CSI (41.37 vs 69.60; $p = 7.8e-05$).

The index of the clinical results was positive (1.46), indicating a generally favorable treatment outcome. Values fluctuated between non-significant results (+1) and good results (+2). We also analyzed the correlation of epidemiological variables, self-assessment scales, and clinical results. The two psychometric scales had no reciprocal correlation. Only TMD subjects exhibited low (0.42 between CSI and DASS total) or ultra-low correlations (0.37-0.36, CSI with D and S), with no other correlation being detected in control subjects. The correlations were evident within the sub-categories of DASS. Thus, the correlations were stronger in control subjects compared to TMD subjects.

When considering the 28 TMD subjects, 18 subjects (64%) obtained results of +3 and +2, three subjects (10%) obtained a result of +1 on the clinical evaluation scale, four subjects (14%) had a null (0) or slightly negative (-1) result, and three subjects (10%) had a negative (-3) result, which is considered a crisis in the dentist/patient relationship (Fig. 1).

The distributions of the CSI values, DASS42 variables (D, A, S) and age were evaluated. CSI was

distributed very differently in the two groups, with a cut-off value of about 30. Similarly, the DASS A and DASS S components were also distribution in a way that separated, at least a part, of the subjects in the two groups, but to a lesser extent. The cut-off for DASS A and DASS S was set to 8 and 11, respectively. In the TMD subjects, DASS values exhibited bimodal behavior, with a peak typically occurring within the general values and a second, less pronounced, peak occurring in the pathological area. One value that was of clinical significance and concern is the DASS D component; unlike the second peaks of the other variables (which were slightly beyond normal values), the DASS D component had a second peak (18) in an area that is considered clinically relevant (14-20 “moderate”).

Finally, while the distribution of the CSI above the cut-off value seemed to collapse in the control subjects, psychometric variables were present for clinical values within the control group. Consequently, clinical disturbances concerning the CSI sphere might not be presented; instead, significant clinical values of changes affecting the psychological sphere were presented. The same phenomenon was obtained for TMD subjects; the CSI distribution score was almost always above the clinical threshold, whereas subjects without significant deviations from the score considered

Table I. Summary table of the results

	CTRL	TMD	P (Wilcoxon test)
Age	43.53 (14.37)	37.82 (13.56)	0.15
CSI	17.60 (7.72)	38.67 (14.35)	<u>2.5e-07</u>
DASS D	5.85 (7.04)	8.42 (6.65)	<u>0.043</u>
DASS A	7.71 (5.09)	10.28 (4.95)	<u>0.049</u>
DASS S	9.25 (5.66)	12.21 (9.25)	0.092
DASS tot	23.44 (15.60)	30.92 (23.44)	<u>0.032</u>
DASS tot CSI sum	41.37 (18.64)	69.60 (25.219)	<u>7.8e-05</u>
Result of dental treatment	1.46 (2.04)		

“normal” were present in the psychometric scales. The distribution of control and TMD subjects scores (CSI and DASS42) was higher than what is considered non-pathological or pre-clinical. Thus, scores lower than the borderline values of CSI and of single DASS42 variables were considered equal to zero. For higher values, the “positive” subjects for that value were evaluated. Subsequently, the positive subjects were counted for the observed variable, and the control and TMD subjects were divided into positive and negative CSI (CSI and no CSI). In each of these groups (CSI and no CSI), positive DAS variables were checked.

For TMD subjects, about 33% had no clinical scores for CSI. The DASS42 scores for 37% subjects did not change. Approximately 10–15% of subjects that were classified as chronic TMD did not present changes to CSI and DAS. In the “TMD no CSI” category, 37% of patients had positive A values. This means that about 10–15% of TMD subjects probably had problems due to anxiety. The other single DAS variables (D, 0%, S, 0%) or combined variables (AS 13%, DA 13%, DAS 0%) were poorly represented in this group, and represented approximately 6% of the total TMD sample. In the TMD CSI group,

50% of patients simultaneously presented altered DAS parameters, while 32% (approximately 21% of the total TMD group) of this group had no altered DAS parameters. The remainder of patients were distributed fairly evenly among DA, AS, and A. Overall, the CSI group represented about 15% of the TMD group.

Only three subjects in the control subjects in the CSI-noCSI categories had positive CSI; consequently, it difficult to draw any conclusions. The “Control no CSI” subjects had a similar distribution to the corresponding “TMD no CSI;” specifically, the two most represented categories were “no DAS” (52%, which represented 45% of the entire CTRL sample) and A (about 22%, which represented 20% of the total CTRL sample). In contrast to TMD subjects, in this group, component D was presented in 9% of “Control no CSI” subjects, with “depression” being a problem of clinical relevance in about 8–9% of the control population overall.

The distribution of the psychometric components evaluated in DASS42 revealed that a high number of subjects (37% “TMD no CSI” and 32% “TMD CSI”, representing more than 30% of the entire TMD group) presented no symptoms (depression, anxiety

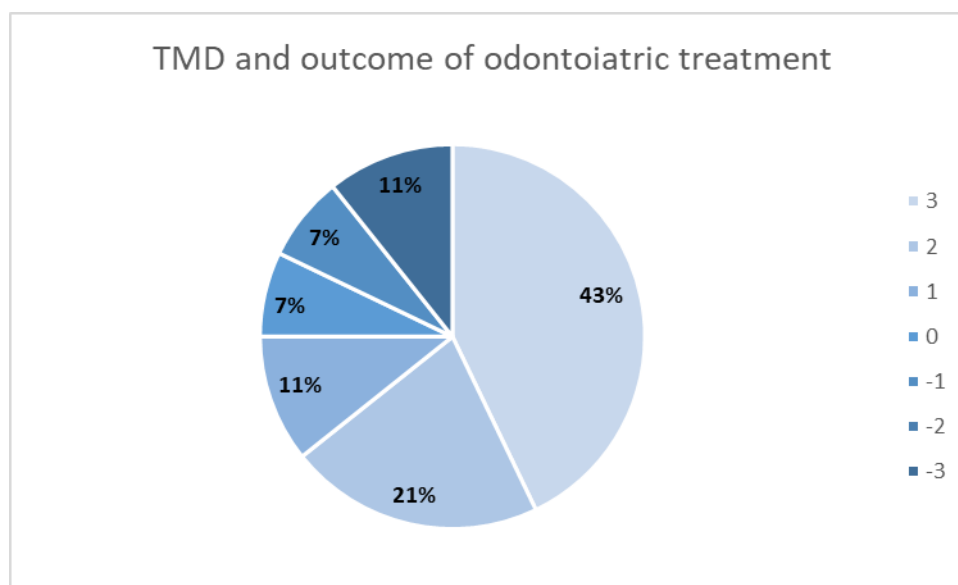


Fig. 1. Pie chart showing the subjects divided according to clinical treatment outcome. Color gradient from lightest to darkest in descending order: +3 to -3. The clinical outcome of treatment -2 is not presented as no subject had this outcome.

or stress). Furthermore, when CS was present in 50% of cases, all of the DASS42 components were entirely altered, indicating the general involvement of the psychological sphere. These psychological characteristics were not specific to TMDs, as they appeared to belong to the Control group.

Based on the assimilated information, a generalized logical model was developed that considered the dependent variable belonging to the control group or to the TMD group according to the series of covariates represented by the considered variables (e.g., CSI, D, A, S, age, and sex). The data demonstrated that variation in CSI was significant. In particular, any increase in the CSI score involved a 23% increase in the risk of belonging to the TMD group. The model confirmed that observed when evaluating the distribution of the variable data; specifically, CSI was an effective parameter for including chronic TMD subjects within the group of so-called Central Sensitization Syndromes. The presence of altered psychometric variables in the test group seemed to be a clinical phenomenon that prevented patients with actual chronic TMD disorder being distinguished from non-real disorders.

DISCUSSION

After the data analysis, the authors confirmed that:

- The chronic TMD group presented CSI and DASS42 values that were significantly higher compared to those of the Control group;
- DASS42 Index scores could not be used to distinguish the TMD group from the Control Group clearly. The CSI index score could be used to distinguish the TMD group from the Control Group.
- The CSI Index scores were not correlated with the DASS42 scores, as a single variable of each index or as the sum of the variables D, A, and S.
- When considering the clinical results, it was not possible to select any psychometric variable or CSI that could be used to predict the result

The TMD group had central sensitization values that were higher than those of the control group. Existing publications demonstrated that, in the pathogenesis of TMD, there is a form of disturbance

when modulating the CS pathways and autonomous control. The present study showed that TMDs have a form of dysregulation at the level of the PAG-RVM circuit (Peri Aqueductal Gray - Rostral Ventral Medulla), which was evident during the activation of this circuit under stress, rather than tonically in the “basic” condition (6). Some TMD subjects appeared to be predisposed to respond to “pre-contact,” anatomical, or functional alterations at the level of the stomatognathic system, triggering hyperactivity. Thus, peripheral sensory input (i.e., occlusal, muscular, articular, and trigeminal) might be processed differently at the central level in different subcategories of subjects, including TMD subjects. The relationship between success and failure determined the average result of therapy (1.45) which was sufficient; however, excellent clinical results were obtained for most subjects, which were offset by poor or bad results in others. This study confirmed a recent analysis of therapeutic results in TMDs; however, the question of which therapy is adequate remains open, along with whether there is therapeutic specificity for TMD (12).

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LETTER TO THE EDITOR

Efficacy of early oral and perioral physiotherapy on feeding autonomy in preterm infants: results of randomized controlled trials

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To the Editor,

Preterm infants can obtain oral feeding late; it is estimated that around 30–40% of them may have oral feeding problems (1) and it is an important outcome for the timing of discharge from the hospital (2). Several treatments have been proposed to facilitate the achieving of Full Oral Feeding (FOF) and reduce hospitalization times.

A blind, randomized clinical trial (Trial registration: UMIN000026533) was performed in the Neonatal Intensive Care Unit (NICU) of University Polyclinic Foundation A. Gemelli IRCCS, Rome, Italy, from January 2016 to July 2017. Primary outcome was timing to reach full oral feeding (FOF). Secondary outcomes were hospitalization time, gain of body weight from birth to discharge, and Preterm Oral Feeding Readiness Assessment Scale (POFRAS) (3) score assessment. The inclusion criteria were gestational age GA < 32 weeks and/or birth weight < 1500 g. Newborns that presented congenital malformations and genetic syndromes were excluded. After birth, the neonates were randomized into an experimental group (newborns

undergoing the oral/perioral facilitation program; the physiotherapists performed a physiotherapy program of a codified series of oral and perioral stimulations 3 times a day for 6-7 minutes for six days/week) (4) and a control groups (usual care).

Physiotherapists and parents were aware of the group assignment, but neonatologists collecting data and the statistician were blinded. The study had no influence on the attending physician's clinical and nutritional decision nor on the infants' hospital discharge date. The criteria for discharge were reaching weight > 1600 g, resolution of the acute pathology and reaching full oral feeding without problems for at least 48 hours. Written informed consent was obtained from the parents of the newborns for their participation in the study. One hundred and five newborns were enrolled, and their characteristics are described in Table I. The groups were homogeneous at baseline. Oral feeding was attained significantly earlier in the experimental group (n. 53; GA: 28.8±2.2 weeks) than in the control group (n. 52; GA 29.5±2.0 weeks) (Table II)

Key words: preterm infant, oral feeding, early physiotherapy, outcome

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DISCUSSION

Various studies suggest that oral stimulation improves in preterm infants sucking and feeding skills and shortens hospitalization, time to achieve feed autonomy, and the huge cost of preterm care.

Evidence shows that preterm infants who received oral and sensory-motor intervention demonstrate more advanced nutritive sucking and suck-swallow-respiration coordination (5). Our results showed that an early structured intervention accelerated the transition from tube to FOF in preterm infants. This

Table I. Baseline characteristics of premature infants in both groups

Variables	Experimental Group N=53	Control Group N=52	P value
Gestational age in weeks	28.8±2.2	29.5±2.0	0.23
Birth weight in grams	1146±365	1148±368	0.89
Weight on discharge (g)	2455.5 (±754)	2456 (±744)	0.91
Male sex n (%)	23 (43.4%)	23 (44.2%)	1
Caesarean delivery n (%)	41 (77.4%)	47 (90.4%)	0.68
APGAR score 1 min	6.7 (±1.8)	6.6 (±1.7)	0.9
APGAR score 5 min	8.1 (±1.2)	8.1 (±1.1)	1

Data are expressed as mean±SD. n (%) or median

Table II. Comparison of clinical outcomes between both groups

Outcomes	Experimental Group	Control Group	P value
FOF (PMA)	35.4 (± 2.8)	36.7 (±3.4)	0.013*
POFRAS T0	17.6 (±4.9)	18.1 (±5.6)	0.58
POFRAS Tf	32.4 (±2.8)	31.7 (±3.1)	0.09
POFRAS T0-Tf difference	15 (±6)	13.6 (±6.1)	0.11
Weeks of assessment (wk)	7.5 (±3.9)	7.4 (±3.7)	0.97
Weight Z-Score difference	-0.9 (±1)	-0.71 (±1.3)	0.78
Total hospital days (d)	61.7 (±45.8)	62.1 (±47.1)	0.87

Data are expressed as mean±SD; FOF: full oral feeding; PMA: postmenstrual age; wk: weeks; d: day

observation corroborates several studies that tested similar methods of oral stimulation in premature babies (6-7). Compared to the other studies, in our work we considered newborns with an extremely low birth weight and the treatment was started very early, on average within the first week of life, as soon as the babies were clinically stable and not sedated for need of invasive respiratory assistance. Moreover, unlike the other experiences, in the present study, the start of treatment was independent from the beginning of enteral feeding. We did not have a significance impact in terms of weight gain and length of stay. These findings are similar to some previous studies (8-9). Instead, it is in contrast with other Authors, who reported that the oral stimulation protocol decreases length of hospital stay (7). These results may have been influenced by different factors. Usually every hospital follows its own discharge criteria that can be different from center to center. Moreover, in our study we have also included preterm infants with major morbidities. This can be a strong point of the study but can also lead to confounding factors influencing the length of hospital stay. In this regard, a multicenter trial it would be advisable. Rocha et al. (7). found a significant effect on the hospital stay but not on the weight at discharge and the weekly weight gain, respectively. Our results confirmed the validity of POFRAS in very low birth weight infants and in infants with major morbidities. An early oral facilitation program accelerates the achievement of the FOF in preterm infants. The infant with necrotizing enterocolitis, bronchopulmonary dysplasia or severe neurological injury could benefit from the program and should be the subject of dedicated studies. The treatment did not significantly influence weight gain or time of hospital stay although to confirm this data it would be necessary to conduct further multicenter studies that have homogeneous definition of these outcomes.

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LETTER TO THE EDITOR

Knockdown of Leucine-rich pentatricopeptide repeat motif-containing protein suppresses the proliferation and migration of ECA-109 cells

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To the Editor,

Esophageal carcinoma (EC) is one of the most common carcinomas worldwide, and the sixth most common cause of carcinoma-associated deaths (1). Esophageal squamous cell cancer (ESCC) remains the predominant histological subtype, accounting for more than 80% of ECs (2). Despite progress in early detection and treatment, the prognosis of EC is far from satisfactory. Metastasis is the main cause of death and poor prognosis in EC patients (3). In recent years, an increasing amount of research has focused on identifying biomarkers and targets of EC to develop new therapeutic strategies.

Mitochondria play an important role in cell survival. At present, it has been found that carcinogenic factors or reactive oxygen species produced in the process of cell metabolism can affect the stability of the mitochondrial genome. Alterations in the mitochondrial apoptosis pathway can affect the body's ability to remove malignant transformed cells, resulting in tumor progression. Leucine-rich pentatricopeptide repeat motif-containing protein (LRPPRC) is a 130-kDa RNA-binding protein that is primarily located within the

mitochondrial inner membrane, where LRPPRC can regulate mitochondrial protein expression at both the transcriptional and translational level. LRPPRC also plays a vital role in regulating mitochondrial function (4). Recently, Zhou et al. discovered that LRPPRC is highly expressed in prostate carcinoma and promotes tumor proliferation and metastasis (5). Prior studies have established that LRPPRC is highly expressed in ESCC (6), however, whether LRPPRC is related to the occurrence and development of EC is unknown. In this study, we are the first, to our knowledge, to report the effect of LRPPRC knockdown on cell viability, proliferation, migration, and invasion of EC cells using an *in vitro* model.

MATERIALS AND METHODS

Cell culture and transfection

ECA-109 cells, a human EC cell line, were obtained from Procell Life Science and Technology Co. Ltd. (CL-0077; China), and TE-1 (ZQ0235) and KYSE-150 (ZQ0235) cell lines were obtained from Cell Research (China). Cells were maintained in RPMI-1640 medium (Gibco; Life Technologies Corporation) supplemented

Key words: esophageal carcinoma; LRPPRC; migration

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with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) and 1% penicillin (Invitrogen, CA, USA) in a 5% CO₂ humidified incubator at 37°C. Small interfering RNAs (siRNAs) were then transfected with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc. MA, USA) according to the manufacturer's guidelines. The LRPPRC siRNA was purchased from JTS Scientific. The siRNA sequences were as follows: siRNA-1: 5'-GGAGGAAGCAAACAUAUUAATT-3', siRNA-2: 5'-CCAGUUACAGAGGCAGUAUTT-3', and siRNA-3: 5'-CCAUCUCGCUGCAGUCUAUTT-3'. The sequence of the negative control (NC) siRNA was: 5'-UUCUCCGAACGUGUCACGUTT-3'. Cells that were not transfected served as the control group.

Western blot analysis

Cultured cells were washed with ice-cold phosphate buffered saline (PBS) and then lysed on ice in RIPA lysis buffer (Sigma, MO, USA). Protein concentration was calculated using the BCA kit (Thermo Fisher Scientific, Inc.). Subsequently, 40 µg protein were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking in 5% nonfat milk in ice-cold PBS for 1 h, membranes were incubated with primary antibodies targeting LRPPRC (1:2,000; 21175-1-AP; Proteintech), E-cadherin (1:500; WL01482; Wanleibio), Nm23 (1:1,000; AF0222; Affinity), vascular endothelial growth factor (VEGF) (1:500; WL00009b; Wanleibio), and β-actin (1:1,000; WL01845; Wanleibio) at 4°C overnight. Next, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody (1:5,000; WLA023; Wanleibio) at room temperature. Signals were detected using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) per the manufacturer's instructions.

Real-time fluorescence quantitative polymerase chain reaction assay (RT-qPCR)

Total RNA was isolated from cells using an ultrapure RNA extraction kit (Wanleibio, China). The RNA was reverse transcribed into cDNA. LRPPRC expression was identified using SYBR Premix Green I (Solarbio, USA). The primers were synthesized by GENSCRIPT (Nanjing, China) as follows: LRPPRC:

F: 5'-GAGAGATGCCGGAATTGAGC-3', R: 5'-CTCGGACTTCTCCACCTTCT-3'. β-actin served as the internal control: F: 5'-GACCTGACCTGCCGTCTAG-3', R: 5'-AGGAGTGGGTG7CGCTG T -3'. Relative gene expression was measured using the 2^{-ΔΔCt} method.

MTT assay to measure cell viability

Cells were transfected with LRPPRC siRNA-3 or control siRNA after being plated in 96-well plates for 24 h (1 × 10⁴ cells/well). Cells treated with 0.9% NaCl were used as the control group. After 12, 24, 36, 48, and 60 h of incubation at 37°C and 5% CO₂, 10 µl of the MTT reagent (Sigma, USA) was applied to every well and the cells were cultured at 37°C for 2 h. The optical density (OD) value was calculated at 570 nm using a microplate reader (Biotek, USA).

Transwell assay

Transwell chambers (Corning, Tewksbury, MA, USA), treated with or without Matrigel (BD Bioscience, San Jose, California), were prepared to assay cell invasion or migration. The upper chambers were coated with 100 µl of 5 µg/ml Matrigel. Cells suspended in 0.2 ml serum-free medium were added to the upper wells (3 × 10⁴ cells/well) and 0.8 ml medium with 10% FBS was added to the lower chamber. The chambers were maintained at 37°C overnight. Cells penetrating through the chambers (with 8-µm-pore size filters) were observed using crystal violet staining, and the invasive cells were visualized (magnification, x200) using a light microscope (Olympus IX53, Japan). The protocols for the migration and invasion assays were similar, except that the Transwell chambers were not coated with Matrigel for the migration assay.

Wound healing assay

ECA-109 cells were plated onto a 6-well plate (5 × 10⁵ cells/well). Once the cells reached 70-80% confluence, a scratch wound was made in the center of the well using a 200 µl pipette tip. Cells were maintained for 24 h in serum-free medium at 37°C and then imaged (magnification, x100; Olympus IX53). Wound closure was calculated using Image J software (version 1.5, National Institutes of Health, Bethesda, Maryland, USA).

