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Mast cell activation and inhibition from cytokines secreted by macrophages

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Mast cells (MCs) are ubiquitous immune cells that participate in allergic reactions through the activation of the FcεRI receptor, but also in inflammatory processes induced by various biological and non-biological compounds, neurotransmitters, and cytokines. Activation of MCs can lead to the immediate release of chemical mediators of inflammation, but it can also result in the secretion of pro-inflammatory cytokines without degranulation. In the inflammatory network, macrophages cross-talk with MCs by producing IL-1, which stimulates MCs to secrete pro-inflammatory cytokines including IL-6, TNF, IL-33 and other cytokines and chemokines. IL-37 and IL-38 are anti-inflammatory cytokines implicated in the suppression of the immune and inflammatory system. Therefore, it is pertinent to think that these cytokines can open new pathways in the field of inflammatory and immune diseases. Here we report the relationships between macrophage cytokines, MCs, and inflammation.

The innate immune system is heavily involved with inflammatory responses induced by microorganisms. Inflammation is a process that must always be eliminated to avoid more serious situations. The most common anti-inflammatory drugs are mainly divided into steroidal and non-steroidal compounds. Steroidal anti-inflammatory compounds are part of the cortisone family and mostly block lipoxygenase A2, while the non-steroidal block cyclooxygenase, and subsequently prostaglandins, which are mediators of inflammation and pain. Cytokines are powerful and critical mediators of inflammation and represent a large network of proteins with the ability to send stimulatory and suppressor signals and may represent therapeutic targets (1). They exert by toxicity. Cytokines can also intervene in the

maturation process of different cell types including immune cells.

In the past 25 years another group of anti-inflammatory compounds has emerged, called cytokine inhibitors. These are biological-proteins that block pro-inflammatory cytokines, an effect that should be specific, but often a specific anti-inflammatory cytokine acts on various pro-inflammatory cytokines with a redundant action.

Medullary progenitors of peripheral white blood cells can evolve into distinct phenotypic mature cells with different functions. Some of these cells mediate the innate immune response; while others participate in acquired immunity.

Mast cells (MCs) are derived from hematopoietic stem cells and are found in vascularized tissues and

Key words: mast cell, inflammation, cytokine, macrophage, anti-inflammatory

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serous cavities (2). They are exposed in the tissues in contact with the external environment such as airways, skin, mucous membranes, and the gastrointestinal tract, where they can encounter microorganisms and allergens, causing an immune response. MCs can participate in the innate and acquired immunity by the immediate release of biologically active compounds that can perform immunosuppressive, pro-inflammatory and anti-inflammatory functions. Furthermore, activated MCs can later release pro- and anti-inflammatory cytokines. In IgE-associated immune responses, MCs are activated through the high affinity of IgE to its FcεRI receptor located on the cell surface.

The granules of MCs contain several proteases including tryptases, chymases, and carboxypeptidases. MCs MCT are tryptase positive, while MCTC are chymase and tryptase positive. In addition, stem cell factor (SCF) promotes the differentiation and development of MCs through the c-KIT receptor. MCs are recruited on allergic tissues by SCF of epithelial cells. Furthermore, activated MCs contribute to allergic inflammation by secreting cytokines IL-4, IL-5, and IL-13. The allergic inflammation is also mediated by cytokines T2: IL-9 and IL-13, while the cytokines T1, such as TNF and IL-1, cause an increase of inflammation in severe pathologies. Like macrophages, MCs express toll-like receptors (TLRs) that can be activated by certain cytokines including IL-1 and microorganisms, thereby starting the inflammatory process. Many specific ligands can activate MCs by binding to their receptors. In addition, when an allergen bridges between two IgE which are bound on the FcεRI fragment of MCs, activation occurs with the release of a series of compounds including cystenyl leukotrienes and prostaglandine PGD2 from arachidonic acid cascade, cytokines and chemokines, which in turn activate different immune cells and participate in inflammatory responses.

IL-1

IL-1 is a fundamental cytokine mediator of the innate immune response that manifests itself with inflammation, a defense mechanism of living organisms.