Cell cycle analysis

ECA-109 cells transfected with either control or

LRPPRC siRNA-3 for 24 h were collected and fixed in 70% ice-cold ethanol for 2 h. The cells were centrifuged at 1,000 g at 4°C for 5 min, treated with 100 µl RNaseA for 30 min at 37°C, and re-suspended in 500 µl propidium iodide (WLA010; Wanleibio) at 4°C for 30 min. The labeled cells were measured utilizing the BD Accuri C6 flow cytometer (BD Biosciences).

Statistical analysis

The data were acquired from triple independent assays and are represented as mean ± standard deviation. Statistical analyses were carried out using SPSS version 21.0 (SPSS, Inc.). The variation among groups was examined using one-way analysis of variance (ANOVA), after which the least-significant difference (LSD) post hoc test was conducted. $P < 0.05$ represents statistical significance.

RESULTS

LRPPRC knockdown suppresses proliferation of EC cells

To investigate the role of LRPPRC in EC progression, we used siRNA to knockdown LRPPRC expression in EC cell lines. Following knockdown, we measured LRPPRC protein expression in a panel of EC cells. We observed the highest knockdown efficiency in the ECA-109 cell line and therefore used this cell line in all subsequent experiments (Fig. 1A). Three siRNA sequences were transfected into ECA-109 cells, and using RT-qPCR we found that LRPPRC mRNA levels were significantly reduced by siRNA-1 ($P < 0.05$) and siRNA-3 ($P < 0.05$) (Fig. 1B). siRNA-3 had the greatest knockdown efficiency compared to the NC ($P < 0.05$; Fig. 1C) and was therefore used to determine the effects of LRPPRC knockdown on cell proliferation. Using the MTT assay, we found that siRNA-3-mediated LRPPRC knockdown markedly suppressed ECA-109 cell proliferation (Fig. 1D).

LRPPRC knockdown blocks cell cycle in ECA-109 cells

Since we found that LRPPRC knockdown inhibited ECA-109 cell proliferation, we next sought to determine whether LRPPRC knockdown affected cell cycle in ECA-109 cells. As shown in Fig. 2, the

percentage of cells in the G2/M phase of the cell cycle was increased after siRNA-3-mediated LRPPRC knockdown ($P < 0.05$). The ratio of cells that transitioned from G0/G1 to S phase was decreased, although this change was not significant ($P > 0.05$). These data indicate that LRPPRC promotes cell proliferation by regulating cell cycle progression in ECA-109 cells.

LRPPRC knockdown decreased the invasion and metastasis of ECA-109 cells

We evaluated the effects of LRPPRC knockdown on the invasive and migration ability of ECA-109 cells using the wound-healing and Transwell assays. siRNA-3-mediated LRPPRC knockdown significantly decreased the metastasis of ECA-109 cells compared to NC and control cells ($P < 0.05$, Fig. 3A). The invasive ability of ECA-109 cells was also significantly inhibited following siRNA-3-mediated LRPPRC knockdown ($P < 0.05$, Fig. 3B). We next measured protein expression of VEGF, E-cadherin, and Nm23 following LRPPRC knockdown in ECA-109 cells. As shown in Fig. 3C, LRPPRC knockdown markedly down-regulated expression of the pro-metastatic marker VEGF, and up-regulated expression of the inhibitors of metastasis, E-cadherin and Nm23. Taken together, these data indicate that LRPPRC plays a role in the invasion and metastasis of EC cells by modulating several metastasis-associated proteins.

DISCUSSION

LRPPRC is generally regarded as an effective tumor marker in several cancers (5), however, it is unclear whether or not LRPPRC is related to EC development. In the current study, we explored the biological role of LRPPRC in modulating EC progression and the underlying mechanisms. We found that LRPPRC knockdown not only inhibited proliferation by blocking the G2/M phase of the cell cycle, but also inhibited the migratory and invasive properties of ECA-109 cells by modulating the protein expression of several metastasis-related markers. To the best of our knowledge, this is the first study to establish that LRPPRC plays a role in EC pathology.

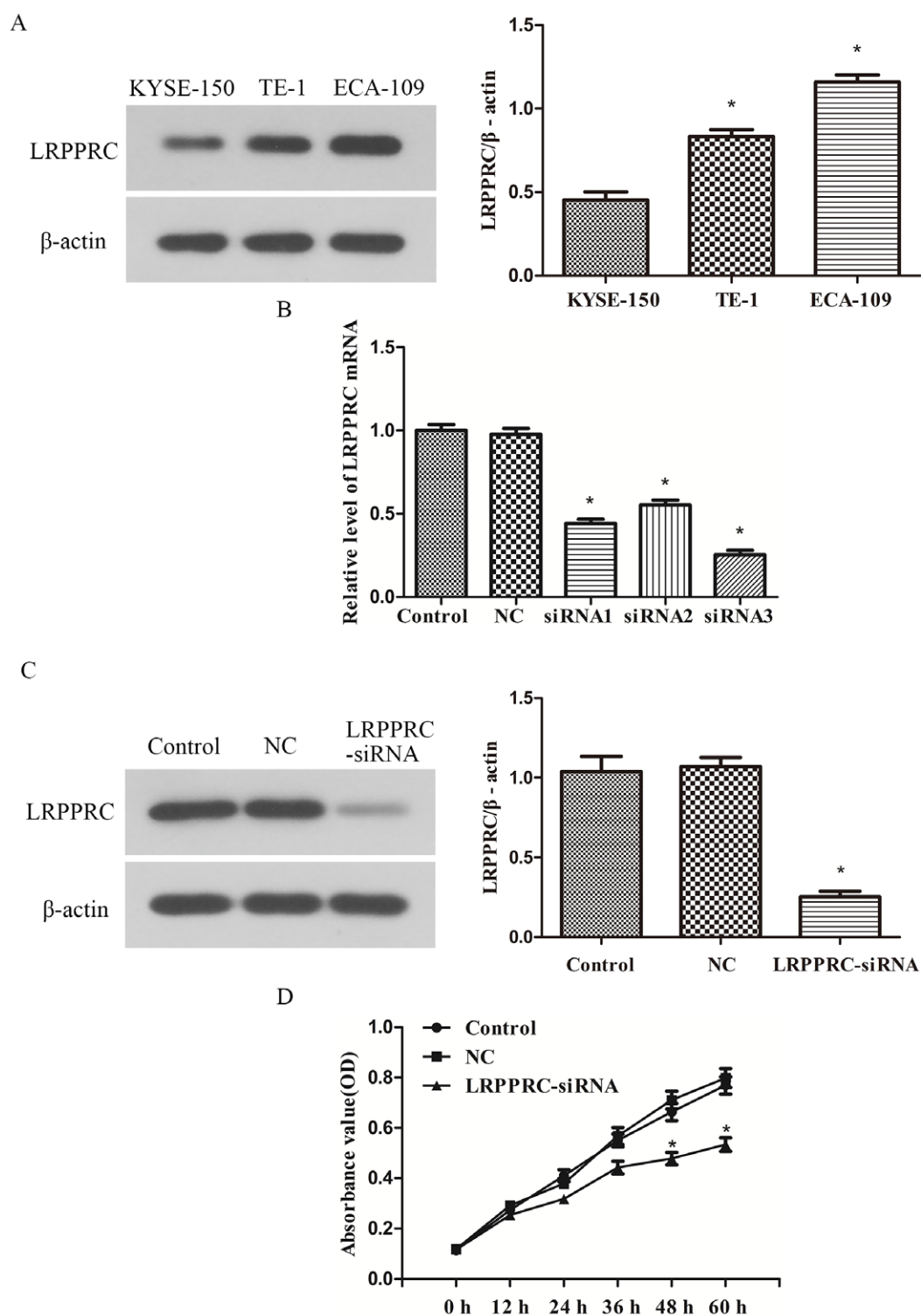


Fig. 1. LRPPRC knockdown inhibits EC cell proliferation. **A)** LRPPRC protein expression in EC cell lines was determined using western blot analysis. β -actin was used to normalize LRPPRC expression. **B)** LRPPRC mRNA levels in ECA-109 cells were measured using RT-qPCR. β -actin was used to normalize LRPPRC mRNA levels. **C)** siRNA-mediated LRPPRC knockdown efficiency was determined using western blot analysis. * $P < 0.05$ vs control. **D)** Knockdown of LRPPRC protein expression inhibited ECA-109 proliferation as determined using the MTT assay. LRPPRC, Leucine-rich pentatricopeptide repeat motif-containing protein; siRNA, small interfering RNA; NC, negative control; OD, optical density.

Metastasis is a significant cause of ESCC-related mortality. In addition to metastasis, angiogenesis is an important prognostic factor in ESCC patients. Angiogenesis furnishes growth nutrients and excretes metabolism products that promote metastasis (7). VEGF is the most pro-angiogenic stimulus in cancer angiogenesis and metastasis (8), and E-cadherin is an important biomarker that regulates adhesion and mobility of epithelial cells (9). Down-regulation of E-cadherin can promote epithelial cells to invade and metastasize, and are considered to be important for epithelial-to-mesenchymal transition (EMT) in cancer (10). Nm23 is a protein that encodes nucleoside diphosphokinase and is regarded as a suppressor gene in cancer metastasis (11). To the best of our knowledge, there are no reports regarding the interaction between LRPPRC and the pro-metastatic gene VEGF, as well as the metastasis suppressors E-cadherin and NM23, in EC. We demonstrated that LRPPRC knockdown inhibits the invasive and

metastatic nature of EC cells, likely by decreasing VEGF and increasing E-cadherin and Nm23 expression.

In conclusion, the present study revealed that LRPPRC plays a role in promoting EC progression by promoting cell metastasis. LRPPRC may be a possible biomarker and therapeutic target in EC. However, since we only investigated the effects of LRPPRC in one EC cell line, future studies are needed to further confirm the role of LRPPRC in multiple EC cell lines, as well as in *in vivo* models.

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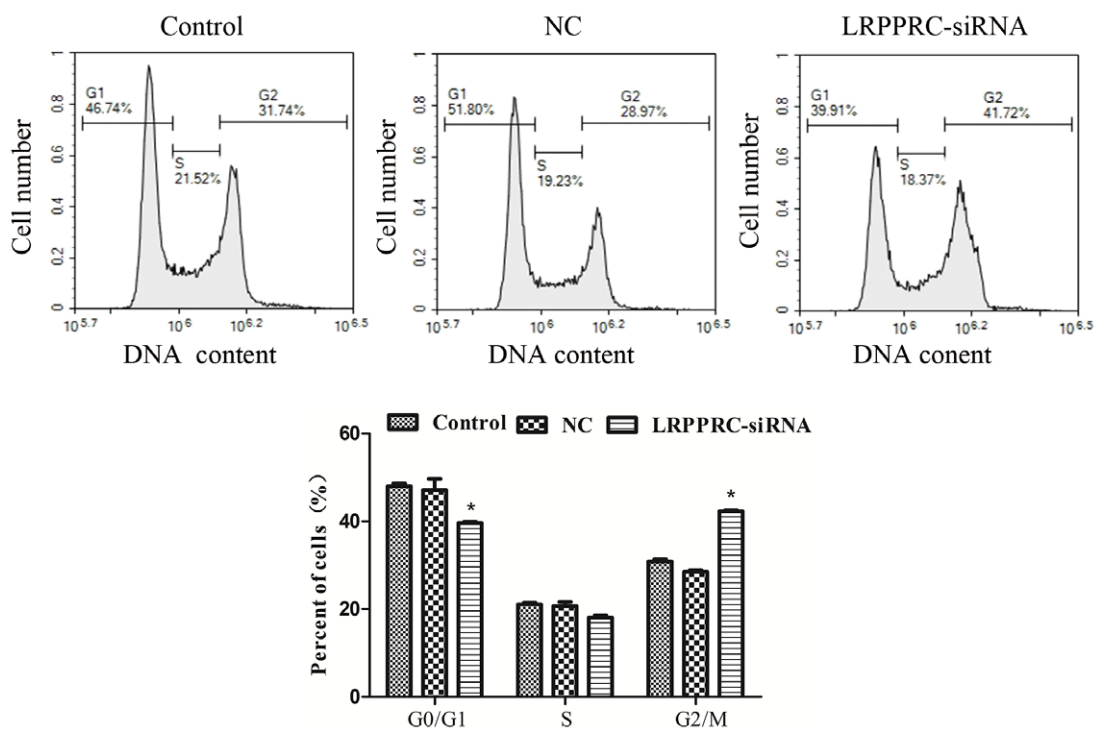


Fig. 2. LRPPRC knockdown induces G2/M cell cycle arrest in ECA-109 cells. ECA-109 cells transfected with LRPPRC siRNA-3 or NC for 48 h were collected and the cell cycle profile was measured using flow cytometry. * $P < 0.05$ vs control. LRPPRC, Leucine-rich pentatricopeptide repeat motif-containing protein; siRNA, small interfering RNA; NC, negative control.

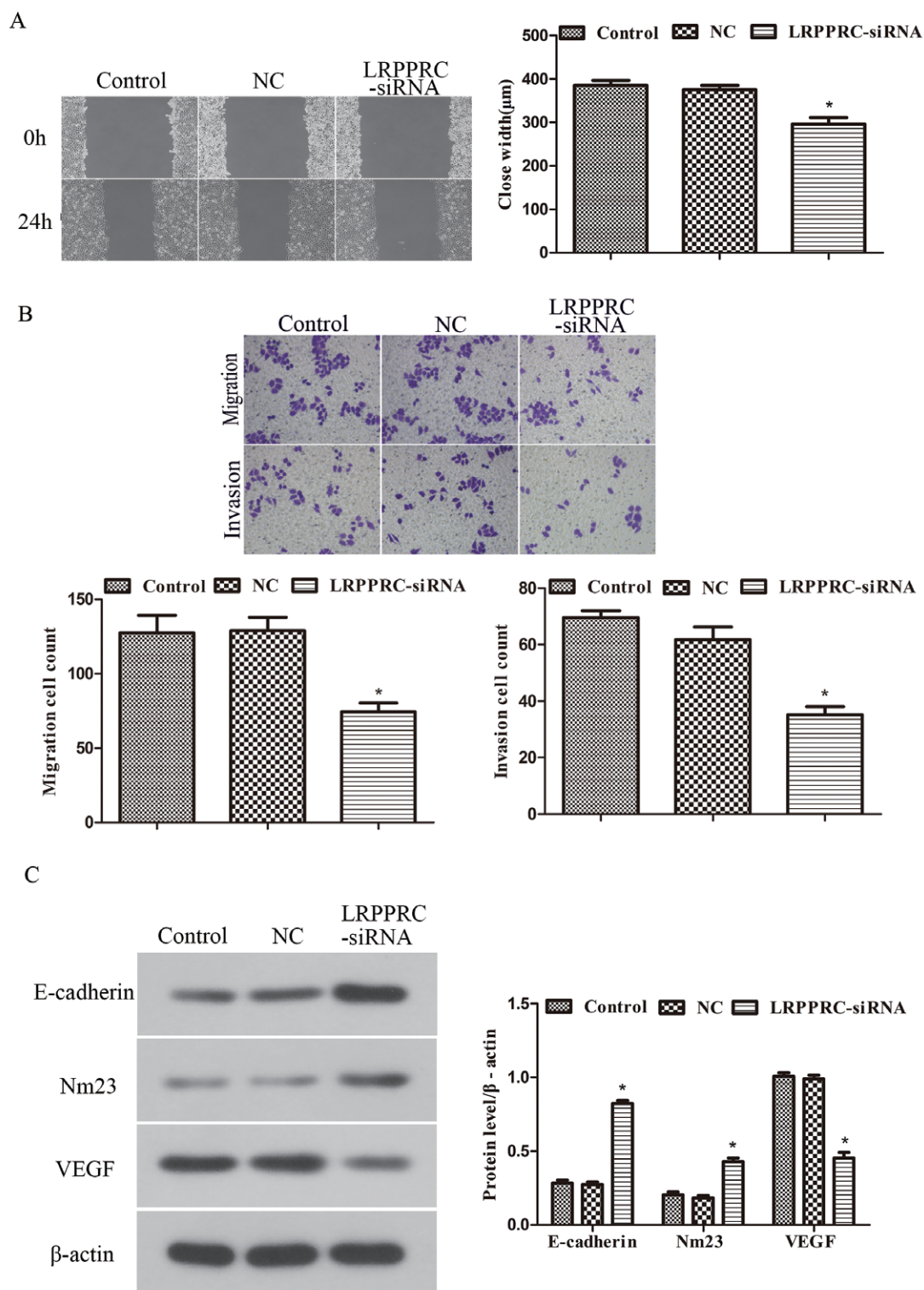


Fig. 3. LRPPRC knockdown inhibits invasion and metastasis of ECA-109 cells. **A)** The metastasis of ECA-109 cells was investigated using the wound-healing assay following LRPPRC knockdown. **B)** Migration and invasion were measured using the Transwell assay following LRPPRC knockdown. **C)** LRPPRC knockdown down-regulated VEGF protein expression and up-regulated E-cadherin and Nm23 protein expression. * $P < 0.05$ vs control. LRPPRC, Leucine-rich pentatricopeptide repeat motif-containing protein; siRNA, small interfering RNA; NC, negative control.

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LETTER TO THE EDITOR

Application of high-frequency jet ventilation via oropharyngeal catheter in fiberoptic bronchoscopy under general intravenous anesthesiaHT. Tian^{1#}, PJ. You^{1#}, Y. Su¹, CW. Song¹, HO. Li¹ and M. Wang^{2*}¹Department of Anesthesiology, Jining NO.1 People's Hospital, Jining, China; ²Department of Anesthesiology, Maternity and Child Health Care of Zaozhuang, Zaozhuang, China*Received January 7, 2021 - Accepted July 20, 2021*

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To the Editor,

Fiberoptic bronchoscopy is a deeply invasive procedure for patients with airway irritation, and under local anesthesia brings severe discomfort to patients (1). Fiberoptic bronchoscope in the airway makes it difficult for anesthesiologists to manage breathing. Elderly patients have poor cardiopulmonary function, which usually causes hypoxemia (2). Multi-fiber bronchoscopy mediated by a traditional endoscopic mask, trachea catheter and laryngeal mask occupies the ventilation path of the patient for a long time which may lead to airway stenosis, therefore it has limitations in the application of intravenous general anesthesia (3). Although the endoscopic mask, trachea catheter and laryngeal mask can significantly improve the ventilation effect as mediation, they still do not completely relieve the ventilation pressure, which easily leads to hypoxia caused by catheter front displacement, ventilation tools or fiberoptic bronchoscopy (4).

Anesthesiologists and endoscopic specialists have found that high-frequency jet ventilation (HFJV) can

be directly inserted into the sputum suction tube through the mouth to the pharynx cavity. This method can effectively prevent the occupation of airway space by fiberoptic bronchoscopy, the inhibition of respiration by anesthetics and the occurrence of hypoxemia (5). This study was designed to analyze the application of HFJV via oral and pharyngeal catheter in fiberoptic bronchoscopy under general intravenous anesthesia. The postoperative conditions of patients treated with HFJV of the oropharyngeal catheter and nasal catheter oxygen inhalation were observed.

MATERIALS AND METHODS

A total of 269 patients treated with painless fiberoptic bronchoscopy in our hospital from March 2014 to December 2016 were selected; 138 patients treated with HFJV were enrolled as the research group, and 131 patients treated with nasal catheter oxygen inhalation were enrolled as the control group. Patients with severe liver and kidney dysfunction, coagulation dysfunction,

Key words: high frequency jet ventilation via oropharyngeal catheter; fiberoptic bronchoscopy; nasal catheter oxygen inhalation; heart rate; mean arterial pressure; partial pressure of oxygen

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or patients who did not cooperate with the examination, or with cognitive and communication disorders were excluded from the study. All the subjects voluntarily participated in the experiment and signed informed consent, and cooperated with medical staff to complete the diagnosis and treatment.

All the patients in the two groups were treated with routine fasting before the operation. Oxygen inhalation was used prior to the operation. Patients in the control group were treated with nasal catheter oxygen inhalation with oxygen flow of 5~10 L/min, and patients in the research group were treated with HFJV via oropharyngeal catheter. After vein channel establishing, 0.5 mg of atropine was given intravenously (SFDA Approval Number H33020086, Jiangsu Enhua Pharmaceutical Industry) to reduce oral and respiratory secretions. 0.02 mg/kg of imidazepam (SFDA Approval Number H20090270, Yichang Renfu Pharmaceutical Co., Ltd.), 2 µg/kg of Fentanyl (SFDA Approval Number H20030197, Pfizer USA), 1~2 mg/kg of propofol (SFDA Approval Number H20051843, Xi'an Libang Pharmaceutical Co., Ltd.), and 1.5~2 mg/kg of Scoline (SFDA Approval Number H11021581, Beijing Shuanghe Pharmaceutical Co., Ltd.) were given in turn. When the muscle relaxed, fiberoptic bronchoscope was inserted through the mouth or nose, and then 5 ml of 2% lidocaine (SFDA Approval Number H20043676, Pfizer USA) was injected to reduce stimulus. At a frequency of 65 times/min, the sputum suction hole was connected with a high-frequency ventilator to maintain the injection volume I:E of oxygen supply of the body at a rate of 1:1, 10 L/min or above. During the operation, 50 µg/(kg·min) of propofol and 0.05 mg/(kg·min) of Scoline were continuously injected intravenously by micropump.