For more than 25 years, it has been known that MCs can generate and release IL-1 after stimulated

cells through the FcεRI receptor, but also some chemicals compounds can activate MCs to produce IL-1, such as 48/80, LPS, ATP, calcium ionophore and others. In releasing IL-1, MCs contribute to the inflammatory process that occurs in autoimmune diseases such as rheumatoid arthritis and various skin diseases. Bronchial asthma, a disease where MCs play an important role, characterized by excessive proliferation of muscle cells and airway fibroblasts, can cause pulmonary fibrosis to occur, a disease mediated by IL-1 secreted by macrophages. Macrophages are distinct in M1 and M2 derivatives from peripheral monocytes which generate pro-inflammatory and anti-inflammatory cytokines. They can also be activated, at low activity, by IgE on the FcεRII receptor. Macrophages with the pro-inflammatory M1 phenotype secrete innate cytokines and later, with the M2 phenotype, produce anti-inflammatory cytokines which play a role in tissue repair. IL-1 secreted by macrophages can stimulate the production of TH2 cytokines in activated MCs without inducing or potentiating the degranulation. Histamine released by MCs, and IL-1 secreted by macrophages, participate in chronic inflammatory diseases and allergic reactions. Histamine alone does not induce IL-1 synthesis, but a few decades ago it was reported that the stimulation of IL-1 alpha on some type of cells caused the generation of IL-1 beta, an effect which was enhanced by histamine. This interaction suggests that there is a close relationship between acute and chronic inflammation. In addition, the function of MCs in angiogenesis has long been known. In fact, they release factors that activate angiogenesis such as TNF (secreted also by macrophages) and some chemokines (CXCL8), as occur in skin diseases. Furthermore, MCs activated with IL-1 can release IL-6, contributing to inflammation and angiogenesis. Thus, between macrophages and MCs there is cross-talk leading to the release of histamine, tryptase, IL-6, TNF, and IL-1, all compounds which can intervene in many cellular functions, including inflammation.

TNF

In the 1970s, a new macrophage-derived protein, tumor necrosis factor (TNF) was identified and

found to cause hemorrhagic necrosis of cancer. TNF was subsequently cloned, and it was found to induce cachexia and to have pro-inflammatory properties. TNF acts positively in immunity by activating the innate response, but its gene overexpression and production causes strong inflammation. In fact, TNF is one of the fundamental pleiotropic proteins secreted in host defense through inflammation. In allergic and inflammatory processes, TNF is overexposed, and its inhibition represents an important therapeutic action for several inflammatory diseases.

MCs are immune cells that act against pathogens, such as microbes and allergens, by releasing soluble mediators including TNF. This is the only cytokine contained in the cytoplasmic granules of the MC. TNF is produced and released by macrophages after activation with the bacterial lipopolysaccharide, while MCs also generate TNF which is expressed in their constitutively granules and *de novo* synthesis (Galli SJ et al.). Therefore, TNF from the granules of MCs can be released immediately (in few minutes) after activation, while it can also be produced by MCs later (in several hours) after gene activation through mRNA.

The over expression of this immune-inflammatory cytokine is involved in many diseases, causing

pathologies not yet resolved. MCs activated with IgE or with some neurotransmitters, such as substance P, can release TNF through the inflammatory process. TNF is secreted by MCs as an immune response to the causes of inflammation. In fact, it has the ability to attract immune cells in the inflammatory reaction, including that mediated by IgE. Therefore, MCs-derived TNF causes chemoattraction of neutrophil granulocytes and mediates the response of dendritic cells (DC). TNF secretion from MCs can influence the adaptive immunity, but it can also affect non-immune cells.

In a recent article, our group reported that the peptide substance P, that is implicated in inflammation, administered in combination with IL-33 strongly increases (1000 times) the gene expression of TNF in human umbilical cord mast cells and LAD2 cells, indicating a synergistic effect of these two cytokines amplifying inflammation.

IL-33

The inflammatory reaction in the tissues is mediated by leukocytes that try to eliminate the harmful reaction. Usually, pathogenic microorganisms initiate innate immunity by producing pro-inflammatory cytokines such as TNF and IL-1. Macrophages and

Table I. Mast cells activated by IgE on the FcεRI fragment: secretion of cytokines and chemokines that activate the respective immune cells.