Observation indexes

General information of the two groups; hemodynamic changes including heart rate (HR), mean arterial pressure (MAP) and PaO₂ before and after the operation of both groups; the time of microscopy examination; and at different time points before and after operation; time of microscopy examination; Adverse Reaction and satisfaction of the patients in the two groups.

In the current study, HR, MAP and PaO₂ were measured at three different time points: T0 (before anesthesia), T1 (5 minutes after anesthesia) and T2 (10 minutes after anesthesia).

Statistical analysis

SPSS 17.0 software system (Beijing Sichuang Weida Information Technology Co., Ltd.) was used for statistical analysis. Counting information was expressed as [n(%)]. Measurement information was expressed as ($\bar{x} \pm s$). Independent sample *t*-test was used for comparing the information between the two groups. χ^2 test was used for counting information. The information between groups were compared by One-way ANOVA. When *P* value was less than 0.05, the difference was statistically significant.

RESULTS

Comparison of the general information between the two groups was not statistically significant ($p > 0.05$) (Table I).

Hemodynamic changes between groups before and after surgery

HR at T0, T1 and T2 of the research group were (80.12±4.31) times/min, (79.00±4.34) times/min, and (80.52±4.01) times/min, respectively. HR of the control group at each time point were (79.82±4.61) times/min, (81.00±4.62) times/min, and (79.12±4.91) times/min, respectively. There was no significant difference in HR between the two groups at each time point ($p > 0.05$). HR at T1 in the research group was lower than that at T0. At T2, HR returned to the same level as that at T0, the differences at each time point were not statistically significant ($p > 0.05$). HR at T1 of the control group was significantly higher than that at T0, with statistically significant difference ($p < 0.05$). HR at T2 returned to the level as at T0, without statistically significant difference ($p > 0.05$). It is suggested that both ventilation schemes affect the HR of patients (Fig. 1).

Changes of MAP between groups

MAP of the research group at each time point were (81.12±4.31) mmHg, (74.00±4.34) mmHg, and (81.10±4.01) mmHg, respectively. MAP of the control group at each time point were (80.82±4.03) mmHg, (70.49±35) mmHg, and (80.52±4.31) mmHg, respectively. There was no significant difference in MAP between the two groups at T0 and T2 ($p > 0.05$). MAP of the research group at T1 was significantly

higher than that in control group, with statistically significant difference ($p < 0.001$). MAP of the two groups at T1 were lower than that at T0, and MAP at T2 returned to the level as at T0. The difference between the two groups was statistically significant ($p < 0.001$). It is suggested that both ventilation schemes could affect the MAP of patients (Fig. 2).

Changes of PaO₂ between groups

PaO₂ of the research group at each time point were (863 ± 14.32) mmHg, (105.30 ± 10.74) mmHg,

and (98.22 ± 125) mmHg, respectively. PaO₂ of the control group at each time point were (86.29 ± 14.45) mmHg, (87.01 ± 14.28) mmHg, and (87.46 ± 14.36) mmHg, respectively. There was no significant difference in PaO₂ between the two groups at T0 ($p > 0.05$). PaO₂ of the research group at T1 and T2 were significantly higher than those in the control group, with statistically significant difference ($p < 0.001$). PaO₂ in the research group at T1 was significantly higher than that at T0, and PaO₂ at T2 was significantly lower than that at T1, which

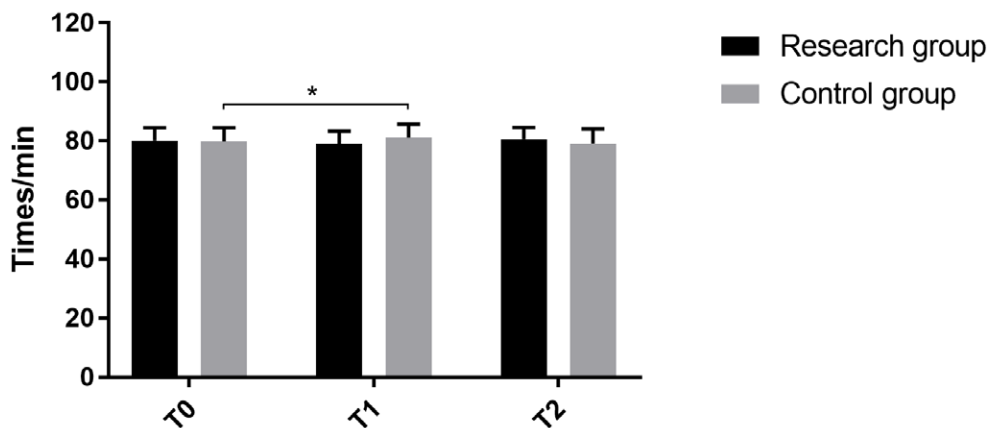


Fig. 1. Changes of HR before and after operation in research group and control group. * indicates that HR at T1 of the control group was significantly higher than HR at T0, with statistically significant difference ($p < 0.05$).

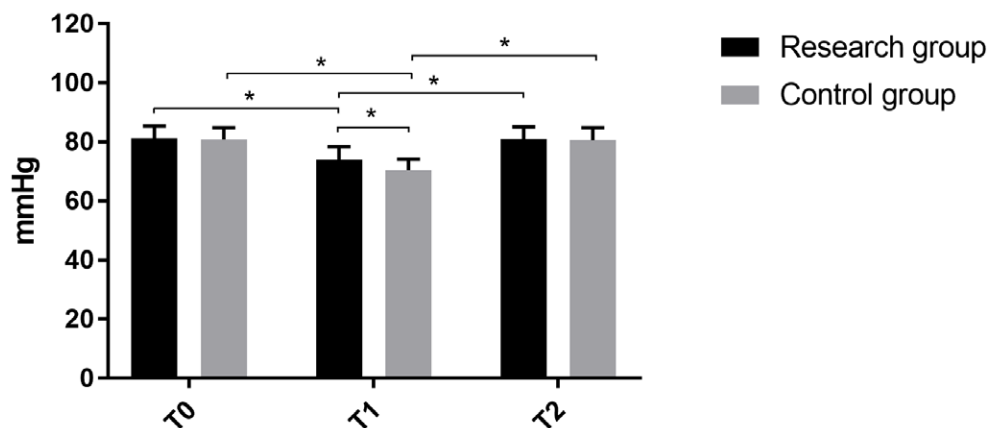


Fig. 2. Changes of MAP before and after operation in the research group and control group. * indicates that, MAP of the research group at T1 was significantly higher than that in the control group, with statistically significant difference ($p < 0.001$).

was still significantly higher than that at T0, with statistically significant differences ($p < 0.001$). PaO₂ in the control group at T1 and T2 were higher than those at T0, but there was no statistically significant difference at each time point in the group ($p > 0.05$). It is suggested that both ventilation schemes could affect PaO₂ of patients (Fig. 3).

Comparison of time of microscopy examination in both groups

The time of microscopy examination of the research group was (9.20 ± 3.80) min, the time of microscopy examination of the control group was (16.30 ± 2.80) min. Compared between the two groups, the time of microscopy examination in the

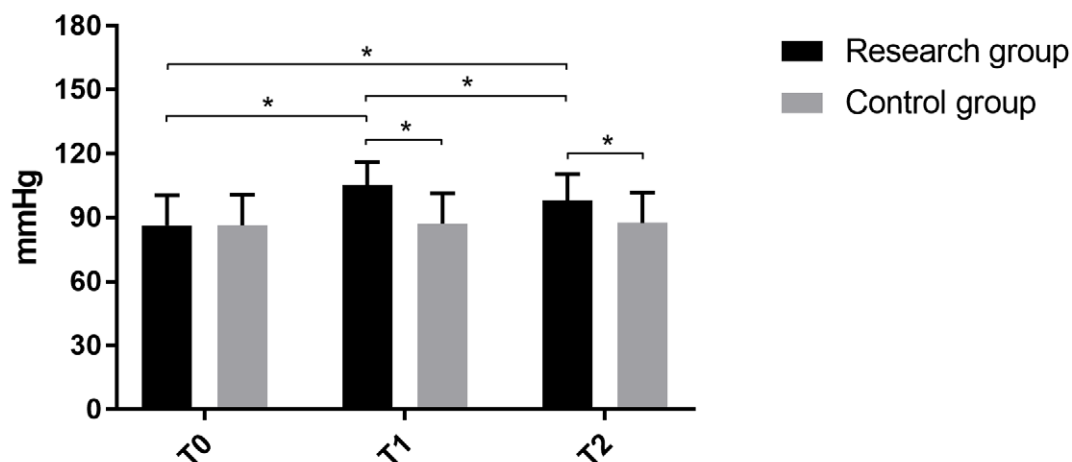


Fig. 3. Changes of PaO₂ before and after operation in the research group and control group. * indicates that PaO₂ of the research group at T1 and T2 was significantly higher than that in the control group, with statistically significant difference ($p < 0.001$).

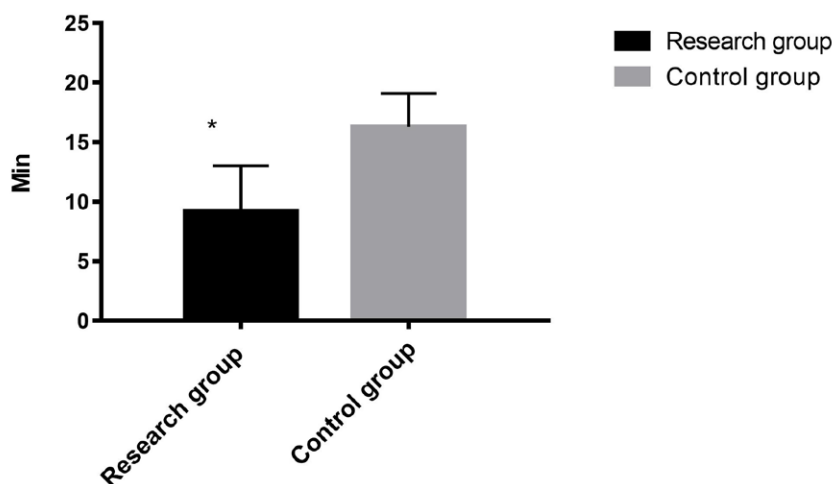


Fig. 4. Comparison of time of microscopy examination in the research group and control Group. * indicates that the time of microscopy examination of patients in the research group was significantly less than that in the control group, with statistically significant difference ($p < 0.001$).

research group was significantly lower than that in the control group ($p < 0.001$) (Fig. 4).

Comparison of adverse reaction and satisfaction between groups

Comparing between the two groups, the incidence of nausea and vomiting, catheter ectopic, cough and elevated blood pressure were lower in the research group than those in the control group, and

the difference between the groups was statistically significant ($P < 0.05$). Patients satisfied in the research group were significantly more than those in the control group, with statistically significant difference ($p < 0.001$) (Table II).

DISCUSSION

During surgical anesthesia, the patient's heart rate

Table I. Comparison of general information between two groups

Group		Research Group (n=138)	Control Group (n=131)	t/χ^2	p
Gender	Male	80	72	0.248	0.619
	Female	58	59		
Age (Years)	≤ 47	74	73	0.112	0.730
	> 48	64	58		
BMI (kg/m ²)	≤ 18.25	64	58	0.112	0.730
	> 18.25	74	73		
Types of bronchoscopy operations	Identifying the causes and confirming diagnosis	57	66	0.143	0.418
	Etiological detection	29	31		
	As means of treatment	47	31		
	Others	5	3		

Table II. Comparison of adverse reaction and satisfaction between two groups [n(%)]

Group	Research Group (n=138)	Control Group (n=131)	χ^2	p
Nausea and vomiting	8 (5.80)	15 (11.45)	2.747	0.097
Ectopic catheter	0 (0.00)	15 (11.45)	16.730	< 0.001
Coughing	2 (1.45)	10 (7.63)	6.031	0.014
Pneumothorax	0 (0.00)	1 (0.01)	1.004	1.000
Elevated blood pressure	2 (1.45)	10 (7.63)	6.031	0.014
Satisfaction	130 (94.20)	60 (45.80)	75.900	< 0.001

fluctuates to different degrees due to stress response (6). If the HR fluctuation is out of the normal reference range, it greatly affects the recovery of patients (7). Therefore, we believe that both ventilation schemes could affect HR of patients. However, changes of HR of patients treated with fiberoptic bronchoscopy under HFJV via oropharyngeal catheter were less fluctuant, thus the stress response of patients would be lower than that of patients treated with nasal catheter oxygen, and the comfort would greatly increase. MAP is the functional pressure of the arterial system in each phase of the heart and is an index to evaluate tissue perfusion (8). In similar hemodynamic studies, the importance of MAP stability maintenance has been shown (9). We believed that both ventilation schemes have a significant impact on MAP and the improvement effect of PaO₂ on patients. The above results indicate that HFJV has less impact on MAP in the anesthetic period of patients and more significant improvement on PaO₂. Other studies on the application of HFJV in fiberoptic bronchoscopy have confirmed that the patient's hemodynamic stability has a great impact on improving his comfort and subsequent treatment (10).

To sum up, HFJV via oropharyngeal catheter under intravenous general anesthesia could be used in the treatment of fiberoptic bronchoscopy, with better hemodynamic stability. It greatly shortens the time of microscopic examination and improves the comfort of patients. It is worth widely popularizing in a clinical setting.

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LETTER TO THE EDITOR

Cannabinoid type-1 receptor on glutamatergic neurons differentially controls gender difference of emotional behaviors in mouseL-X. Wu¹, M-Y. Dai¹, K-Q. Yu¹, X. Yu² and T-H. Ye^{3*}¹*Department of Rehabilitation, the Second People's Hospital of Lishui, LiShui, Zhejiang, China;*²*Department of Psychiatry, the Second People's Hospital of Lishui, LiShui, Zhejiang, China,*³*Department of Rehabilitation, People's Hospital of Suichang, LiShui, Zhejiang, China**Received April 26, 2021 – Accepted August 2, 2021*

To the Editor,

Epidemiological studies frequently indicate that emotional disorders, such as anxiety and depression, are more common in women than men; however, the mechanisms underlying such a gender difference remain unclear. Notably, the endocannabinoid system (ECS) appears to be differentially regulated in men and women. The cannabinoid type-1 receptor (CB1R) is the primary mediator of the ECS signaling in the central nervous system, and the presence of a relationship between gender and the cannabinoid receptor system has been frequently reported (1). Sex differences in this system may appear early in development. The experimental studies showed that CB1R in the rat's brain demonstrates a progressive elevation in number during early adolescence and the pre-weanling period, with receptor pruning and a reduction to adult levels during later adolescence. Moreover, a higher density of CB1R has been detected in male rodents in the prefrontal cortex, hippocampus, and hypothalamus, compared to females. Males also exhibit lower levels of CB1R binding in the amygdala and higher levels of binding in the hypothalamus, compared to females. Sex differences in the cannabinoid receptor system can be also reflected by the gender-specific effects of the cannabinoids concerning psychopathology,

emotion, and stress (2). There is evidence that shows the constitutive global knockout of CB1R exerts a differential sex-dependent effect on anxiety-like behavior in mice (3). The role of the forebrain and dorsal telencephalic regions, such as the hippocampus and prefrontal cortex, in anxiety and depression is well established. In this brain region, CB1R is located on the GABAergic and glutamatergic axon terminals where underlies the inhibition of neurotransmitter release. Both gamma-aminobutyric acid (GABA) and glutamatergic neurotransmissions have a role in anxiety and depression. GABA is the main suppressive mediator of cortical interneurons, which can regulate a broad spectrum of local neurotransmitter counterparts, such as the glutamatergic excitatory system. GABA interneurons target somatic domains of adjacent glutamatergic neurons and regulate the excitatory output to projecting regions, thus handling complex cognitive and emotional processes in the brain (4). In addition to GABAergic and glutamatergic neurons, CB1R is also highly expressed in raphe nuclei, where its deficiency has been found to increase anxiety (5). Of note, the CB1R activation on dorsal telencephalic glutamatergic neurons and forebrain GABAergic neurons mediates the opposite anxiolytic- and anxiogenic-like functions, respectively. Of note, it

*Key words: anxiety; cannabinoid-1 receptor; depression; GABAergic neurons; glutamatergic neurons***Corresponding Author:*

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has been shown that CB1R on cortical glutamatergic terminals mediates anxiolytic-like effects of the low dose of cannabinoids, while CB1R on the GABAergic terminals is required to induce an anxiogenic-like effect under a high-dose treatment (6). Possibly, CB1R activation on glutamatergic neurons, as excitatory cells, can inhibit glutamate release and thus provide an anxiolytic effect, while CB1R activation on GABAergic neurons, as inhibitory cells, would lead to an anxiogenic response via a decrease in GABAergic transmission (6). Accordingly, we hypothesized that cell-specific knockout of CB1R on dorsal telencephalic glutamatergic neurons and forebrain GABAergic neurons may have a differential sex-dependent effect on anxiety/depression-like behavior in experimental mice. In the present study, we used three different transgenic mouse lines, including global-CB1 knockout (KO) mice, Glu-CB1-KO mice, and GABA-CB1-KO mice, and corresponding littermate controls, to evaluate the role of forebrain GABAergic- and dorsal telencephalic glutamatergic-CB1R in sex-related differences of anxiety and depression behaviors. For this purpose, we studied the emotional responses of the aforementioned mutant mice in different behavioral models of anxiety and depression.

MATERIALS AND METHODS

Animals

This study was carried out on the global and conditional CB1R mutant mice and their littermate control mice (8–10 weeks old, 24 ± 3 g), including CB1^{-/-} mice with the whole body knockout of CB1R (CB1-KO; 10 males and 10 females) and littermate wild type CB1^{+/+} mice (CB1-WT 10 males and 10 females), Glu-CB1^{-/-} mice with conditional knockout of CB1R on dorsal telencephalic glutamatergic neurons (Glu-CB1-KO; 10 male, 10 female) and littermate wild type NEX-Cre mice (Glu-CB1-WT; 10 male and 10 females), and GABA-CB1^{-/-} mice with conditional knockout of CB1R on forebrain GABAergic neurons (GABA-CB1-KO; 10 males and 10 females) and littermate wild type Dlx5/6-Cre mice (GABA-CB1-WT; 10 males and 10 females). The CB1-KO breeding pairs who do not express the receptor at the whole body, derived from a stock obtained from the Jackson Laboratory

(Sacramento, California, USA), were bred in the Bioray Laboratories (Shanghai, China) as previously explained (7). Conditional CB1R mutant mice were generated at the Bioray Laboratories by crossing CB1^{flox/flox} mice (8) with the respective transgenic mice expressing Cre-recombinase obtained from The Jackson Laboratory. In brief, CB1^{flox/flox} mice were crossed with the transgenic NEX-Cre mice to generate CB1^{f/f}; NEX-Cre mice (herein termed Glu-CB1^{-/-} or Glu-CB1-KO mice lack the CB1R primarily in dorsal telencephalic glutamatergic neurons), using a previously-described breeding protocol (9). CB1^{flox/flox} mice were also crossed with the transgenic Dlx5/6-Cre mice to generate CB1^{f/f}; Dlx5/6-Cre mice (herein termed GABA-CB1^{-/-} or GABA-CB1-KO mice lacking the CB1R mainly in forebrain GABAergic neurons), using previously-described breeding protocol (9). To homogenize the genetic background of the mice, the mutant lines were bred for 10–12 generations on the background of C57BL/6 mice. The wild-type (WT) littermate controls, including the CB1^{+/+} mice, the NEX-Cre mice, and the Dlx5/6-Cre mice, were bred and used in all experiments. Genotyping was performed at the Bioray Laboratories by polymerase chain reaction (PCR) using specific primers as explained for the CB1^{-/-} and CB1^{+/+} mice (7, 8), for Glu-CB1^{-/-} and NEX-Cre mice (9), and for GABA-CB1^{-/-} and Dlx5/6-Cre mice (9). The mice were housed under standard environmental conditions (humidity 50–60%; temperature 23–24 °C; 12-h light/dark cycle) with free access to standard diet and water. According to previous power analysis, we implicated the number of mice at the absolute minimum, enough to obtain significant group differences.