	<u>Secretion</u>	<u>Immune cell activation</u>
<i>Chemokines:</i>	CCL1,2,3,4,7,8,9,17, CCL20, CXCL2,8	Eosinophils, Basophils, T cells, DC, Monocytes
<i>Cytokines:</i>	TNF, IL-1,2,3,4,5,6,9,10,11,13 TGF-β1, SCF, GM-CSF, FGF-2, VEGF, IFNγ	T cells, Neutrophils, Eosinophils, Monocytes, Macrophages, B cells
	<u><i>Cytokines that activate mast cells</i></u>	
	IL-3,4,6,9,33, IFNγ, SSF, TGF-β1, VEGF, NGF	

******However, many of these compounds have been found in rodents but not in humans.

MCs play a sentinel role and emit danger signals such as the cytokine IL-33, also called “alarmin”. IL-33 is a member of the IL-1 subfamily and exerts a pleiotropic activity in several cell types. IL-33 is released into the extracellular space in response to tissue damage. IL-33 activates many cell types including MCs and participates in allergic inflammation. This cytokine activates MCs and other immune cells through its ST2 receptor, magnifying the pro-inflammatory action. The precursor of IL-33 is inactivated by caspase-1, with subsequent production of the active form “alarmin” as it is released after cell damage.

Proteases of MCs cleave IL-33, which translocates into the nucleus. IL-33 plays a role in T helper 2 (Th2) cells, but also in Th1 inflammatory diseases and increases IFN γ production. Therefore, IL-33 mediates Th2 cells in diseases, while another pro-inflammatory cytokine, IL-18, macrophage derived, participates in Th1 reactions. MCs activated with IL-33 release histamine and chemokines proving to be an important protein in allergic processes. IL-33 has been reported to be elevated in synovial fluid *in vivo*, while *in vitro* activated synovial fibroblasts from patients with rheumatoid arthritis results in an increase in IL-33. This shows that this cytokine mediates inflammatory rheumatoid disease.

About 11 years ago we reported that substance P (SP), a neuropeptide implicated in inflammatory diseases where MCs play an important role, induces VEGF gene expression in human MCs, a greatly enhanced effect by the pro-inflammatory cytokine IL-33, demonstrating that the inflammatory cooperation between SP and IL-33 can be important in understanding inflammatory diseases. Moreover, in 2018 we reported that SP and cytokine IL-33 administered in combination in cultured MCs markedly stimulate intracellular levels of the pro-inflammatory cytokine IL-1, demonstrating a relationship between the nervous system and inflammation induced by IL-33.

IL-37

Among the anti-inflammatory cytokines today, IL-37 emerges as a member of the IL-1 family and is mostly generated by macrophages. IL-37 is one of the 11 pro- and anti-inflammatory members of the IL-1 family and suppresses innate inflammation. This

novel cytokine is a natural inhibitor of IL-1, which is the most important cytokine in inflammatory processes. IL-37 is activated by cleavage of caspase-1 and by translocating to the nucleus, it inhibits pro-inflammatory genes. IL-37 binds to the IL-18Ra receptor, inhibiting the cascade and leading to NF- κ B. In treated rodents, IL-37 has been reported to acquire anti-inflammatory protection with inhibition of the delayed immune response.

IL-37 opens new avenues in the therapy of inflammatory diseases mediated by pro-inflammatory cytokines. In inflammatory models in mice, IL-37 reduces joint inflammation and pain induced in the synovial by improving the pathological status of rheumatoid arthritis. It has long been known that MCs produce and are activated by IL-6 and TNF, an effect that amplifies inflammatory and allergic processes. Recombinant IL-37 administered in mice suppresses IL-6 and TNF by inhibiting inflammation. In the adaptive immune response where there is cross-talking between MCs and T cells, IL-37 has been found to be able to influence the activation of T lymphocytes. IL-37, as well as IL-38, exert anti-inflammatory power, not on the chemical mediators released immediately by the MCs after activation with IgE, but on the pro-inflammatory cytokines secreted after a few hours by these interesting immune cells. In allergic diseases where MCs play a predominant role, endogenous IL-37 contributes to the reduction of the development of the pathological state. These concepts open new frontiers in the field of allergic and inflammatory disease therapy.

IL-38

Another anti-inflammatory cytokine is IL-38, a member of the IL-36 family which is, by contrast, pro-inflammatory. IL-38 suppresses inflammation by binding IL-1R6 receptor. Stimulated white peripheral blood cells treated with IL-38 are inhibited in the production of IL-17 and IL-22. IL-38 produced by B cells and macrophages is present in the synovial tissue of rheumatoid arthritis as a response to the pro-inflammatory stimulus. IL-38-deficient transgenic mice exhibit inflammatory allergy MCs mediated with an increase in pro-inflammatory cytokines IL-1 and IL-6, confirming that IL-38 acts as a brake

in inflammatory processes and can represent a therapeutic target. Other cytokines such as IL-17, IL-22 and IL-23 are also inhibited by IL-38, with relief of the pathological state of disease. Furthermore, it has also been observed that in some autoimmune diseases the concentration of IL-38 is greatly increased. This leads us to think that IL-38 is important in these pathologies. IL-38 inhibits the activation of T cells and therefore manifests itself as an immunosuppressant with therapeutic implications in this field.

Recently our group highlighted that neurotensin-stimulated and treated microglia cultures produce pro-inflammatory cytokines such as IL-1 and chemokine CXCL8, which can be inhibited by IL-38. Inhibition of IL-38 on microglia opens a new therapeutic strategy towards brain diseases where microglia cells are involved.

Hence, MCs involved in allergic reactions and innate immunity and treatment *in vitro* with the pro-inflammatory cytokine IL-1, causes the *de novo* release of IL-6 without tryptase synthesis and in the absence of degranulation (), amplifying innate immunity. Since IL-38 blocks the activity of IL-1, it is pertinent to think that in inflammatory diseases mediated by innate immunity this inhibition may represent a new therapeutic strategy in allergic inflammation mediated by MCs.

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Sleep quality in parents with children affected by psoriasis, psoriatic arthritis or atopic dermatitis: a multicenter cross-sectional study

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Background: Psoriasis (PsO), Psoriatic arthritis (PsA), and Atopic dermatitis (AD) patients have an increased risk of sleep disorders. Although sleep disturbances are well-known among patients, caregivers' onerous evaluation remains poorly assessed. The objective was to quantify the sleep burden of parents with affected children.

Methods: In this multicenter cross-sectional study, we enrolled sex-age matched parents with children affected by PsO, PsA, and AD. Both parents underwent the Pittsburgh Sleep Quality Index to determine their sleep quality.

Results: We enrolled a total of 90 children (age 12.36 ± 1.83 years, 45 male and 45 female) with psoriasis (n=30, PASI 7.00 ± 2.49), atopic dermatitis (n=30, SCORAD 33.13 ± 10.03), and psoriatic arthritis (n=30, DAPSA 26.40 ± 10.94). Patients' Parents (age 49.83 ± 6.69 years, 45 male and 45 female) had a PSQI of 6.17 ± 1.91 ; hence 70.0%, 73.3%, and 96.7% had a bad quality of sleep, respectively, with children suffering from atopic dermatitis, psoriasis, and psoriatic arthritis. Parents with children affected by psoriasis (6.23 ± 2.46) or by psoriatic arthritis (6.73 ± 1.42) had lower PSQI than the ones with

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atopic dermatitis (5.53 ± 1.55). Interestingly, children with PsA had parents with higher risk of sleep disturbances (OR 52.25 [95%CI 1.92-1,422.66], $p=0.0189$), and male gender was protective (OR 0.14 [95%CI 0.02-0.86], $p=0.0337$).

Conclusions: The sleep quality of parents is deeply influenced by the dermatological/rheumatological disease in children but not by its duration. Thus, sleep evaluation in caregivers should be part of routine patient check-ups with PsO, PsA, and AD.

Key words: psoriasis; psoriatic arthritis; atopic dermatitis; sleep quality

Psoriasis (PsO) is a chronic, immune-mediated disorder (1) that combines autoimmunity- and auto-inflammation-related features (2, 3). From a clinical point of view, psoriasis patients present demarcated, erythematous, infiltrated, large edges squamous plaques. From an epidemiological standpoint, it has a prevalence of 2–3% without gender preference (4-6). It is estimated that approximately 10-30% of psoriasis patients will develop psoriatic arthritis (PsA) (7).