Behavioral testing

Mice were not single-housed before testing to avoid confounding influences of the isolation on anxiety and depression levels. The anxiety-like phenotype of the mice was studied by behavioral tasks in the following order: the elevated plus-maze (EPM) and open-field test (OFT) tasks on separate days in one week. The depression-like phenotype of the mice was tested by the sucrose preference test (SPT) and the forced swimming test (FST). All behavioral tests were performed during the light phase in an experimental room with constant noise and a temperature-controlled environment ($23 \pm 1^\circ\text{C}$). Each behavioral test was performed within at least one

day of each other. Mice were tested in a random order by the same trained observer blind to mouse genotype throughout the entire course of the analysis. After the tests, the equipment was cleaned with 70% ethanol and super-hypochlorous water to eliminate olfactory cues.

EPM task

The EMP is a simple and reliable behavioral test widely employed to evaluate anxiety-related behavior in laboratory animals, particularly rodents. The EMP test is based on a strategy-avoidance conflict, that is, the animal comes into a struggle between a tendency to find a new environment and an unconditioned fear of open and high spaces. Eventually, an anxiety-like state is revealed by elevated open arm avoidance, compared to control subjects. We used the EPM test on an apparatus composed of wood covered with laminate and with four elevated arms (70 cm above the floor) 50-cm long and 10-cm wide. The arms were arranged in a cross-like disposition, with two opposite arms being enclosed (by 40-cm high walls) and two being open, having at their intersection a central platform (10 cm×13.5 cm) which gave access to any of the four arms. The closed arms were surrounded by 20-cm high walls, while the open arms were surrounded by a raised ledge (1-mm thick and 5-mm high) to avoid mice falling off the arms. Each mouse was put in the central square of the apparatus and allowed to freely tour the maze. After several entries into open arms, the total number of entries into open arms and the time spent on open arms (sec) were recorded during 5 min. The latency of the first entry into the open arms (full entry of 4 paws into the open arm), the percentage of entries into open arms, the percentage of time spent on open arms were measured as parameters for the anxiety-like phenotype. The total number of entries into arms was assessed as a parameter representing the general locomotor activity. Time spent in the arms was tracked by Any-Maze software (Stoelting Co., Wood Dale, IL, USA) and entries of all four paws into each arm were manually counted using a mechanical counter by a trained observer blind to the genotype.

OFT task

The OFT is a common test employed in experimental animals (usually rodents) to measure exploratory behavior and anxiety. Rodents naturally have an aversion to open areas because open areas leave them exposed to predators,

but they also want to explore new areas. This conflict allows us to measure their anxiety levels. Indeed, reduced levels of anxiety result in increased exploratory behavior, and increased anxiety will lead to less locomotion and a preference to stay close to the walls of the field. In the present study, the OFT was performed in a square box made of wood covered with laminate, which had a square floor of 100cm×100cm (divided by black lines into 25 squares of 20cm×20cm) and walls, 40-cm high. Each mouse was placed in the center of the box and permitted to freely tour the open field for 10 min. After a 30 s adaptation, the time spent in the central square and the total distance traveled were tracked and calculated during 10 min using Any-Maze software.

SPT task

The SPT is a reward-based task that provides a simple and reliable indicator of depression and associated anhedonia. Anhedonia, or the reduced ability to experience pleasure, shows one of the core symptoms of depression. Rodents are inherently fond of sweet solutions and foods. Decreased preference for sweet solution in the SPT task indicates anhedonia. In the present study, the depression-like phenotype was tested using the SPT based on a modified model of the previously explained test. Each mouse was housed for 48 hours in an individual cage equipped with two bottles containing 200 ml of 2% sucrose (w/v) or 200 ml of tap water. To avoid potential position preference of drinking, the location of the bottles was altered every 12 hours. After 48 hours, the bottles were checked and the volume of consumption was determined. The sucrose preference was measured as the percentage of 2% sucrose solution intake to the total amount of solution intake.

FST task

In this study, the FST task was also performed to test depression-like behavior. This task has been widely employed because it includes the exposure of the animals to stress, which is known to exert a role in the tendency for major depression. The FST is based on the hypothesis that once putting a mouse in a water-filled container, it will first try to escape but finally will leave movement that may be regarded to represent a measure of behavioral despair. The FST is a two-day approach in which mice swim under conditions where escape is not possible. On the first day, mice are forced to swim for 15 min. Mice initially struggle

to escape from the water, but eventually, they adopt a posture of immobility characterized by few movements necessary to keep heads above the water. When mice are tested again 24 h later, immobility is increased if animals show depressive-like behavior. On the first day, mice were put in a Plexiglas cylinder (25 cm [height] $\times$ 15cm [diameter]) filled with water (25°C) to a depth of 9 cm. After 15 min of forced swimming, the mice were removed from the water, dried with towels, and placed under a warming lamp until dry. The cylinder was emptied and cleaned between mice. The test was videotaped from above the cylinders, and the behavior measures were scored and defined as follows: (i) immobility: the lack of motion of the whole body, except for small movements necessary to keep the animal's head above the water; (ii) climbing: vigorous movements with the forepaws in and out the water, usually directed against the wall of the cylinder; (iii) swimming: they were making active swimming movements to the center of the cylinder. The time that mice spent in each of these behaviors was recorded using chronometers (1/100 s Stop Watch, Primex).

Statistical analyses

Data were analyzed using Prism 9.0 software (GraphPad Software, Inc.). As analyzed by the Kolmogorov-Smirnov test, all data were normally distributed. Accordingly, the Two-way ANOVA followed by Tukey's *post-hoc* test was employed to measure statistical differences between mean values of the different groups. Differences with a *p*-value less than 0.05 were considered statistically significant. Results are expressed as the means $\pm$ standard deviation (SD).

RESULTS

Anxiety-like behavior

The EPM and the OFT tasks were used to evaluate anxiety-like behavior (Fig. 1). In the EPM, Two-way ANOVA analysis showed the significant main effects of the sex and genotype on anxiety-related responses including the latency to the first open-arm entry [F (1, 108) = 11.41, *p* < 0.01 and F (5, 108) = 149.5, *p* < 0.01], the percentage of open-arm entries [F (1, 108) = 7.94, *p* < 0.01 and F (5, 108) = 172, *p* < 0.01], and the percentage of the time spent on open arms [F (1, 108) = 0.2294, *p* < 0.01 and F (5, 108) = 117.7, *p* < 0.01]. Moreover, the significant genotype by sex interactions was observed in the latency to the first

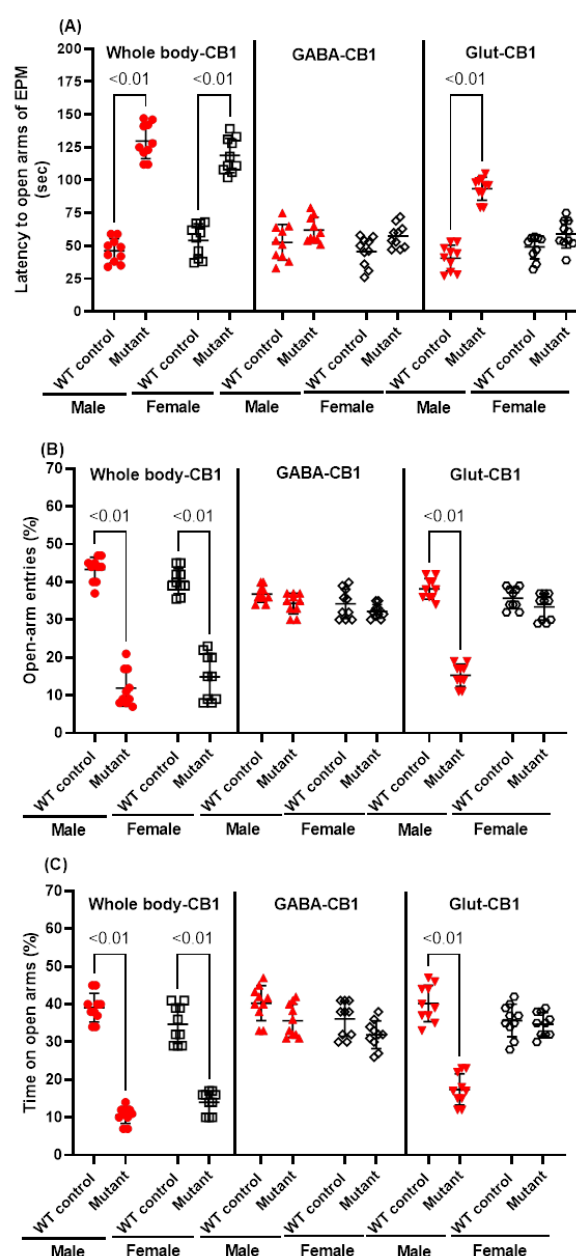


Fig. 1. The effect of the cannabinoid-1 receptor (CB1R) on the anxiety-related behaviors in the elevated plus-maze (EPM) test. Data of the latency of the first entry into the open arms (A) percentage of entries into open arms (B) and the percentage of time spent on open arms (C) are presented for mutant mice lines including the whole body CB1R knockout (CB1-KO) mice, GABAergic CB1R knockout (GABA-CB1-KO) mice, and glutamatergic CB1R knockout (Glut-CB1-KO) mice in both genders. Wild type (WT) controls represent corresponding WT littermate mice including CB1-WT mice, GABA-CB1-WT mice, and Glut-CB1-WT mice. Data are presented as the mean $\pm$ SD of the behavioral scores of 10 mice per group. Numbers on the lines show *p*-values of differences between groups.

open-arm entry [$F(5, 108) = 10.32, p < 0.01$], the percentage of open-arm entries [$F(5, 108) = 27.9, p < 0.01$], and the percentage of the time spent on open arms [$F(5, 108) = 22.51, p < 0.01$]. Remarkably, the sex [$F(1, 108) = 0.2, p = 0.65$], genotype [$F(5, 108) = 0.58, p = 0.71$], and genotype by sex interactions [$F(5, 108) = 0.52, p = 0.76$] had no significant effect on total arm entries (Table I). Since the “total arm entries” parameter is usually considered as an indicator of the general locomotion that may confound anxiety-like behavior, our finding confirms that the interpretation of the anxiety-related results has not interfered with the general locomotion. Of note, Tukey’s *post-hoc* test revealed the anxiety-like phenotype in the male and female CB1^{-/-} mice

and male Glut-CB1^{-/-} mice with the significantly ($p < 0.01$) higher latency (Fig. 1A) as well as the significantly ($p < 0.01$) lower percentage of entries into the open arms (Fig. 1B) and the significantly ($p < 0.01$) lower percentage of the time spent on open arms (Fig. 1C) than the corresponding littermate controls. There were no significant differences in the tested behaviors in both male and female GABA-CB1R^{-/-} mice as well as female Glut-CB1^{-/-} mice when compared with the corresponding littermate controls. Consistent with EMP data, there were the main significant effects of the sex and genotype on anxiety-related parameters in the OFT task, including total distance traveled [$F(1, 108) = 17.86, p < 0.01$ and $F(5, 108) = 134.0, p < 0.01$], the time spent in

Table I. Total number of entries into arms in the EPM test.

	Genotype	Sex	Total number of entries (mean ±SD)	Two-way ANOVA analysis	
Mutant mice	CB KO mice	Male	34.3 ±12	The sex effect	<i>p</i> -value
		Female	35.5 ±6.1	F (1, 108) = 0.2	0.61
	GABA-CB1 KO mice	Male	38.1 ±13.6		
		Female	36.7 ±10.4	The genotype effect	<i>p</i> -value
	Glu-CBR1 KO mice	Male	37.9 ±8.5		
		Female	43 ±13	F (5, 108) = 0.58	0.71
Control mice	CB1 ^{+/+} mice	Male	38.6 ±12.8		
		Female	37.1 ±9.5	The genotype and sex interaction	<i>p</i> -value
	Dlx5/6-Cre mice	Male	39.3 ±10.6		
		Female	36.3 ±8.8	F (5, 108) = 0.52	0.76
	NEX-Cre mice	Male	36.5 ±10.2		
		Female	41.5 ±10.1		

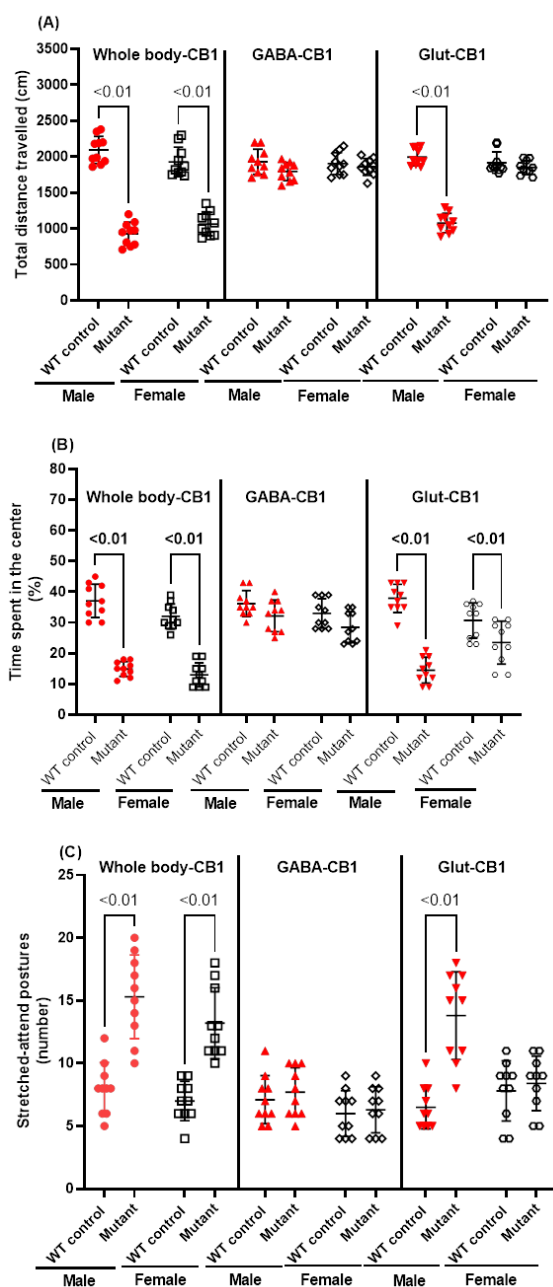


Fig. 2. The effect of the cannabinoid-1 receptor (CB1R) on the anxiety-related behaviors in the open-field test. data of total distance traveled (A) and the time spent in the center zone (B), and the number of stretched-attend postures (C) are presented for mutant mice lines including the whole body CB1R knockout (CB1-KO) mice, GABAergic CB1R knockout (GABA-CB1-KO) mice, and glutamatergic CB1R knockout (Glut-CB1-KO) mice in both genders. Wild type (WT) controls represent corresponding WT littermate mice including CB1-WT mice, GABA-CB1-WT mice, and Glut-CB1-WT mice. Data are presented as the mean $\pm$ SD of the behavioral scores of 10 mice per group. Numbers on the lines show p -values of differences between groups.

the center zone [$F(1, 108) = 5.222, p < 0.02$ and $F(5, 108) = 70.16, p < 0.01$], and the SAP [$F(1, 108) = 14.38, p = 0.02$ and $F(5, 108) = 34.93, p < 0.01$]. The significant interaction of genotype and sex was also found in total distance traveled [$F(5, 108) = 24.59, p < 0.01$], the time spent in the center zone [$F(5, 108) = 6.933, p < 0.01$], and the SAP [$F(5, 108) = 4.355, p < 0.01$]. As shown by Tukey's *post-hoc* test, both male and female CB1^{-/-} mice as well as male Glut-CB1^{-/-} mice exhibited more anxiety-like behavior on any of the measures in the OFT (Fig. 2), such that they showed the significantly lower total distance traveled (Fig. 2A) and the time spent in the center zone (Fig. 2B) as well as the significantly higher SAP (Fig. 2C) than the corresponding littermate controls ($p < 0.01$). Notably, both male and female GABA-CB1^{-/-} mice as well as female Glut-CB1^{-/-} mice showed no significant differences ($p > 0.05$) in the tested behaviors when compared to their corresponding control mice. Altogether the EMP and OFT results suggest that male and female CB1^{-/-} mice and male Glut-CB1^{-/-} mice had increased anxiety-like phenotype compared to littermate control mice, whereas female Glut-CB1^{-/-} mice as well as male and female GABA-CB1^{-/-} showed similar levels of anxiety-like phenotype compared to their corresponding control mice. Overall, the results indicate that despite the strong role in male mice, dorsal telencephalic glutamatergic CB1R did not show a clear role in anxiety-like behavior observed in female CB1-KO mice; in female Glut-CB1-KO mice, the increased anxiety-like behavior observed in CB1-KO mice was reversed to normative anxiety-like behavior.

Anhedonia

A main symptom of depression is anhedonia, which decreases the responsiveness to pleasurable stimuli. Reduced sucrose intake and preference is a measure of anhedonia. Using Two-way ANOVA analysis, we found (Fig. 3) the significant sex effect [$F(5, 108) = 98.15, p < 0.01$], the significant genotype effect [$F(1, 108) = 535.8, p < 0.01$], and the significant genotype by sex interactions, [$F(5, 108) = 101.5, p < 0.01$] on the sucrose preference. Further statistical analysis using Tukey's *post-hoc*

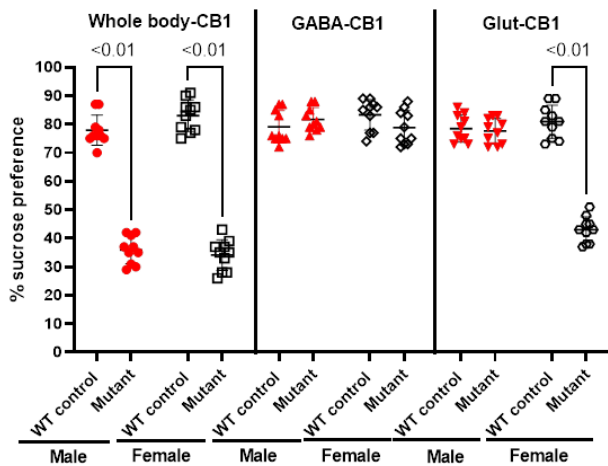


Fig. 3. The cannabinoid-1 receptor (CB1R) and anhedonia. Percentage of the sucrose preference in mutant mice lines including the whole body CB1R knockout (CB1-KO) mice, GABAergic CB1R knockout (GABA-CB1-KO) mice, and glutamatergic CB1R knockout (Glut-CB1-KO) mice in both genders. Wild type (WT) controls represent corresponding WT littermate mice including CB1-WT mice, GABA-CB1-WT mice, and Glut-CB1-WT mice. Data are presented as the mean $\pm$ SD of the behavioral scores of 10 mice per group. Numbers on the lines show *p*-values of differences between groups.

test showed that both the male and female CB1^{-/-} mice had a significantly ($p < 0.01$) lower sucrose preference than the littermate control mice. Male and female GABA-CB1^{-/-} mice as well as male Glut-CB1^{-/-} mice exhibited no significant differences in the sucrose preference from the littermate control mice, whereas female Glut-CB1^{-/-} mice showed a significantly ($p < 0.01$) reduced amount of sucrose preference when compared with male Glut-CB1^{-/-} mice and the littermate female control mice.

Depression-like behavior

The FST provides a measure of depressive-like behavior. After Two-way ANOVA analysis, we detected the significant main effects of the sex and genotype on depression-related behavior in the FST task, including the immobility time [F (1, 108) = 31.51, $p < 0.01$ and F (5, 108) = 140.0, $p < 0.01$], the swimming time [F (1, 108) = 0.8139, $p = 0.04$ and F (5, 108) = 36.68, $p < 0.01$], and the climbing time [F (1, 108) = 17.57, $p < 0.01$ and F (5, 108) = 28.30, $p < 0.01$]. We also observed the significant genotype

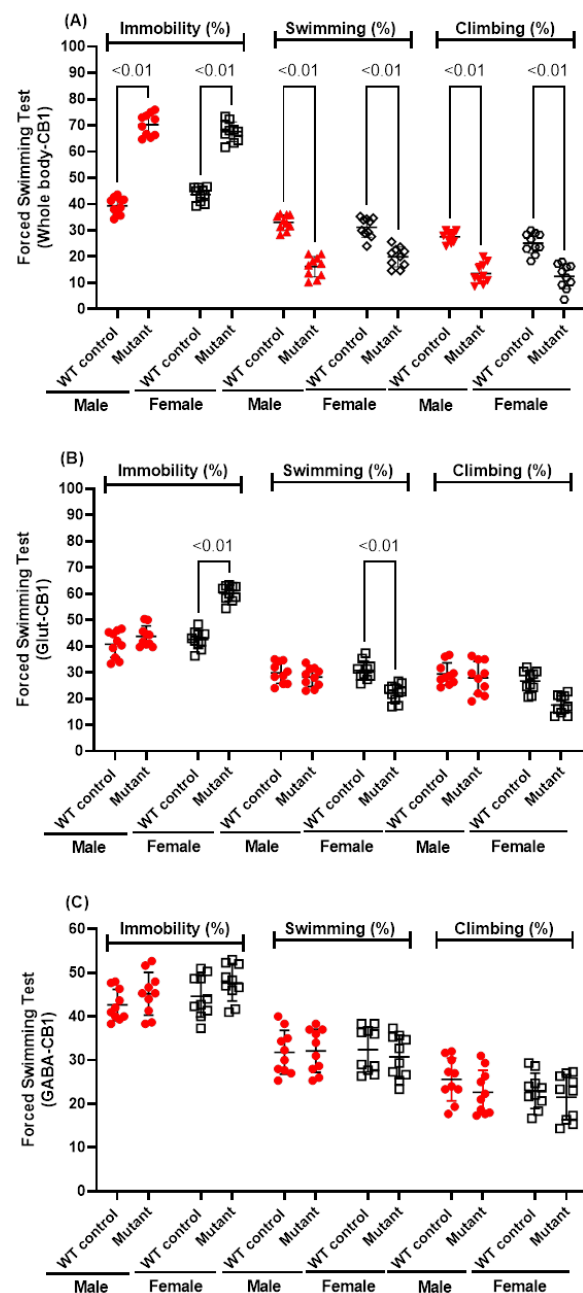


Fig. 4. The effect of the cannabinoid-1 receptor (CB1R) on depression-related behaviors in the forced swimming test. Data of the immobility time, the swimming time, and climbing time of mutant mice lines including the whole body CB1R knockout (CB1-KO) mice (A), GABAergic CB1R knockout (GABA-CB1-KO) mice (B), and glutamatergic CB1R knockout (Glut-CB1-KO) mice (C) in both genders. Wild type (WT) controls represent corresponding WT littermate mice including CB1-WT mice, GABA-CB1-WT mice, and Glut-CB1-WT mice. Data are presented as the mean $\pm$ SD of the behavioral scores of 10 mice per group. Numbers on the lines show *p*-values of differences between groups.

by sex interactions in the immobility time [$F(5, 108) = 13.42, p = 0.01$], the swimming time [$F(5, 108) = 3.188, p = 0.01$], and climbing time [$F(5, 108) = 3.157, p = 0.01$]. *Post-hoc* Tukey test revealed that both male and female CB1^{-/-} mice had depression-like phenotype with significant changes in values of the FST parameters (Fig. 4A), including the significant increase in the immobility time ($p < 0.01$) and the significant decrease in the swimming time ($p < 0.01$) and climbing time ($p < 0.01$), as compared with the littermate control mice. The female Glut-CB1^{-/-} mice displayed a depression-like phenotype with changes of the measures in the FST (Fig. 4B), including the significant increase in the immobility time ($p < 0.01$) and the significant decrease in the swimming time ($p < 0.01$) and climbing time ($p < 0.01$), as compared with the littermate female control mice. There were no similar changes in male Glut-CB1^{-/-} mice. Both male and female GABA-CB1^{-/-} mice showed no depression-like behaviors in terms of the FST parameters when compared with the corresponding control mice (Fig. 4C).

DISCUSSION

The results of the present study indicate that the lack of CB1R on dorsal telencephalic glutamatergic neurons increases anxiety-like behavior (in male mice) and depression-like behavior (in female mice), while deletion of CB1R on forebrain GABA terminals does not alter tested behaviors in both male and female mice, suggesting that dorsal telencephalic glutamatergic CB1R have an important role in anxiety and depression-like behaviors observed in male and female CB1-KO mice, respectively, and can be responsible for gender differences in anxiety/depression-like behavior. To our knowledge, there are not many studies assessing differences in depression- and/or anxiety-like behavior between male and female mice with conditional deletion of CB1R in either glutamatergic or GABAergic neuronal populations, however, several studies using the genetic rescue strategy can support our results on male mutant mice. Of note, the conditional gain-of-function studies on male mice with the rescue of the CB1R on dorsal telencephalic glutamatergic neurons

(Glut-CB1-RS) showed a decrease in the anxiogenic responses observed upon global CB1R deletion (10, 11). Moreover, there are also other preclinical studies that show the activation of CB1R can markedly decrease depressive-like behavior (12, 13). Since CB1R activation on glutamatergic neurons inhibits excitatory neurotransmission and appears to be responsible for the overall network activity-reducing effect of (endo) cannabinoids, glutamatergic CB1R deletion can be expected to increase hyperactivity and hyperexcitability features that are correlated with excessive anxiety or depression. Therefore, it seems that the glutamatergic hyperactivity due to the loss of the inhibitory control of the CB1R signaling over glutamatergic transmission is responsible for the increased anxiety- and depression-like behaviors observed in our study. One potential explanation for the gender-specific impact of glutamatergic CB1R neurons on the anxiety/depression-like behavior can be the possibility of sex-specific differences in the intracellular CB1R signaling, such as G-protein coupling. CB1R as a G protein-coupled receptor (GPCR) regulates various G proteins in different brain cells, and G protein-dependent signaling of CB1R has been found to have high efficacy in hippocampal glutamatergic neurons (4). In addition to stimulatory G-protein coupled signaling, it is noteworthy that CB1Rs are also able to 'sequester' Gi/o proteins (4), making them unavailable to other GPCRs expressed in the same cells; thus, these receptors, despite a high expression, might be silent in certain cell types, such as dorsal telencephalic glutamatergic neurons. Accordingly, it can be concluded that the observed results in male or female Glut-CB1-KO mice might be due to a predominant activity of inhibitory G-protein coupled signaling. Of note, there is also evidence supporting the possibility of a developmental mechanism by which gonadal hormones in females, potentially during the postnatal developmental period, may affect the CB1R signaling to mediate sex-specific anxiety behaviors in adult animals. Moreover, in males, perhaps an interaction between the CB1R signaling and an early development surge in testosterone may be responsible for such an effect (14). However, to determine the exact nature and timing of a Glut-CB1/testosterone or gonadal hormone interaction

in anxiety and depression-like behaviors, future investigations are demanded to study the impact of hormones that are essential for sexual differentiation during early development. In summary, our results demonstrated for the first time that the CB1R signaling in dorsal telencephalic glutamatergic neurons plays an important role in gender-specific differences of the anxiety- and depression-like behavior.

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LETTER TO THE EDITOR

Expression of NLR and IL-1 β and their predictive efficacy value in acute myocardial infarction patients treated with aspirin combined with clopidogrelQ. Ma¹, WN. Li², HY. Liu³, HY. Zhang⁴, JY. Dong⁵ and XL. Tian^{6*}

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To the Editor,

Acute myocardial infarction is a myocardial necrosis caused by acute, persistent ischemia and hypoxia in the coronary arteries, and is the main reason for patients' hospital admission and death around the world (1). Multiple diseases are commonly seen in the primary diagnosis of acute myocardial infarction, posing impacts on the treatment and prognosis of patients (2). Currently, the monitoring system of coronary heart disease is not complete, as subjects are not able to follow-up with the changes of epidemiology in coronary heart disease (5). Neutrophil-to-lymphocyte ratio (NLR) is a simple and convenient indicator of systemic inflammation that has been proved to be closely related to the development and prognosis of tumors in colorectal cancer, non-small cell lung cancer, gastric cancer, pancreatic cancer, hepatocellular carcinoma, and other solid malignant tumors (9-13). Interleukin (IL)-1 β is one of the most effective proinflammatory multifunctional cytokines (14). It is synthesized as a precursor in the cytoplasm. Precursor IL-1 β can obtain biological activity only after it is hydrolyzed and processed by proteins. However, there is still no evidence whether NLR and IL-1 β can be used as observation indicators

and predictive indicators of the efficacy on patients with acute myocardial infarction.

Therefore, in this study, patients with acute myocardial infarction were treated with aspirin combined with Clopidogrel. The expression of NLR and IL-1 β were recorded, and their predictive value for the efficacy on the patients was investigated, in order to provide references for clinical practice in the future.

MATERIALS AND METHODS

Study subjects

In this study, 82 patients with acute myocardial infarction were enrolled as study subjects. They were treated in our hospital between April 2012 and September 2015. The patients who were treated with aspirin combined with clopidogrel were included in a study group (42 patients), in which there were 23 males and 19 females aged 28-65 years, average (50.8 $\pm$ 10.6) years. The patients treated with aspirin alone were included in a control group (40 patients), in which there were 22 males and 18 females aged 29-68 years, average (51.3 $\pm$ 10.2) years. This study was approved by the medical ethics committee of the hospital.

Inclusion criteria: patients diagnosed and treated in our hospital; the clinical general data were complete; age

Key words: NLR; IL-1 β ; acute myocardial infarction; aspirin combined with clopidogrel

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between 25 and 70 years; education level was primary school and above; patients who were able to cooperate with the investigation; patients with no other serious organ diseases that could affect this study; patients or their immediate family members signed an informed consent form.

Exclusion criteria for the patients: died during treatment; complicated with other tumors and cardiovascular and cerebrovascular diseases; disabled; pregnancy; complicated with other autoimmune or chronic diseases; transferred to other hospitals; surgical contraindications, mental illness, language dysfunction and diseases that affected the results of this study.

Treatments

The patients in the control group were treated with aspirin alone (Huayin Shijin Qiancheng Pharmaceutical Co., Ltd., SFDA approval number: H20046739). Aspirin was taken with warm water for 30 consecutive days. The initial dosage was 300 mg, once per day. After 3 days, the dosage changed to 100 mg, once per day.

The patients in the study group were treated with aspirin combined with clopidogrel. The administration of aspirin in the study group was the same as that in the control group. Clopidogrel (Wuhan Wuyao Pharmaceutical Co., Ltd., SFDA approval number: H20123155) was taken with warm water for 30 consecutive days. The dosage was 300 mg per time, once a day. After 3 days, the dosage was reduced to 75 mg, once a day.

Evaluation of clinical efficacy

Markedly effective: precordial pressing pain, arrhythmia, fever and other clinical symptoms of the patient disappeared; the improvement of ECG was obvious. Effective: precordial pressing pain, arrhythmia, fever and other clinical symptoms of the patients basically disappeared; the improvement of ECG could be seen. Ineffective: precordial pressing pain, arrhythmia, fever and other clinical symptoms of the patient were not improved or the patient's condition worsened.

Detection methods

Five mL of fasting venous blood of the patients was collected 1 day before treatment and 2 months after treatment. The blood was left for 30 min, then it was centrifuged at 3000 rpm for 10 min and the serum was

collected. The serum and red blood cells were separated quickly and were stored at -80°C . An Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to detect the serum. IL-1 β test kits were purchased from Shanghai JingKang Bioengineering Co., Ltd., article number: JKSJ--2014. NLR test kits were purchased from Shanghai JingKang Bioengineering Co., Ltd., article number: JK-EA00921. Detection methods of IL-1 β and NLR were performed in strict accordance with the manufacturer's instructions.

Observation indicators

Main observation indicators: The clinical efficacy on the patients in the two groups was observed after treatment. Concentrations of IL-1 β and NLR in serum of the patients in the two groups were compared before and after treatment.

Secondary observation indicators: According to the predictive value of concentrations of IL-1 β and NLR for the efficacy on the patients, the relationship between IL-1 β , NLR, and the clinical efficacy on the patients was analyzed after treatment.

Statistical analysis

All data were processed and analyzed by SPSS 24.0 software system (Beijing Strong Vinda Information Technology Co., Ltd.). All graphics were drawn by Graphpad8 (Shenzhen SOFTHHEAD Inc.), and the results were checked twice. The count data were expressed as rate (%) and were analyzed by a *chi*-squared test, expressed as χ^2 . The comparison between groups was performed by independent sample *t*-test. The comparison in groups was analyzed by paired *t*-test, expressed as mean value $\pm$ standard deviation ($\bar{x} \pm s$), then it was analyzed by non-parametric test, expressed as *Z*. In the analysis of diagnostic value, a ROC curve was used to evaluate diagnostic efficiency and calculate sensitivity, specificity, etc. Spearman correlation test was used to analyze the relationship between IL-1 β , NLR, and clinical efficacy.

RESULTS

General clinical data of the patients

There were no significant differences between the age, gender, BMI, marriage, nationality, residence, smoking history, drinking history, and exercise

habits of the patients in the study group and those of the patients in the control group, indicating that their clinical data were comparable ($p > 0.05$).

Clinical efficacy on the patients in the two groups

In the study group, 23 patients were markedly effective (54.76%), and 12 patients were effective (28.57%). In the control group, 18 patients were markedly effective (45.00%), and 9 patients were effective (22.50%). The treatment effective rate in the study group (83.33%) was significantly higher than that in the control group (67.50%), and the differences were statistically significant ($p < 0.05$) (Table I).

Comparison of concentrations of IL-1 β and NLR in serum of the patients in the two groups before and after treatment

The concentration of IL-1 β in serum of the patients in the study group was (105.16 $\pm$ 28.57) pg/ml before treatment, the concentration of NLR was (8.72 $\pm$ 0.61) pg/ml. The concentration of IL-1 β in serum of the patients in the control group was (104.89 $\pm$ 29.12) pg/ml before treatment, the concentration of NLR was (8.67 $\pm$ 0.75) pg/ml. There were no significant differences between the two groups ($p > 0.05$). The concentration of IL-1 β in serum of the patients in the study group was (84.51 $\pm$ 5.03) pg/ml after treatment, the concentration of NLR was (2.63 $\pm$ 0.59) pg/ml. The concentration of IL-1 β in serum of the patients

in the control group was (97.24 $\pm$ 6.33) pg/ml after treatment, the concentration of NLR was (4.89 $\pm$ 0.84) pg/ml. Concentrations of IL-1 β and NLR in the study group were significantly lower than those in the control group, $p < 0.05$ (Fig. 1).

Predictive value of concentrations of IL-1 β and NLR for the efficacy on the patients after treatment

According to the ROC curve, when the cut-off value was 90.85, the sensitivity of the predictive value of IL-1 β for the efficacy on the patients was

Table II. ROC curve analysis

	IL-1 β	NLR
AUC	0.758	0.777
Std.Error	0.054	0.055
95%5CI	0.653~0.863	0.669~0.882
P	0.001	0.001
Cut-off	90.85	3.739
Sensitivity (%)	61.90	62.50
Specificity (%)	88.10	97.62

Table I. Comparison of the efficacy

	Study group (n=42)	Control group (n=40)	Z/ χ^2	P
Markedly effective	23 (54.76%)	18 (45.00%)	-1.298	0.194
Effective	12 (28.57%)	9 (22.50%)		
Ineffective	7 (16.67%)	13 (32.50%)		
Treatment effective rate	83.33%	67.50%	2.785	0.001

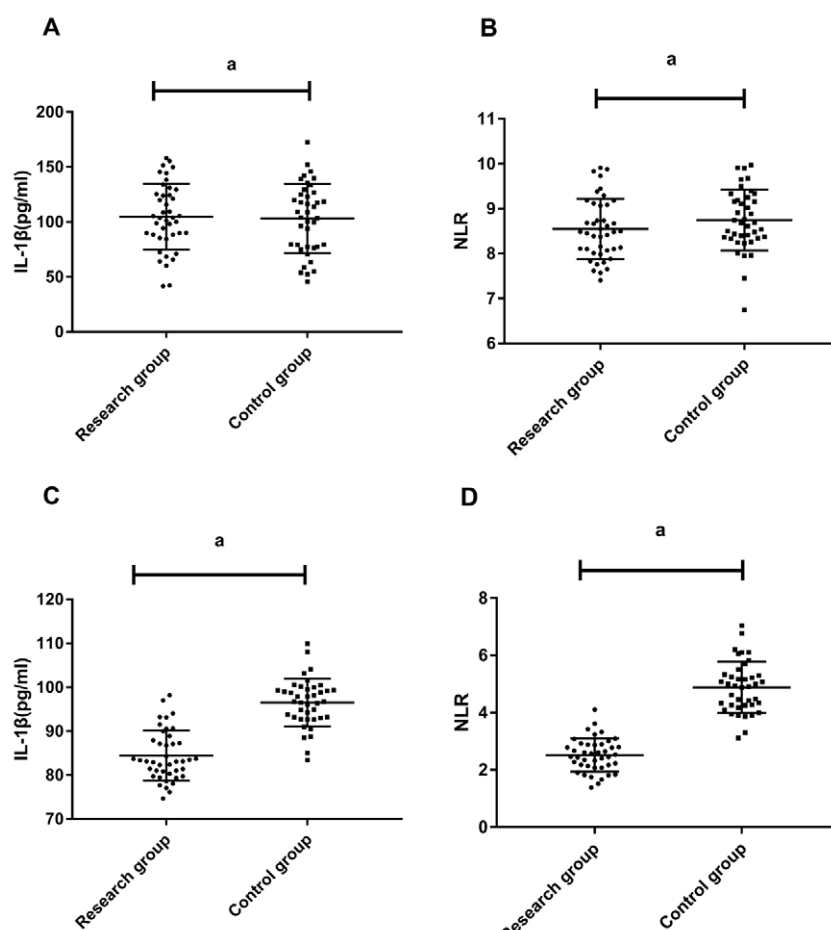


Fig. 1. The comparison of concentrations of IL-1 β and NLR in serum of the patients in the two groups before and after treatment. The concentration of IL-1 β (A) and the concentration of NLR (B) before treatment. There were no significant differences in concentrations of IL-1 β and NLR in serum of the patients in the two groups before treatment, $p > 0.05$. The concentration of IL-1 β (C) and the concentration of NLR (D) after treatment. Concentrations of IL-1 β and NLR in serum of the patients in the study group were significantly lower than those of the patients in the control group after treatment, $p < 0.05$. ^a denotes no significant difference between two groups. * significant difference between two groups.

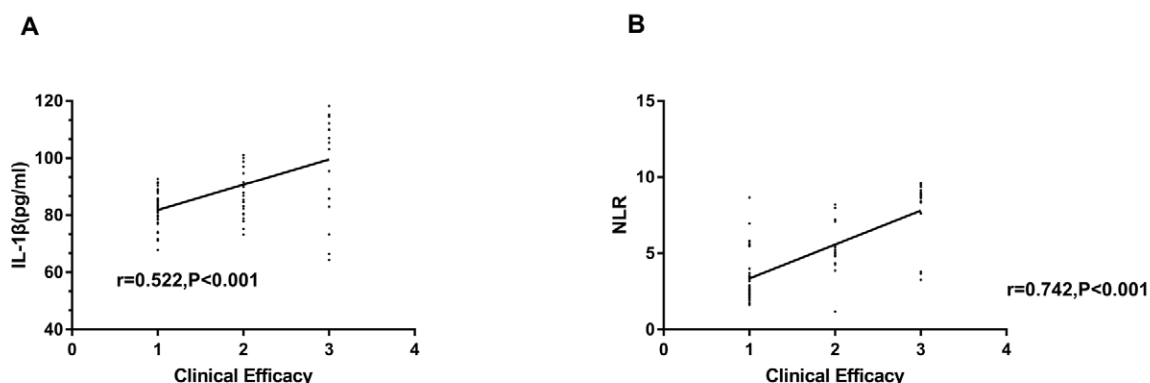


Fig. 2. The predictive value of concentrations of IL-1 β and NLR for the efficacy on the patients after treatment. The predictive value of IL-1 β for the efficacy on the patients (A). When the cut-off value was 90.85, the sensitivity of the predictive value of IL-1 β for the efficacy on the patients was 61.90%, the specificity was 88.10%. The predictive value of NLR for the efficacy on the patients (B). The sensitivity of the predictive value of NLR for the efficacy on the patients was 62.50%, the specificity was 97.62%.

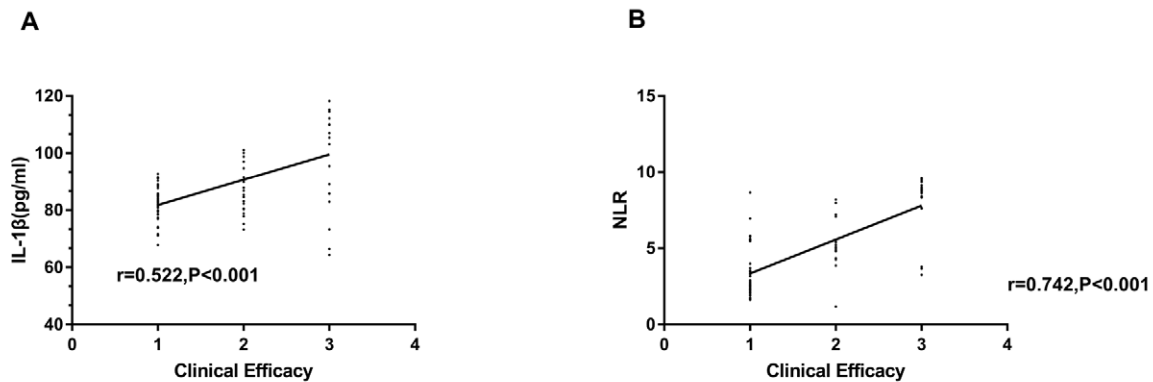


Fig. 3. The relationship among IL-1 β (A), NLR (B), and the clinical efficacy on the patients after treatment. A Spearman correlation test was used to analyze the relationship between IL-1 β , NLR, and the clinical efficacy on the patients. It was found that expression levels of IL-1 β and NLR gradually increased as the clinical efficacy worsened, and they were positively correlated.