PsO is often co-localized with different comorbidities (8-11), included but not limited to sleep disorders (12-14). For instance, PsO patients have a higher risk of developing obstructive sleep apnea (OSA) with a prevalence rate of 36.0-81.8% *versus* 23% and 49% for women and men, respectively in moderate-to-severe OSA (15) in the general population, as well as restless legs syndrome (15.1-18.0% *versus* 5.0-10.0%), whereas the burden of insomnia seems comparable with that of the general population (5.9-44.8% *versus* 10-35%) (12).

Atopic dermatitis (AD) is a chronic, relapsing dermatosis that causes a cutaneous barrier dysfunction (16-18), provoking an increased trans-epidermal water loss (TEWL), dehydration, itch, and finally inflammation (19-21). Disturbed sleep is a common complaint from both parents of children with AD and AD children. Several studies focusing on AD had considered sleep as a notable patient-reported outcome (23-29).

While it is well-established that PsO, PsA and AD patients suffer from poor sleep quality, there is a dearth of data about sleep quality in parents of children affected by psoriasis, psoriatic arthritis, and atopic dermatitis. Therefore, the present study was designed to fill in this gap of knowledge.

MATERIAL AND METHODS

Study design

This is a multicenter cross-sectional study that involved 3 Italian primary referral centers (IRCCS San Donato Milanese, IRCCS San Gallicano, IRCCS Istituto Ortopedico Galeazzi) for psoriasis, psoriatic arthritis, and atopic dermatitis. The enrollment period spaced from December 2018 to December 2019. Both patient's parents signed a consent form; for pediatric patients (> 5 years and < 18 years), data were extracted and/or collected with the signed consensus to the child's legal tutor (*Patria potestate* ownership). Both parents were enrolled, and they were sex and age-matched in the three diseases analyzed. The entire protocol followed the Helsinki Declaration and its most recent amendments; thus, it was approved by the ethical committees of each center involved.

Pediatric patient selection criteria

Psoriatic pediatric patients (<18 yoa) were screened to exclude PsA preliminarily with Psoriasis Epidemiology Screening Tool (PEST) (30) and Classification for Psoriatic Arthritis (CASPAR) (31) and after with sonography to check entheses (32) and magnetic resonance for joints involvement (33). PsO patients were included in case of Psoriasis Area Severity Index (PASI) >10 (moderate to severe), PsA pediatric patients in case of fulfilling CASPAR criteria and Disease Activity in Psoriatic Arthritis (DAPSA) ≥ 5 points, then atopic dermatitis (AD) pediatric patients in case of SCORing Atopic Dermatitis (SCORAD) >15 (moderate to severe). All pediatric patients had to agree i) to participate to the study together their parents or legal tutors, ii) to be undertreatment and iii) to had attended every dermatological visit during the last year.

Exclusion criteria:

Patients were excluded on the basis of i) previously diagnosed sleep disorders, ii) occasionally or routinely use of sleep medications, iii) any psychiatric diagnoses, iv) recent cranial trauma, v) recent jet-lag diagnosis (34), vi) untreated, or they refuse to be treated for PsO/PsA/AD, other rheumatological or gastroenterological autoimmune or systemic inflammatory diseases beside PsO/PsA/AD, vii) drug addictions (35), viii) concurrent chronic or acute infectious diseases (hepatitis B and C, HIV, tuberculosis), ix) concurrent multiple chemical sensitivity (36), x) they did not leave together parents.

Parents selection criteria

Parents were included if they both volunteered to participate, signed the consent form, and at the time of the study were living together with their partner and children.

Conversely, they were excluded if i) the parent was not the natural parent, ii) they did not live together with their partner, iii) they were practicing intermittent circadian fasting during the Pittsburgh Sleep Quality Index (PSQI) (37-40), iv) previously diagnosed sleep disorders, v) occasionally or routinely use of sleep medications, vi) any psychiatric diagnoses, vii) recent cranial trauma, viii) recent jet-lag diagnosis, ix) no signing the consent form.

Clinical measurements

During the dermatological examination, the degree of psoriasis, psoriatic arthritis, and atopic dermatitis severity were determined using the PASI, the DAPSA, and SCORAD scores, respectively.

DAPSA is a composite indicator that takes into account several parameters, including clinical symptoms (tender and swollen joints), laboratory findings (C-reactive protein (CRP)), and patient-reported outcomes. All these measurements were done by two independent board-certified dermatologists.

SCORAD takes into account the severity of atopic dermatitis, giving approximate weights of 60% to intensity and 20% each to spread and subjective signs (including insomnia or other sleep disturbances).

The measurement of sleep quality was conducted using the Pittsburgh Sleep Quality Index (PSQI). The scale comprises 19 items, assessing seven components: namely, subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping

medications, and daytime dysfunctions. Scores in each domain of sleep-quality range from 0 to 3, with the global PSQI ranging from 0 to 21. A total score equal to or greater than 5 has been considered as poor sleep quality.

Statistical analysis

Before proceeding with any statistical processing (including data handling and manipulation), figures were visually inspected to capture any potential outlier. Normality of data distribution was verified by performing the Shapiro-Wilk's test, which was preferred to other tests (including the D'Agostino-Pearson *omnibus* or the Kolmogorov-Smirnov tests) due to the small sample size employed. Continuous data were expressed as means $\pm$ standard deviation, whereas categorical parameters were computed as percentages, where appropriate. Relevant socio-demographic (age, gender) and clinical (body mass index, BMI, child's disease duration, PASI, DAPSA, SCORAD, pharmacological treatment, parent's PSQI, and parent's eventual comorbidities) information was extracted from clinical charts.

Both univariate (Student's t-test, analysis of variance, ANOVA, or their non-parametric versions in case of violation of normal data distribution, and chi-squared test) and multivariate (multivariate logistic regressions) analyses, as well as correlation analysis, were conducted to shed light on the determinants of sleep quality among parents with children affected by dermatological disorders (psoriasis, psoriatic arthritis, and atopic dermatitis).

Figures with p-values equal to or less than 0.05 were considered statistically significant. All statistical analyses were performed by means of the commercial software "Statistical Package for Social Sciences" (SPSS for Windows, version 24.0, IBM, Armonk, NY, USA). Graphs were generated utilizing the commercial software MedCalc version 18.11.3 (MedCalc Software bvba, Ostend, Belgium).

RESULTS

Ninety children were recruited (age 12.36 ± 1.83 years, 45 male and 45 female, average duration 3.84 ± 4.12 years), namely 30 with PsO (PASI 7.00 ± 2.49), 30 with AD (SCORAD 33.13 ± 10.03), and 30 with PsA (DAPSA 26.40 ± 10.94). Clinical and therapeutic information is summarized in Table I.

Ninety parents (45 males and 45 females) had an average of 49.83 ± 6.69 years with a BMI of 26.23 ± 1.73 . Moreover, 15 (16.7%) parents were affected by the same disease as their children. Parents' PSQI was 6.17 ± 1.91 , with 72 (80.0%) parents with children affected reporting a poor sleep quality. In greater detail, the prevalence of poor sleep quality was 70.0%, 73.3%, and 96.7% in the case of children with AD, PsO, and PsA, respectively. Further details are reported in Table I.

A significant correlation was found between parents' PSQI and children's age ($r=0.21$, $p=0.0452$). Parents with children affected by PsO or PsA had a poorer sleep quality with respect-parents with

children suffering from AD (6.23 ± 2.46 and 6.73 ± 1.42 vs 5.53 ± 1.55 , $p<0.001$). Remarkably, the association between parents' PSQI and children's disease duration was not significant ($r=-0.18$, $p=0.0902$); conversely, the correlation between parents' PSQI and their BMI was statistically borderline ($r=-0.21$, $p=0.0516$).

At the univariate analysis, parents with PsA children displayed the poorest quality of sleep ($p=0.0054$) (Fig. 1); evaluating the poor sleep group, female parents had a statistically significant higher PSQI ($n=42$, 58.3% versus $n=3$, 16.7%, 0.0017) (Fig. 2). All other variables under study did not result statistically significant. For further details, the reader is referred to Table II.