61.90%, the specificity was 88.10%, and the AUC was 0.758. When the cut-off value was 3.739, the sensitivity of the predictive value of NLR for the efficacy on the patients was 62.50%, the specificity was 97.62%, and the AUC was 0.777 (Table II).

Relationship between IL-1 β , NLR, and the clinical efficacy on the patients after treatment

A Spearman correlation test was used to analyze the relationship between IL-1 β , NLR, and the clinical efficacy on the patients. It was found that expression levels of IL-1 β and NLR gradually increased while the clinical efficacy worsened, and they were positively correlated (Fig. 2).

DISCUSSION

In this study, the clinical efficacy of the treatment on the patients in the two groups was compared. The results showed that there was no difference between the clinical efficacy on the patients in the study group and the control group. However, the treatment effective rate in the study group was higher than that in the control group. This result indicates that aspirin combined with clopidogrel can increase the treatment effective rate of patients. There were no differences in concentrations of IL-1 β and NLR in serum of the patients in the two groups before treatment. However, concentrations of IL-1 β and NLR in serum of the patients in the study group were

significantly lower than those of the patients in the control group after treatment. This result indicates that concentrations of IL-1 β and NLR can be reduced by adding clopidogrel. The expression levels of IL-1 β and NLR gradually increased as the clinical efficacy worsened. This result indicates that the expression of IL-1 β and NLR is closely related to clinical efficacy. Finally, a ROC curve was drawn and analyzed, it was found that the AUC of the expression of IL-1 β and NLR was 0.758/0.777 in predicting the patient clinical efficacy after treatment, and IL-1 β and NLR had good clinical value.

In summary, aspirin combined with clopidogrel can improve the clinical efficacy on patients, and can increase the overall treatment rate of patients. IL-1 β and NLR in serum of patients with acute myocardial infarction after they are treated have predictive value for the clinical efficacy on patients.

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LETTER TO THE EDITOR

Angiopoietin-like protein 4 serum concentration in colorectal cancer patients – potential metastasis biomarker? Preliminary studyA. Kulig^{1,2}, J. Strzelczyk³, K. Janion¹, M. Skrzypek⁴ and E. Nowakowska-Zajdel^{1,2}¹*Department of Nutrition-Related Disease Prevention, Department of Metabolic Disease Prevention, School of Health Sciences in Bytom, Medical University of Silesia in Katowice, Poland;*²*Department of Clinical Oncology, No. 4 Provincial Specialist Hospital in Bytom, Bytom, Poland;*³*Department of Medical and Molecular Biology, School of Medical Sciences in Zabrze Medical University of Silesia in Katowice, Poland;* ⁴*Department of Biostatistics, School of Health Sciences in Bytom, Medical University of Silesia in Katowice, Poland**Received April 13, 2021 – Accepted August 24, 2021*

To the Editor,

The tumor microenvironment is a site of constant inflammatory processes mediated by its heterogeneous cellular components including immune inflammatory cells, fibroblasts, endothelial cells, pericytes and proliferating cancer cells, as well as the extracellular components containing the extracellular matrix and matricellular proteins (1).

Angiopoietin-like protein 4 (ANGPTL4) is the circulating plasma protein belonging to the angiopoietin family (2). ANGPTL4 expression has been found in many tissues and organs, including the liver, adipose tissue, placenta, and other tissues in terms of ischemia and cancer (3). It does not bind to the angiopoietin receptors (Tie 1 and Tie 2), it is an orphan ligand, thus its role is complex and ambiguous (3). As other angiopoietins, ANGPTL4 plays a role in the regulation of lipid and glucose metabolism (4). Increasing evidence indicates its role as the matricellular protein affecting tissue transformation, tumor progression and metastasis, altered redox regulation, angiogenesis, tumor microenvironment, wound healing, vascular permeability, and inflammation (2, 3, 5). The detailed mechanisms of

ANGPTL4 protein in human colorectal cancer (CRC), its association with inflammatory processes related to cancer and other clinicopathological factors require further investigation. Several studies revealed the high expression of ANGPTL4 in metastatic cancers, suggesting that it may play a role in the metastasis (3). ANGPTL4 overexpression promotes colon cancer cell migration through the cytoskeleton signal pathway, reorganization of F-actin and pseudopodia formation (6, 7). It affects vascular endothelial cell-cell junctions, increases capillary permeability, and facilitates the trans-endothelial migration of tumor cells (6). Studies on CRC cell line indicate the link between ANGPTL4 expression and venous and lymph duct invasion (6). The expression of ANGPTL4 may be induced by hypoxia, resulting from the intensified oxygen consumption and insufficient oxygen supply in rapidly growing tumor cells (6, 8). The development of a hypoxic microenvironment in the tumor promotes ANGPTL4 expression via the transcription factor hypoxia-inducible factor-1 α (HIF-1 α), and further promotes cancer invasion and metastases in CRC (8). ANGPTL4 is postulated to exert a VEGF-independent proangiogenic effect (7).

*Key words: ANGPTL4; biomarker; colorectal cancer; inflammation; metastasis; signaling pathways**Corresponding Author:*

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The aim of this study was to examine the ANGPTL4 serum concentration in patients with CRC and to explore the potential association between ANGPTL4 level and selected clinicopathological features, including selected new systemic inflammatory response (SIR) indicators determined on the basis of peripheral blood morphology.

MATERIALS AND METHODS

The study included 119 consecutive patients hospitalized in the Department of Clinical Oncology in the years 2016-2018 and qualified to the systemic therapy. The inclusion criteria for the study were: histopathologically confirmed colorectal adenocarcinoma; clinical stage (CS) I-IV; age > 18 years; patient's informed consent to perform additional biochemical tests – from a single puncture at a routine blood test; at least 4 weeks after surgical treatment if it was carried out; no chemotherapy for colorectal cancer in the previous 6 months. The exclusion criteria included: other active or past cancer except squamous cell carcinoma of the skin; diagnosed infectious disease (hepatitis B or C, HIV infection or AIDS syndrome, tuberculosis); systemic disease requiring immunosuppression; cancer cachexia. The peripheral venous blood samples (3 mL) were collected from each patient from a single puncture at the routine blood test necessary for qualification for chemotherapy. The blood was centrifuged, and the obtained serum was frozen and stored at -80°C pending assays.

The ANGPTL4 serum concentration was assessed by ELISA method using the KIT-Cloud-Clone Corporation tests, according to the procedure described by the kit manufacturer. Tests were carried out in the Department of Medical and Molecular Biology, Medical University of Silesia in Katowice. The study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. The study received approval from the Medical University of Silesia, Katowice, Poland, Ethics Committee (Resolution no. PCN/0022/KB1/42/V/14/16/18/19/20 dated July 14, 2020). Participation in the study was voluntary. Patients' demographics and clinical and pathological characteristics were collected based on medical history. Additionally, in the subgroup of 65 patients, selected indicators were analyzed of immunological processes based on peripheral blood count

with automatic smear. Neutrophil-to-lymphocyte ratio (NLR) was calculated by dividing absolute neutrophil count by absolute lymphocyte count. Similarly, platelet-to-lymphocyte ratio (PLR) was calculated by dividing platelet count by the absolute neutrophil count. LMR or NMR was calculated by dividing the absolute number of lymphocytes or neutrophils by the absolute number of monocytes, respectively. The cut-off points reported in the literature were used for subgroup analysis: $NLR \geq 3$ vs < 3 (26-28) and $PLR \geq 150$ vs < 150 (13,28-30). NPS was calculated as follows: patients with a neutrophil count $\leq 7.5 \times 10^9/L$ and platelets $\leq 400 \times 10^9/L$ scored 0, patients with neutrophils $> 7.5 \times 10^9/L$ or platelets $> 400 \times 10^9/L$ scored 1 and patients with both neutrophils $> 7.5 \times 10^9/L$ and platelets $> 400 \times 10^9/L$ scored 2 (31). All patients were put on a regular follow-up in the outpatient clinic for every 3–6 months for the first 2 years and every 6 months thereafter. Disease-free survival (DFS) was measured from the start of first cycle of adjuvant chemotherapy to the date of disease recurrence or the date of last follow-up visit in patients without recurrences. Progression-free survival (PFS) was defined as the period from the start of first cycle of first line palliative chemotherapy to the date of disease progression. Overall survival (OS) was calculated from the date of the beginning the first cycle of chemotherapy to the date of death or last follow-up visit. The mean observation period was 25.9 months (median 13.1, range 8.9-30.8). The final assessment of disease status was made on August 15th, 2020.

Statistical analysis

Statistical analysis was carried out using the Statistica version 13 software package (StatSoft, Inc., Tulsa, Oklahoma, United States) and IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, New York, United States). For the ANGPTL4 serum concentration, established thresholds have not yet been established. Hence, the optimal cut-off levels for ANGPTL4 were determined by applying receiver operating characteristic (ROC) curve analysis (based on joint sensitivity and specificity). Continuous variables are represented as the arithmetic mean with a standard deviation for the variables with normal distribution or as the median with an interquartile range for the non-normal distribution. The distribution normality

was assessed using the Shapiro-Wilk test. Continuous variables with normal distribution were compared using the Student's *t*-test, while variables with non-normal distribution were compared using the Mann-Whitney U-test. To compare categorical data χ^2 test and Fisher's exact test were used. Survival analysis was performed using the Kaplan-Meier method and differences between the groups were assessed by the log-rank test.

RESULTS

The study group consisted of 119 patients (64 males, 55 women) aged 39-86 years (mean 65.7 ± 9.6 years). There were no patients diagnosed as the CSI of cancer. CSII of CRC was diagnosed in 37 patients, CSIII in 33 and CSIV in 49 patients. The

ANGPTL4 serum concentrations in the entire study group ranged from 2.51 to 56.55 ng/ml [median 11.25 (4.37 – 17.87) ng/ml]. Patients' age, gender, or BMI showed no difference in terms of the ANGTL4 serum concentration. The ANGTL4 serum level did not differ in terms of tumor grade and location. We noted the relationship between the TNM stage of CRC and ANGPTL4 serum concentration in the analyzed population. ANGPTL4 was higher in patients with metastatic disease compared to CSIII and CSII of CRC cancer patients. The study group characteristic in term of ANGPTL4 serum concentration and clinical stage and the presence of distant metastases are presented in Tables I and II, accordingly.

In the subgroup of patients with CSII and CSIII, there was a trend to higher ANGPTL4 levels in

Table I. Study group characteristics in term of CS of CRC

Variable	Clinical stage			p
	II (n = 37)	III (n = 33)	IV (n = 49)	
ANGPTL4 [ng/ml]	5.92 (2.91 – 13.54)	11.43 (5.0 – 17.87)	13.49 (4.76 – 21.76)	0.0275
Age [years]	66 (61 – 70)	65 (58 – 70)	68 (63 – 74)	0.239
BMI [kg/m ²]	25.8 (21.9 – 29.2)	27.8 (24.5 – 30.6)	52.1 (22.6 – 30.54)	0.107
CEA [ng/ml]	2.8 (2.1 – 3.3)	3.3 (1.5 – 7.0)	60.0 (11.1 – 342.4)	<0.00001
WBC [x10 ⁹ /L]	6.90 (5.86 – 8.50)	6.58 (5.17 – 8.17)	7.3 (6.54 – 9.68)	0.142
Neu* [x10 ⁹ /L]	3.76 (2.96 – 4.54)	3.56 (3.11 – 4.95)	4.63 (3.61 – 6.47)	0.090
NLR*	1.72 (1.31 – 3.07)	2.47 (1.55 – 3.63)	2.52 (1.90 – 3.15)	0.137
NMR*	6.34 (5.54 – 10.97)	8.50 (6.03 – 10.90)	7.83 (6.34 – 9.79)	0.469
LMR*	4.17 (3.92 – 5.46)	3.68 (2.95 – 4.66)	3.28 91.93 – 4.05)	0.025
PLT [x10 ⁹ /L]	294 (260 – 386)	272 (215 – 300)	293 (221 – 354)	0.077
PLR*	128.1 (104.1 – 207.5)	150.8 (111.9 – 237.0)	152.4 (106.8 – 235.4)	0.828
MPV [fl]	7.3 (6.8 – 7.8)	7.7 (7.2 – 7.9)	7.8 (7.1 – 8.8)	0.271
MPV/PLT ratio	0.024 (0.019 – 0.027)	0.030 (0.023 – 0.039)	0.032 (0.020 – 0.038)	0.243

*Data on parameters based on peripheral blood smear data were available for 65 patients. WBC: white blood cell count; Neu: absolute neutrophil count; MPV: mean platelet volume; PLT: absolute platelet count

Table I. Study group characteristics in term of CS of CRC

Variable	Distant metastases		p
	Present (n = 49)	Absent (n = 70)	
ANGPTL4 [ng/ml]	13.49 (4.76 – 21.76)	6.88 (4.34 – 16.56)	0.0494
Age [years]	68 (63 – 74)	66 (58 – 70)	0.092
BMI [kg/m ²]	25.1 (22.6 – 30.5)	26.5 (23.1 – 29.8)	0.550
CEA [ng/ml]	60 (11.1 – 342.4)	2.95 91.60 – 5.35)	0.000001
WBC [x10 ⁹ /L]	7.30 (6.54 – 9.68)	6.70 (5.64 – 8.50)	0.063
Neu* [x10 ⁹ /L]	4.63 (3.61 – 5.11)	3.72 (3.00 – 4.700)	0.0286
NLR*	2.52 (1.90 – 3.15)	1.96 91.34 – 3.27)	0.120
NMR*	7.83 (6.34 – 9.79)	8.32 (5.86 – 10.94)	0.588
LMR*	3.28 (1.93 – 3.05)	3.86 (3.25 – 4.82)	0.0113
PLT [x10 ⁹ /L]	293 (221 – 354)	285 (235 – 335)	0.604
PLR*	152.4 (106.8 – 235.4)	148.2 (108.7 – 213.6)	0.799
MPV [fl]	7.8 (7.1 – 8.8)	7.45 (7.10 – 7.90)	0.200
MPV/PLT ratio	0.031 (0.020 – 0.038)	0.025 90.021 – 0.036)	0.537

*Data on parameters based on peripheral blood smear data were available for 65 patients

patients with higher histological grade compared to lower grade. No differences were observed in ANGPTL4 concentrations depending on tumor size and lymph node invasion in patients with CSII and III.

A ROC curve was used to determine the optimal cut-off value of ANGPTL4 serum concentration. As a result, an ANGPTL4 of 12.63 ng/ml yielded maximum combined sensitivity and specificity (Fig. 1), showing that ANGPTL4 predicted the presence of distance metastasis with a sensitivity of 0.592 and a specificity of 0.429 (AUC = 0.606, 95% CI: 0.501–

0.712, p = 0.0488). On this basis, the patients were classified into two subgroups: those with ANGPTL $\geq$ 12.63 ng/ml and those with ANGPTL < 12.63 ng/ml. The subgroup comparison is presented in Table III.

The subgroup of patients with ANGPTL4 serum concentration $\geq$ 12.63 ng/ml was characterized by higher serum concentration of CEA compared to individuals with lower ANGPTL4. Considering the designated ANGPTL4 cut-off point, no differences were observed in analyzed peripheral blood count parameters and immunological indicators calculated

on the basis of blood count. ANGPTL4 concentration was not associated with the DFS and overall survival (OS) in patients with CSII and CSIII ($p = 0.222$ and $p = 0.906$ in log-rang test, accordingly; Fig. 2). Similarly, no differences were observed in PFS and

OS in patients with metastatic CRC considering the ANGPTL4 levels ($p = 0.208$ and $p = 0.864$ in long-rank test, accordingly; Fig. 3).

DISCUSSION

In the study group, the ANGPTL4 serum concentration in patients with metastatic disease was significantly higher compared to patients with CSII and CSIII of CRC. The literature data on the relation between the intensity of ANGPTL4 expression and tumor staging or recognized prognostic factors are conflicting. Nakayama et al. observed the association between positive expression of ANGPTL4 in the tumor tissues and larger tumor volume in CRC patients, however the ANGPTL4 mRNA expression was not correlated with tumor grade, lymph node metastasis and TNM stage (6). The studies of the colon cancer cell line support these observations (7). Other results indicate that overexpression of ANGPTL4 may inhibit tumor growth, metastasis and angiogenesis in selected types of neoplasms –

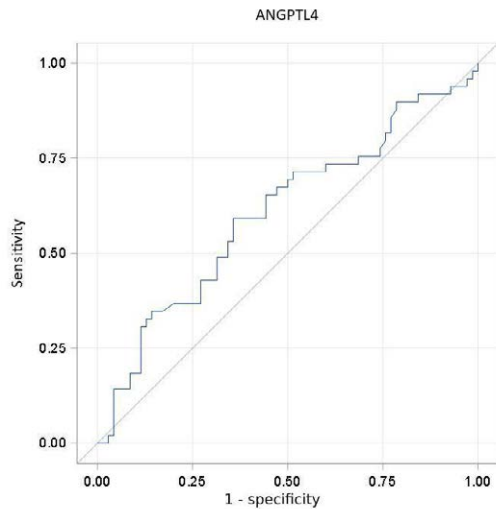


Fig. 1. Optimized cut-off was determined for ANGPTL4 serum concentration using standard ROC curve analysis.

Table III. The relationship between ANGPTL4 serum concentration and selected clinical parameters and indicators of inflammation

Variable	ANGPTL4 [ng/ml]		p
	<12.63	≥12.63	
Age [years]	66 (58 – 73)	67 (61 – 73)	0.934
BMI [kg/m ²]	25.86 (23.11 – 29.72)	26.1 (22.5 – 30.3)	0.783
CEA [ng/ml]	2.3 (1.5 – 7.0)	14.4 (3.3 – 191.4)	0.0179
WBC [x10 ⁹ /L]	7.30 (6.10 – 8.80)	6.92 (5.84 – 8.84)	0.748
Neu [x10 ⁹ /L]	3.61 (2.94 – 4.59)	4.43 (3.33 – 5.23)	0.172
NLR	1.76 91.30 – 2.66)	2.49 (1.77 – 3.28)	0.131
NMR	6.90 (5.04 – 11.60)	8.22 (6.20 – 10.04)	0.757
LMR	4.07 (3.42 – 4.73)	3.54 (2.65 – 4.44)	0.165
PLT [x10 ⁹ /L]	291 (236 – 343)	298 (217 – 339)	0.668
PLR	176.8 (99.40 – 228.3)	151.2 (110.5 – 213.5)	0.634
MPV [fl]	7.3 (7.2 – 7.7)	7.7 (7.1 – 8.1)	0.748
MPV/PLT ratio	0.025 (0.020 – 0.039)	0.028 (0.021 – 0.037)	0.956

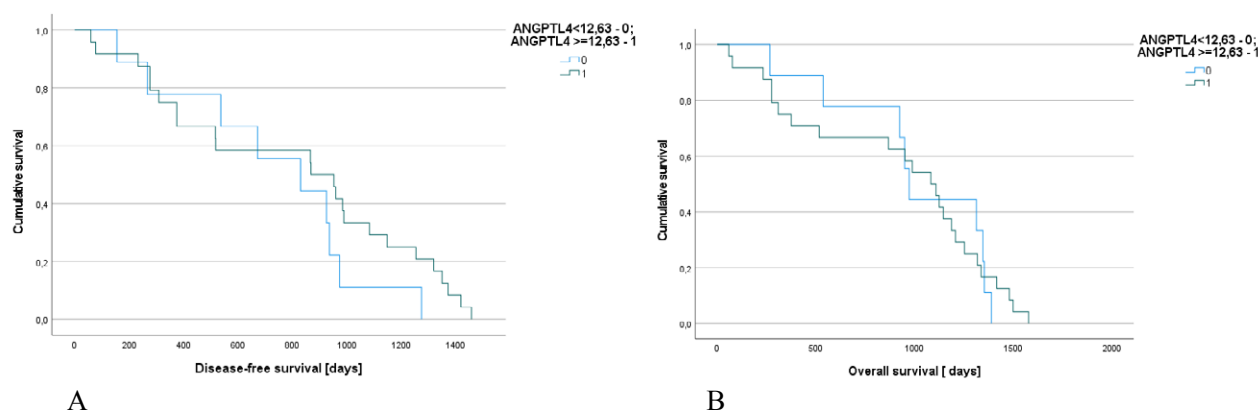


Fig. 2. DFS (A) and OS (B) of patients with CRC stage II and III according to ANGPTL4 serum concentration.