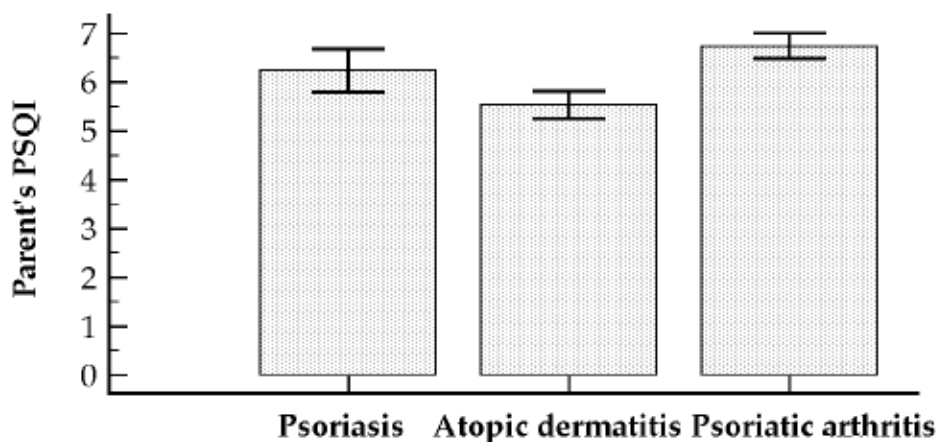


Fig. 1. Sleep quality in parents with children affected by dermatological disorders broken down according to child's disease. PSQI: Pittsburgh Sleep Quality Index.

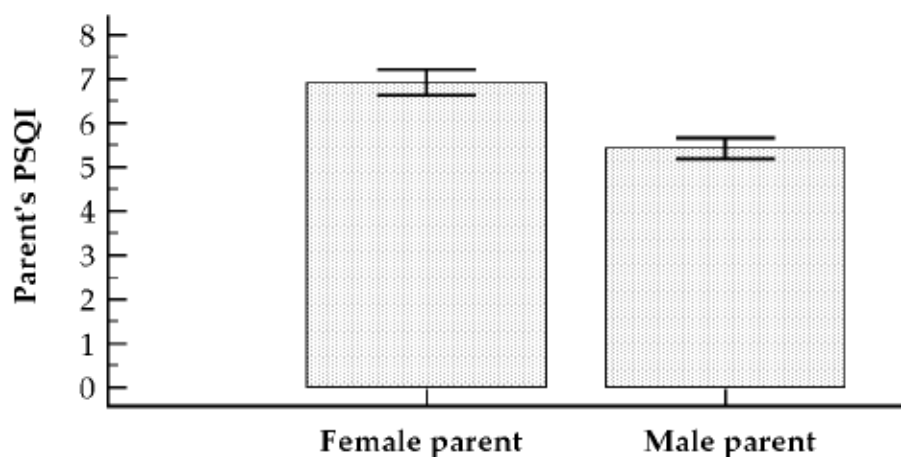


Fig. 2. Sleep quality in parents with children affected by dermatological disorders broken down according to parent's gender. PSQI: Pittsburgh Sleep Quality Index.

Table II. *Univariate analysis assessing differences between parents with children affected by dermatological disorders reporting poor and good sleep quality.*

Parameter	Poor sleep quality (n=72)	Good sleep quality (n=18)	Statistical significance (p-value)
Child			
Age, mean±SD, years	12.40±1.81	12.17±1.92	NS
Gender, N (%)			NS
Male	34 (47.2%)	11 (61.1%)	
Female	38 (52.8%)	7 (38.9%)	
Disease			
Disease duration	3.44±4.17	5.44±3.57	NS
Mild severity, N (%)	15 (20.8)	5 (27.8)	NS
Moderate severity, N (%)	44 (61.1%)	10 (55.6%)	NS
Severe severity, N (%)	13 (18.1%)	3 (16.7%)	NS
Psoriasis, N (%)	22 (30.6%)	8 (44.4%)	NS
PASI, means±SD	7.00±2.47	7.00±2.73	NS
Atopic dermatitis, N (%)	21 (29.2%)	9 (50.0%)	NS
SCORAD, means±SD	31.95±8.93	35.89±12.36	NS
Psoriatic arthritis, N (%)	29 (40.3)	1 (5.6)	0.0054
DAPSA, means±SD	26.14±1.04	34.00±0.00	NS
Tender joints, means±SD	3.55±1.96	3.00±0.00	NS