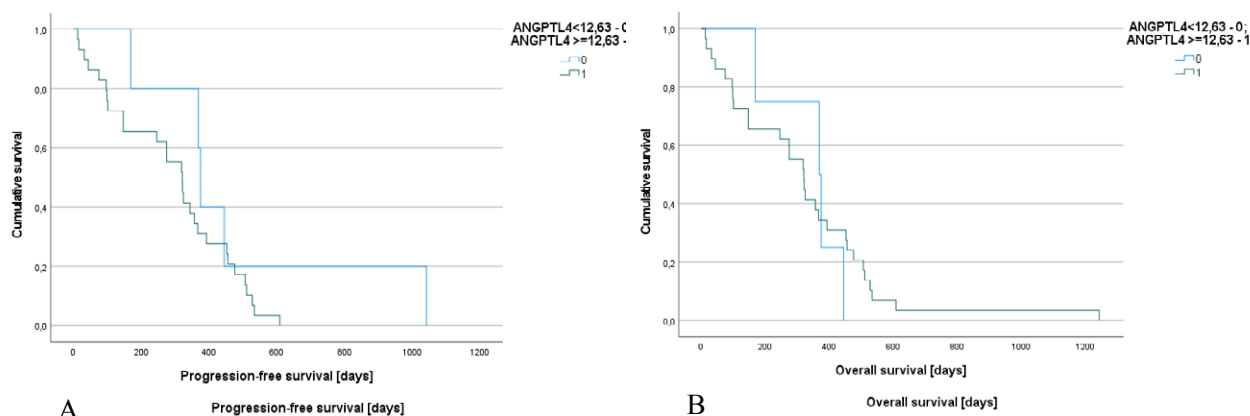


Fig. 3. PFS (A) and OS (B) of patients with CRC stage II and III according to ANGPTL4 serum concentration.

CRC, lung cancer, melanoma (3, 9). The reason for such conflicting results is unclear. The expression of ANGPTL4 and its transcriptional regulation are affected by the nuclear hormone receptors of the PPAR family, as well as hypoxia and fasting (3, 7). ANGPTL4 is involved in regulation of inflammatory signaling. Hypoxic conditions in malignant solid tumors derives gene and protein expression and secretion of ANGPTL-4, promoting chronic inflammation and macrophage infiltration (10). The local cancer-related inflammation seems to be associated with SIR. To the authors' knowledge, this is the first analysis to compare ANGPTL4 concentrations with new inflammatory factors based on the peripheral blood smear. We found the

significant higher absolute neutrophil count lower LMR in patients with metastatic disease compared to non-metastatic CRCs. Neutrophils may trigger a pro-tumoral environment by inhibition of lymphocyte-mediated cytotoxicity and release of cytokines and chemokines that stimulate angiogenesis and tumor cell proliferation and metastasis (11). Thus, the high neutrophil count may correlate to negative prognosis. Lymphocytes promote the antitumor immune response via inducing cytotoxic cell death and inhibition of tumor cell proliferation and migration. Declined lymphocyte counts (thus lowering the LMR) can be associated with downregulation of the anti-cancer immune response. On the other hand, circulating monocytes may reflect the formation of

tumor-associated macrophages which is attributed to accelerate angiogenesis, invasion, migration, and tumor growth (12). A low LMR, which is the result of low lymphocyte count and high monocyte count, reflects insufficient antitumor immunity and a high tumor burden (12). Our analysis of ANGPTL4 serum concentrations in relation to the new indicators of SIR based on a peripheral blood smear revealed no statistically significant dependence. Moreover, the relation between ANGPTL4 serum concentration and survival was not confirmed. However, the size of the subgroups was small, and the analyses should be repeated on a larger study group. As usual, several various clinical factors may influence the status of serum level of proteins, which are known as important in carcinogenesis and disease progression. The search for correlations between ANGPTL4, the inflammatory markers and other clinical factors may contribute to understanding interactions for axes of carcinogenesis and cancer-related inflammation. Further studies are needed to determine the precise roles of ANGPTL4 in colorectal cancerogenesis.

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LETTER TO THE EDITOR

Is chemical plaque control, carried out with chlorhexidine, a successful requisite for cause-related therapy outcomes foregoing periodontal surgery? A controlled, randomized study

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To the Editor,

Specific bacteria and immuno-inflammatory mechanisms have been connected to the pathogenesis of periodontitis (1-3). The microbiota activation of immuno-inflammatory pathways induces the loss of periodontal attachment leading to periodontal pocket formation (3). Substantially, little is known of the specific inflammatory alterations and pathogenic mechanisms involved in periodontal disease onset, thus cause-related therapy, aiming to correct the causes that lead to periodontal disease, is a ‘must do’ and focuses on controlling the “external” risk and prognostic factors of periodontal disease (such as microbiota or lifestyle). The cause-related therapy is more often than not able to resolve the periodontal defects, otherwise it is followed by an adjunctive surgical periodontal therapy.

The additional preventive protocols applied during the cause-related therapy (in addition to mechanical plaque control) quite often include fluoridated or chlorhexidine (CHX) toothpastes or rinses. However, the use of CHX is burdened by some side effects, mainly related to stains, alterations in taste and erythematous–desquamative lesions of the oral mucosa, parotid swelling, and increase of calculus formation in long-term use. Among these

factors, the most frequent is brown pigmentations that appear on the dental surfaces, prosthetic and composite restorations, and tongue after prolonged use, whereas other consequences are fairly rare or exceptional (4). Tooth surfaces, increasing their own roughness with pigmentation, increase plaque deposition. The evidence that these CHX-based approaches offer tangible benefits, however, remains debatable (4).

The aim of this controlled, randomized cohort study is to compare the clinical outcomes obtained after the cause-related therapy on patients with periodontal disease using two different additional preventive regimens: (A) fluoridated toothpaste and mouth rinses; and (B) 0.05% chlorhexidine toothpaste + chlorhexidine-based oral rinse 0.12% with an anti-discoloration system (CHX+ADS).

MATERIALS AND METHODS

Study population

This study was performed at the Periodontology Unit of Dentistry and Oral-Maxillofacial Surgery of Modena University Hospital (Periodontology Unit of Dentistry and Oral-Maxillofacial Surgery). All procedures of this clinical were approved and supervised by the local ethics

Key words: cause-related therapy; chlorhexidine; fluoride ions; oral hygiene products

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committee of the Health Service of the Emilia-Romagna region (University Hospital of Modena, protocol number 4406/CE, registration number 369/17). All the patients considered in this study signed an informed consent detailing all procedures of the treatment, as requested by Helsinki protocols.

Subjects referred for dental and periodontal therapy were selected between December 2017 and January 2021. Healthy patients, affected by moderate-to-advanced periodontitis, were screened during the first step of therapy for the presence of at least twenty teeth. At end of the first step, inclusion criteria for the patients were as follows: 18 years or older; non-pregnant or lactating; non-smokers and without a history of alcohol abuse; medical history of good health (5, 6); and high levels of compliance (4, 7). Moreover, the selected patients had to show an extent and/or a typology of periodontal lesions, at the end of the first step, that would be treated most likely with periodontal surgical adjunctive therapy after a more specific phase of cause-related therapy (the second step). Exclusion criteria were as follows: diagnosis of bone diseases; rheumatoid arthritis; diabetes mellitus; unstable or life-threatening conditions; health conditions requiring antibiotic prophylaxis; and the presence of dental implants or more than four prosthetic crowns and 2 pontics (4).

Periodontal procedures and clinical measurements

The study design was set out in three consecutive steps. The first step comprised the patient evaluation, case history, dental screening and a first phase of cause-related therapy which included patient motivation, professional oral hygiene, gingivitis therapy, taking care of caries, of defective contours of restorations, required endodontic therapies, and extractions of hopeless teeth. Emergencies, such as pain or acute dental-periodontal infections, were also treated to provide a stable dental-periodontal condition.

The second step was re-evaluation and qualification of the patients' state of oral health after the first step of cause-related therapy. After accomplishing the first step, patients' dental pathology and their respective compliance were better known and the remaining periodontal damage was presumed to derive mainly from periodontal disease, therefore the consequent second phase of cause-related therapy was specifically aimed to deal with it. Periodontal conditions were therefore carefully evaluated and specifically treated with cause-related therapy in the

second step and the randomized patients' assignment (patients who met the inclusion criteria) in the two different study cohorts were performed at the beginning of this step. The enrolled patient pool had most likely to be treated by surgical therapy because of the severity of the residual periodontal lesions at the end of the first step. The cause-related therapy performed during the second step had to be extremely effective, in order to be preliminary to the subsequent highly probable adjunctive therapy, and also effectively reduce the bleeding. Randomization was performed by computer-generated random codes, using random permuted blocks with a block size of 4 to avoid uneven splits. Allocation was hidden, by using opaque envelopes, which were opened after randomization and at the beginning of the second phase of cause-related therapy for each respective patient. All clinical measurements (baseline included) were performed by a double-blind examiner.

The full-mouth plaque score (FMPS) was assessed by using a plaque disclosing gel. The percentage of total tooth surfaces (6 aspects per tooth) displaying the presence of plaque was recorded. Using a periodontal probe with 1-mm marks (modified Click-Probe, Kerr Corp., Bioggio, Switzerland), the full-mouth bleeding score (FMBS), probing pocket depth (PPD) and recession depth (REC) were assessed, 6 aspects per tooth, and were calculated on full mouth (full-mouth PPD-FMPPD and full-mouth REC-FMREC, respectively). The gingival bleeding (GB) index was calculated 4 aspects per tooth using a gum stimulator (gum stimulator – Sunstar Americas Inc., Chicago, USA) as the percentage of sites displaying bleeding on stimulation of total tooth surfaces (gingival bleeding). FMPS, FMBS and GB were considered as percentages of all the tooth sites considered, while FMPPD and FMREC were the resulting mean of the respective recordings carried out on all the teeth. All the indices were recorded at the beginning (baseline data-T1) and at the end of the second step (T2). The T2 recordings were performed after about 5 weeks from the last scaling-root planing and three months after the beginning of the second step.

During the first step, clinicians and hygienists clearly educated patients that supragingival and subgingival dental plaque is the primary etiologic factor for periodontal diseases (as well as dental caries). By means of models and intraoral demonstrations, patients were taught how to use a toothbrush, tongue brush as well as interproximal

cleaning aid (i.e. dental floss, interproximal toothpick, interproximal brush), and had to demonstrate their competency in using these tools properly and effectively. These hygienic indications were reinforced during the second and the subsequent steps.

During the second step, a cohort of patients (A cohort – control cohort) used fluoridated toothpaste and rinse (Elmex toothpaste and rinse®, Colgate-Palmolive Company, Roma, Italy), and the other cohort (B cohort) used CHX toothpaste and rinse (Curasept toothpaste 0.05%+ ADS® and rinse + ADS 0.12%®, Curasept s.p.a., Saronno, VA, Italy). Except for the CHX+ADS use as an alternative to fluorine use, the oral hygiene protocol applied was the same in the two cohorts of patients (implemented with the aid of dental floss, interdental brushes and tongue brushes). None of the full-mouth disinfection protocols was ever applied.

Consequently, the third step varied on the basis of the clinical outcome obtained with cause-related therapy. Forty patients, 30 women and 10 men, aged 33-69 years fulfilled the entry criteria of the retrospective study. Twenty patients, 16 women and 6 men, aged 42-69 years (mean±SD 55.4±12.9 years) were enrolled in A cohort and 20 patients, 14 women and 6 men, aged 33-69 years (53.3±11.3 were enrolled in B cohort. At the end of the second step, only 1 patient of the A cohort and 2 of the B cohort showed such a reduction of periodontal defects that they did not have to undergo periodontal surgery. At the end of follow-up all the patients were included in the study.

Statistical analysis

The primary outcomes considered were the FMPS, FMBS, GB, FMPPD and FMREC comparisons between the A and B cohort in T2. Moreover, the comparison between the two cohorts in the difference of FMPPD recorded in T1 and T2 was considered a further primary outcome. The secondary outcome was the intra-group change analysis of the same indices between T1 and T2. The adjusted Fisher–Pearson standardized moment coefficient G was calculated, and skewness was refused if $-2 < G < +2$ (8). Statistical analysis was performed using the variance analysis or t -test for the comparison of two cohorts of patients in the same phase (T1 or T2), and paired t -test for the clinical data taken before and after the second step of cause-related treatment. The χ^2 test (nominal data) was applied to compare the gender distribution between

the two treated groups (6, 8). For all measured variables, the null hypothesis (H_0) of no difference among groups was rejected for a critical significance level of $p < 0.05$, as previously described (6, 8).

RESULTS

At baseline (T0), the adjusted Fisher–Pearson test, calculated from numerical data, always resulted in $-2 < G < +2$, so skewness hypothesis was refused (8). At baseline (T0), the FMPS and FMBS were respectively $42 \pm 13.2\%$ and $30.5 \pm 6.5\%$ in A cohort, and $41 \pm 13\%$ and $29.8 \pm 6.3\%$ in B cohort. GB resulted in $20.4 \pm 9.5\%$ and $21.3 \pm 8.9\%$ respectively in the A and B cohort. FMPPD resulted in $2.4 \pm 0.2\text{mm}$ in A cohort and $2.3 \pm 0.2\text{mm}$ in B cohort. FMREC was $2.2 \pm 0.9\text{mm}$ in A cohort and $2.2 \pm 0.9\text{mm}$ in B cohort.

The comparison between A and B cohorts at baseline (T1) was not statistically significant for age, FMPS, FMBS, GB, FMPPD, FMREC, gender ($p \geq 0.05$, t -test analysis and χ^2 test for gender).

At the end of the second step (T2), the adjusted Fisher–Pearson test always resulted in $-2 < G < +2$, so the skewness hypothesis was again refused. FMPS and FMBS were respectively $16 \pm 3\%$ and $9.2 \pm 3.8\%$ in A cohort and $14.4 \pm 2.9\%$ and $11.4 \pm 4.1\%$ in B cohort. GB resulted in $2 \pm 1.5\%$ and $4.5 \pm 2.2\%$, respectively, in the A and B cohorts. FMPPD resulted in $2.1 \pm 0.3\text{mm}$ in A cohort and $2 \pm 0.4\text{mm}$ in B cohort. FMREC was $2.3 \pm 0.9\text{mm}$ in A cohort and $2.3 \pm 0.8\text{mm}$ in B cohort. The difference of FMPPD between T1 and T2 were $0.3 \pm 0.1\text{mm}$ in A cohort and $0.3 \pm 0.2\text{mm}$ in B cohort.

The statistical comparison between A and B cohorts at T2 always resulted as not significant for all the indices considered ($p \geq 0.05$, t -test analysis) with the exception of GB ($p < 0.05$) that resulted greater in B cohort. The FMBS comparison between A and B cohorts was not significant but a trend for a greater FMBS score in B than in A cohort was observed ($p=0.096$).

The intra-group comparisons between baseline (T1) and the end of the second step (T2) were statistically significant for FMPS, FMBS, GB, FMPPD and FMREC in both A and B cohorts ($p < 0.05$ paired t -test). The indices measured significantly

decreased during the second step in all the cases with the exception of FMREC that significantly increased.

DISCUSSION

Tissue healing depends on several variables, such as materials, devices and techniques used (9-11). Nevertheless, the risk factor control is first rate in periodontal disease therapy. Some risk factors, such as the presence of dental plaque or detrimental lifestyles, have to be strictly controlled by cause-related therapy. However, to obtain satisfactory outcomes, it is necessary to obtain an excellent patient compliance (7). Together these agents can reduce inflammation that represents the uncontrolled physiopathologic factor characterizing periodontal disease and causing periodontal pocket and periodontal damage (2, 3, 11).

In this study, only compliant patients with a set of periodontal defects most likely requiring subsequent periodontal surgery were considered, therefore, the individual cause-related therapy of the second step had to be very effective to avoid, or prepare each patient to the adjunctive therapy. Full-mouth indices were always considered since they were more representative of full-mouth periodontal damage than the index in reference to the single tooth during the cause-related therapy. A significant clinical improvement of all the measured periodontal indices at T2 compared with the baseline values at T1 was always observed in both cohorts. Increased FMREC could be due to gingival healing and the subsequent gingival shrinkage.

The comparison between A and B cohorts at T2 resulted always not significant in relation to the measured periodontal indices except for GB that was significantly greater in the B cohort. However, the deep bleeding index (FMBS), that means periodontal inflammation, did not show significant differences between cohorts at T2 and it was clinically optimal, around 10%. Although GB was significantly greater in cohort B, it was about 4.5% and clinically ideal. Therefore, the difference between the two cohorts regarding GB has only mathematical, but not clinical significance of gingival inflammation.

The enrolled patients were compliant during

the entire study follow-up, and periodontal indices showed a clinical improvement with the usual healing pattern after the periodontal cause-related therapy. Chemical control of oral ecosystems performed by CHX (with ADS system) did not play a significant role in the periodontal indices considered. However, no full-mouth-disinfection protocol was applied to these compliant cohorts of patients. The full-mouth disinfection protocols with the contribution of CHX appear to be effective in compliant patient populations. On the other hand, plaque control performed by mechanical procedure is widely carried out, and is considered greatly significant because it also allows for the physical disruption of the structured oral ecosystems. The chemical plaque control could play an additional role or even no clinical role (4, 12).

In conclusion, this study shows that periodontal cause-related therapy performed on compliant patients carried out by a correct mechanical plaque control with fluoridated toothpaste and rinses produces excellent clinical results that are comparable to those obtained by combining mechanical and chemical control by toothpaste and mouth rinses with CHX+ADS. No clinically significant differences were demonstrated between the two cohorts of patients at the end of the follow-up period, therefore it was possible to obtain the same clinical outcome by cause-related therapy without the side effects of CHX that could lead to gingival inflammation, especially with prolonged use, explaining the greater GB highlighted in cohort B.

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LETTER TO THE EDITOR

Thromboprophylaxis with low molecular weight heparin in major orthopedic surgery: a review regarding the time of administrationF. Raggini¹, G. Placella², V. Pace³, A. Belluati⁴, V. Salini² and P. Antinolfi³

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To the Editor,

Major orthopedic surgery (MOS), such as total hip arthroplasty (THA) and total knee arthroplasty (TKA) procedures, represents an increased risk of developing venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE). Without any VTE prophylaxis, symptomatic DVT and PE occur with a frequency of approximately 0.24-1.60% for THA and 0.45-5.30% for TKA (1). VTE prophylaxis in patients undergoing MOS are divided in two groups: mechanical (graduated compression stockings and intermittent pneumatic compression); and pharmacological (vitamin K antagonists, low-molecular-weight heparins (LMWH), aspirin, and the novel oral anticoagulants (NOAC) or indirect thrombin inhibitor unfractionated heparin (UFH). Before starting any prophylactic pharmacological treatment, the assessment of both thrombotic and bleeding risks of patients undergoing surgery is of crucial importance as pharmacologic thromboprophylaxis itself can also cause increased risk of major bleeding (2). It has been shown that anticoagulants can significantly reduce the risk of thromboembolic events (in a range of approximately 50-80%) when prescribed prophylactically, and

for this reason they are frequently recommended after MOS. The safety and efficacy of LMWH for thromboembolic prophylaxis are already well established, as reported by several studies. LMWH has some accepted advantages compared to other thromboembolic prophylaxis options: a half-life with good bioavailability, and it can be administered once a day subcutaneously without laboratory monitoring or dose adjustment as well as being safe and effective for extended out-of-hospital prophylaxis after MOS. Maximum efficacy levels are observed 3-4 hours after subcutaneous administration and elimination occurs after 4-6 hours from administration in those with normal renal function (3).

Over the last 20 years, several studies reported data in favor of LMWH over other thromboembolic prophylaxis strategies. It has been hypothesized that LMWH administered closer to surgery, either immediately preoperatively or early postoperatively once daily, may be more effective than the present clinical practice according to the “just-in-time” concept which underlines that the risk of thrombosis starts perioperatively and it takes into consideration the increasing risk of bleeding. Although a broad consensus for some aspects related to pharmaceutical prophylaxis in this field already exist (such as the

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wide use of LMWH), the best timing for initial administration remains unclear (2, 3).

Therefore, when should thromboembolic prophylaxis with LMWH be initiated to balance the benefits (prevention of VTE, which is a major cause of perioperative morbidity and mortality) and risks (major bleeding and epidural hematoma)?

Preoperative or postoperative initiation of thromboprophylaxis?

Orthopedic surgery itself can be considered a prothrombotic event, hence the preoperative approach is based on the assumption that the surgical procedure and the associated reduced mobility status are together the main cause of thrombosis in this cohort of patients. The perioperative period always represents a critical moment for the patient undergoing MOS. Therefore, the main idea to take into consideration is that linked to the international "Enhanced Recovery After Surgery (ERAS) protocol" in order to reduce the response to surgical stress, relieve pain during the perioperative period, reduce the incidence of complications, accelerate functional recovery and improve patient satisfaction. The goal of ERAS in MOS is to improve surgical techniques and optimize perioperative management, including reduction of trauma and bleeding, optimization of pain and blood management and overall prevention of infections and deep vein thrombosis and early mobilization (3).

The American Society of Hematology (ASH) guidelines suggest using combined mechanical and pharmacological prophylaxis or mechanical prophylaxis alone for patients undergoing major surgery, depending on the risk of VTE and bleeding based on the individual patient and the type of surgical procedure. For patients considered at high thrombosis risk and low bleeding risk, combined mechanical and pharmacological prophylaxis should be considered. For patients at high bleeding risk, mechanical prophylaxis methods alone may be preferred (4).

Clinical practice regarding the initiation time of antithrombotic prophylaxis in MOS differs between North America and Europe. The European approach recognizes the hypothesis that DVT

typically originates perioperatively and that preoperative prophylaxis, frequently initiated 12 hours preoperatively, may optimize antithrombotic effectiveness, leading to the assumption that the surgery itself and the accompanying immobility are the main cause of thrombosis. The European guidelines on perioperative prophylaxis of VTE emphasize on the management of elderly patients. These guidelines suggest early identification of comorbidities that increase the risk of VTE and carry out the appropriate optimization, if present (e.g. anemia, coagulopathy), to calculate and measure the pharmacological prophylaxis of VTE as occurs in the non-elderly population (modulating the therapy in elderly patients with renal insufficiency, for whom low-dose UFH or weight-adjusted LMWH administration may be used. When LMWH is the drug of choice for the thromboprophylaxis regimen, the first dose may be administered before surgery (approximately 12 hours before the procedure starts) or after surgery (optimal time 6 to 8 hours after the end of the procedure) which seems to be the preferred option (5).

In the United States and Canada, LMWH prophylaxis is administered through a delayed initiation (12-24 hours postoperatively) in order to minimize the bleeding risk. Several American and Canadian Authors tend to emphasize the importance to reduce the risk of bleeding over the effectiveness of preoperative VTE prevention (6).

While some studies, in the past, demonstrated that the preoperative administration of LMWH can provide a significant degree of protection against the systemic activation of surgical coagulation, at the same time other US studies investigated postoperative thromboprophylactic strategies, thereby establishing their efficacy and safety (7).

This latter approach addresses concerns over bleeding complications, while the use of a higher than usual total daily dose of LMWH recognizes that some thrombi may have already formed and that their growth may have slowed, allowing for fibrinolysis. This has been proven on the basis of the importance of LMWH half-life and the overall consideration of studies and experience in the USA, which supported the postoperative start of thromboprophylaxis as a

safe, effective and convenient regimen for patients undergoing MOS (8). Therefore, with regard to the currently used LMWH regimens, there is no evidence that starting the prophylaxis 12 hours preoperatively is associated with a lower risk of VTE than when prophylaxis is started 12 to 24 hours postoperatively. For these reasons, postoperative initiation of LMWH appears acceptable for the majority of patients, also taking into account the reduced costs, decreased risk of anesthesiology complications, and the possibility to plan same-day admissions (6, 7).

In contrast, other Authors showed that when thromboprophylaxis is started either soon before (2 hours) or after (4 hours) surgery, even with reduced doses, there is a trend for a lower risk of DVT. However this benefit appears to be counterbalanced by a marked increase in the risk of major bleeding (8). Strebel et al. presented a systematic review on the comparison among preoperative (at least 12 hours pre-op, usually defined as the evening before surgery), postoperative (12-24 hours after surgery), and perioperative (2 hours before to ≤ 4 hours after) initiation of LMWH. Perioperative initiation of LMWH resulted in major bleeding rates up to 5-7%, whereas rates were in the 1-3% range with preoperative and postoperative administration. Preoperative initiation of the same regimen of LMWH (dalteparin) increased major bleeding rates, without improving antithrombotic efficacy compared to the early postoperative regimen. The clinical research evidence supported the following general conclusions on the relationship between the timing of the first dose of prophylactic LMWH and the efficacy and safety of prophylaxis: (a) preoperative initiation is not required for good efficacy and, moreover, when begun within 2 hours from surgery, increases major bleeding; (b) initiation at 6 hours postoperatively is effective and not associated with increased major bleeding complications; (c) initiation < 6 h postoperatively increases major bleeding, without improved efficacy on VTE prophylaxis. Thus, the timeframe of 6 hours appears to be the threshold for early postoperative administration; (d) initiation 12 to 24 hours postoperatively may be less effective than initiation at 6 hours, but further randomized trials comparing the same regimen

initiated at different times postoperatively (e.g. 6 hours vs 12 hours) are required to establish the definitive optimal timing of the first administration of LMWH. In that review, the Authors showed that starting prophylaxis ~ 12 hours before surgery is no more effective in preventing DVT than starting 12 hours postoperatively. Moreover, the increased risk of major bleeding outweighed any potential benefit despite a trend of lower VTE rates associated with perioperative initiation (8).

However, it must be said that if the prophylactic treatment is initiated 12 hours before surgery, a significant amount of the administered LMWH is eliminated by the time of the surgery. In fact enoxaparin has a half-life of 4 hours after a single subcutaneous dose and 7 hours after a repeated dose. Therefore, the desired preoperative LMWH antithrombotic effect is minimal as the protective effect is expected to be given after surgery (1).

In cases where local anesthesia is indicated and used (according to each patient's condition and comorbidities), it has been proved that the preoperative initiation of LMWH can increase the risk of the occurrence of compressive spinal hematoma. Therefore, the risk of bleeding with a consequent compressive epidural hematoma could outweigh the potential benefits of VTE prophylaxis of preoperative initiation of LMWH (9).

Furthermore, during recent years, it has been widely debated at the SIOT (Italian Society of Trauma & Orthopedics) annual congress how the effectiveness of postoperative VTE and bleeding prevention regimens are strictly associated with the safety of this practice (10). In fact, following these precautions on the timing in the use of LMWH, the incidence of devastating complications related to the occurrence of an epidural hematoma that can occur under spinal or epidural anesthesia has decreased preoperatively compared to post-operatively (also including the removal of catheter analgesics). There is fair level of evidence that the risk of thromboembolic events is higher (and has a higher impact on the outcome) than the risk of epidural hematoma formation with the utilization of LMWH preceding interventional anesthetic techniques; although both risks are significant. To prevent the occurrence of

these complications, prophylactic LMWH should be administered at least 4 hours after spinal anesthesia; on the other hand, an epidural anesthesia cannot be performed within 12 hours from the administration of prophylaxis (11).

According to the different previous guidelines, the best regimen in terms of efficacy is reported to be the one called “just-in-time” and requires the administration of LMWH from 6 to 8 hours after surgery. In fact, if LMWH prophylaxis is given 4 to 6 hours after surgery, it is as effective as preoperative LMWH given 2 hours before surgery, without any relative increase of perioperative bleeding risks (6).

Even the administration of small preoperative doses (2-4 hours pre-op), which were previously thought to decrease the incidence of DVT during surgery and in the early postoperative hours, showed a significant increase in bleeding complications. Moreover, the occurrence of neuraxial compressive hematoma after anesthetic procedures and the risk of bleeding increase in elderly patients if prophylaxis is initiated too close to the start of surgery (for example, between 2 and 4 hours from surgery). This protocol has been confirmed by several clinical trials over time and has allowed the orthopedic surgeons to refine the blood-saving techniques for MOS procedures that led to the wide use of Tranexamic Acid (TXA) today, which is now seen as an important agent to decrease postoperative bleeding and need of transfusions. Fraval et al. reported that patients undergoing THA treated with enoxaparin and who received TXA exhibited lower transfusion rates and a reduction in their length of stay. Other prospective studies have shown that TXA can reduce the blood loss and postoperative transfusion rate mostly without major complications for MOS procedures (12).

DISCUSSION

Different data suggest that both preoperative and postoperative initiation of thromboprophylaxis are safe and effective regimes to reduce the risk of VTE in patients undergoing MOS. Systematic reviews and meta analyses comparing these different pharmacological approaches have found no consistence differences in efficacy and safety (6, 8).

Taking into account all the aforementioned studies, guidelines and conclusions, it seems that LMWH should be administered about 6 hours postoperatively (in accordance with the “Just in time” protocol) and be prolonged for 5-6 weeks post-op in order to decrease the risk of VTE, which is in accordance with the ACCP guidelines (10, 11). This regimen, in association with the use of intravenously-administered tranexamic acid, allows a decreased need for blood transfusions together with a reduction of VTE and bleeding complications, indirectly resulting in lower costs for national health systems. In addition, the strategy of starting VTE prophylaxis after MOS (and not before) allows to hospitalize about 30% of patients on the day of the operation and perform a lower number of LMWH administrations overall. This consequently results in a cost saving practice with greater convenience for patients (and outcomes) and healthcare organizations. In conclusion, it can be stated that the postoperative approach is a safe and effective strategy for VTE prophylaxis in MOS, decreases the risks related to neuraxial anesthesia, facilitates same-day admission and has potential for overall cost reduction (6, 10-12).

An appropriate thromboprophylaxis assessment and regimen are among the most important milestone interventions that must be offered to patients requiring trauma and orthopedic surgical procedures (both in a trauma and elective setting), particularly for MOS of the lower limbs.

It is likely that with the increasing use of regional anesthesia, the preoperative initiation of thromboprophylaxis will gradually disappear and that future research will be directed towards the optimal timing of postoperative prophylaxis. It is also possible that the optimal timing of administration might differ, depending on the mechanism of action of the agent in question.

Moreover, the timing of pharmacological prophylaxis initiation should be based on the most up-to-date guidelines and on an individualized risk-benefit balance for each patient, especially the elderly. This assessment should take into account both individual patient's relative risk of bleeding complications and their risk factors for VTE, both of which should be established prior to surgery.

Perioperative thromboprophylactic regimens should be unified by the same intent to avoid well-known foreseeable risks and complications, but further research is still required to definitively validate accepted guidelines and better define their precise roles in the MOS setting.

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LETTER TO THE EDITOR

Vocal fold paralysis: a rare complication of measles encephalitisS. Passanisi¹, C. Mignosa¹, J. Trombatore¹, P. Di Bella², G.B. Pajno¹ and M. Valenzise¹

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To the Editor,

Vocal fold paralysis (VFP) is characterized by reduced or absent function of the vagus nerve or its distal branch, the recurrent laryngeal nerve (RLN) (1). To date, the true incidence of VFP in children is unknown. Bilateral VFP seems to be less frequent than unilateral VFP. The causes of VFP in children differ from adults. The Arnold-Chiari malformation is the most common cause of pediatric bilateral VFP. Other neurological conditions that lead to bilateral VFP include cerebral palsy, myelomeningocele, spina bifida, or hypoxia. Birth trauma, particularly with forceps, breach, or vertex delivery, are potential but rare causes among neonates (2, 3). Drug toxicity is another increasingly recognized cause of pediatric VFP. Idiopathic bilateral VFP is rare. During the adult period, the major causes of VFP include laryngeal or extra-laryngeal cancers, thyroid diseases or associated surgery, intubation-related injury, neurodegenerative disorders, and autoimmune diseases (4). Infectious causes are very rarely described in both children and adults. Clinical manifestations of VFP include dyspnea, dysphonia, and dysphagia, and their severity varies according to the underlying etiology, vocal fold position, and compensatory behavior (5). Particularly, focalising the paralysis is crucial to determining further intervention. For example, a medial paralysis may

protect the airways during swallowing, but this could adversely impact breathing and might require tracheostomy.

Case presentation

We present the case of a ten-month old baby, the third born from non-consanguineous Romanian parents belonged to low socioeconomic strata, who was admitted to the Pediatric Intensive Care Unit (PICU) of our hospital due to the following symptoms which were felt to be suggestive of encephalitis: respiratory distress, drowsiness and lethargy. His past medical history was unremarkable for any underlying diseases. Three weeks prior to admission, he had presented fever, vomiting and diarrhea and, a few days after, he had developed a diffuse maculo-papular rash all over the body. The recent family history was positive for measles (one of his older brothers had been affected). All the members of his family had missed both recommended and compulsory vaccinations according to the Italian regulation. During his PICU hospitalization, intravenous antibiotic therapy with ceftazidime and dexamethasone therapy was practiced to treat his brain injury. Full respiratory support was successfully practiced for 48 hours due to severe dyspnea. After recovery from acute respiratory conditions, he was admitted to our Clinic. At that

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time he presented with a mildly depressed level of consciousness. On physical examination, he had mild breathing difficulty and persistent hoarseness. Skin examination revealed millimeter light brown spots. The intake of liquid foods aggravated his respiratory distress. Serologic tests for measles evidenced IgM antibody 32.74 Index (n.v. > 11), revealing a recent infection. Electroencephalogram (EEG) with computerized spectral analysis resulted altered and showed a prevalence of low-frequency delta activity (Fig. 1). The suspicion of acute post-measles encephalitis complicated by VFP was supported by the constellation of his recent history, clinical examination, as well as by instrumental findings. A flexible fiberoptic nasolaryngoscopy examination showed bilateral vocal cord paralysis and incomplete glottis closure during phonation confirming the diagnosis (Fig. 2). Particularly, nasolaryngoscopy identified a paramedian position

of the paralyzed vocal folds, which is strongly associated with impaired airway patency, thus, resulting in breathing difficulties, stridor and snoring sounds, and fast physical exhaustion. Speech therapy was started once a week to monitor voicing and speech development. During the following days, no breathing or swallowing concerns were further noted and the child had a progressive improvement and his state of consciousness normalized. Periodic fiberoptic examinations revealed mild improvements of vocal cord mobility in accordance with normalization of the patient's voice. At discharge, he underwent electrophysiological study which detected no anomalies (Fig. 3). A magnetic resonance imaging of his brain, performed approximately 1 month after the insult, was normal.

At the 10-month follow-up visit, fiberoptic endoscopy showed normal vocal cord mobility and complete glottis closure during phonation. Written

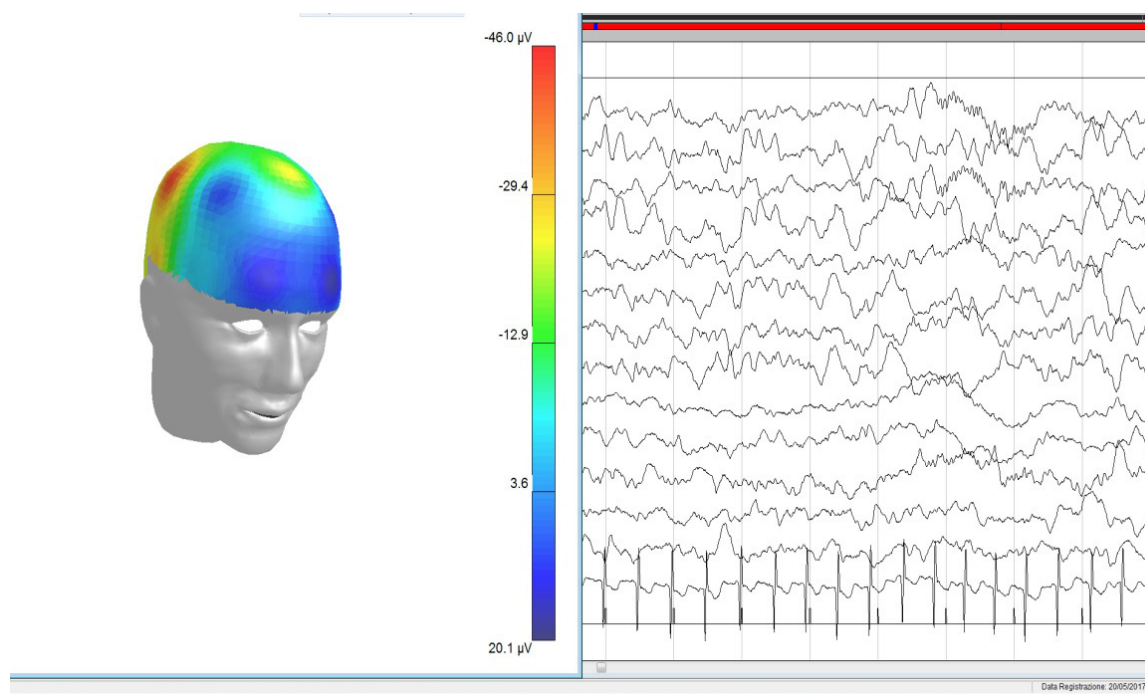


Fig. 1. The first EEG examination showed medium intensity, symmetrical theta rhythm. During falling asleep there were short bouffes of generalized, cuspid morphology slow waves. Spectral graphics showed a prevalence of low-frequency delta activity.



Fig. 2. Flexible fiberoptic laryngoscopy showed bilateral vocal cord paralysis and incomplete glottis closure during phonation.

informed consent was obtained from the patient's parents for the publication of this case report.

DISCUSSION

Measles is a highly contagious, vaccine-preventable, acute febrile disease that is caused by

measles virus infection (6). In 2012, The World Health Organization (WHO) drafted the "Global Measles and Rubella Strategic Plan: 2012-2020", which aimed to achieve the elimination of measles in Europe by 2015 (7). Nevertheless, in 2018 more than 80,000 cases of measles in almost all European countries were notified by WHO, three times the total reported in 2017 and 15 times the total recorded in 2016 (8). This increasing incidence rate seems to be related to the reduction of vaccinations against measles over recent years, mostly induced by vaccine hesitation. Measles complications can occur more commonly in young children and immunocompromised or undernourished patients (9). C-reactive protein has been recently identified as a fairly good sensitivity and specificity marker for predicting outcome, being associated with an increased risk of developing organ complications (10). Neurologic complications are relatively rare but may lead to disability and death. No previous cases of post-measles bilateral vocal cords paralysis in children have been previously reported to the best of our knowledge. We hypothesize that VFP may be the result of the viral invasion of basal meninges and the consequent involvement of the recurrent laryngeal nerve (RLN), a distal branch of the vagus

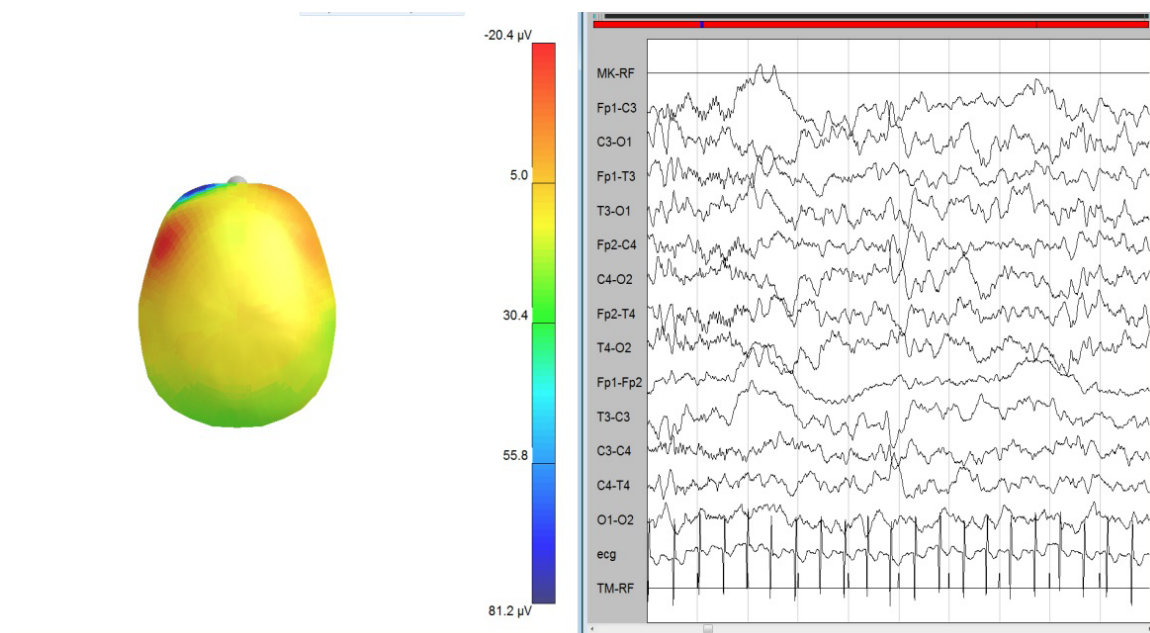


Fig. 3. The last EEG showed synchronous and symmetrical medium voltage activity, with frequencies between 6 and 12 Hz.

nerve. The RLN controls all the intrinsic muscles of the larynx, except for the cricothyroid muscle. These muscles include the posterior cricoarytenoid muscles, which are responsible for opening the vocal cords. Damage to both right and left recurrent nerves may lead to vocal cord paralysis. Flexible fiberoptic laryngoscopy is considered the gold standard diagnostic investigation. Airway management is the major concern in treatment of bilateral VFP. Once the airways are stable, treatment options, which vary from speech therapy to endoscopic and surgery techniques, may be employed.

Our case highlights how the threatening increasing trend of measles cases caused by the recent massive vaccine hesitation may be related to a higher risk of potentially fatal complications.

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