Swollen joints, means±SD	1.03±1.09	2.00±0.00	NS
Activity, means±SD	4.38±1.68	5.00±0.00	NS
Pain, means±SD	3.69±2.52	7.00±0.00	NS
CRP, means±SD, mg/L	13.48±7.53	17.00±0.00	NS
Pharmacological treatment			NS
Adalimumab, N (%)	19 (26.4%)	1 (5.6%)	
Cyclosporine, N (%)	3 (4.2%)	1 (5.6%)	
Dupilumab, N (%)	14 (19.4%)	4 (22.2%)	
Etanercept, N (%)	17 (23.6%)	5 (27.8%)	
Methotrexate, N (%)	7 (9.7%)	1 (5.6%)	
NB-UVB, N (%)	4 (5.6%)	4 (22.2%)	
SB4, N (%)	5 (6.9%)	1 (5.6%)	
Secukinumab, N (%)	3 (4.2%)	1 (5.6%)	
Parent			
Age, mean±SD, years	49.58±6.45	50.83±7.68	NS
Gender, N (%)			0.0017
Male	30 (41.7%)	15 (83.3%)	
Female	42 (58.3%)	3 (16.7%)	
BMI, mean±SD, kg/m ²	26.07±1.73	26.89±1.60	NS
Affected, N (%)	11 (15.3%)	4 (22.2%)	NS

BMI: Body mass index; **CRP:** C-reactive protein; **DAPSA:** Disease Activity in PSoriatic Arthritis; **NB-UVB:** Narrow band ultraviolet-B; **NS:** Nonsignificant; **PASI:** Psoriasis area and severity index; **PSQI:** Pittsburgh Sleep Quality Index; **SCORAD:** SCORing Atopic Dermatitis.

At the multivariate logistic regression analysis, having a child with PsA (OR 52.25 [95%CI 1.92-1,422.66], $p=0.0189$) resulted in a risk factor for poor sleep quality, whereas being a male parent (OR 0.14 [95%CI 0.02-0.86], $p=0.0337$) was a protective factor (Table III).

DISCUSSION

The present study showed that parents with children affected by AD or PsO or PsA had a high prevalence of poor sleep quality, especially in the case of female parents with children affected by atopic dermatitis.

Sleep disorders in a pediatric contest are usually studied at the patient level, without extending this screening to the caregivers or family members. In fact, the family is an open, highly dynamic system that is not immune to the influence of external factors, including stressors (27). Children with

chronic disorders represent a major source of concerns for their caregivers and their sleep. For instance, Meltzer and Booster (28, 29) studied the sleep quality in caregivers of children with AD and asthma, finding a higher prevalence of insomnia and chronic partial sleep deprivation in these caregivers than in parents with unaffected children.

Similarly, Moore and colleagues (41) recruited 92 parents of 55 children with moderate-to-severe atopic eczema and/or asthma. Mothers and fathers caring for AD children lose 39 and 45 minutes of sleep *per* night, respectively, independently of children's age and family composition (single-parent *versus* two-parent family). It is interesting to compare our findings with those of this study: while at the correlation analysis, we found an association between sleep quality and child age, with no correlation between parental PSQI and disease severity, Moore et al. (41) reported opposite trends.

Table III. Multivariate logistic regression analysis shedding light on the co-variables associated with poor sleep quality.

Variable	Coefficient	SE	Wald	P	OR	95% CI
Child's age	-0.03	0.24	0.02	0.8845	0.97	0.61-1.53
Male child	-0.74	0.83	0.79	0.3731	0.48	0.09-2.42
Child's disease duration	0.13	0.25	0.30	0.5857	1.14	0.71-1.85
Severity class						
Mild	REF.					
Moderate	0.71	0.83	0.73	0.3939	2.03	0.40-10.27
Severe	-1.22	1.34	0.84	0.3599	0.29	0.02-4.05
Child's disease						
Psoriasis	REF.					
Atopic dermatitis	-0.93	1.79	0.27	0.6011	0.39	0.01-13.02
Psoriatic arthritis	3.96	1.69	5.51	0.0189	52.25	1.92-1422.66
Parent's age	-0.03	0.06	0.23	0.6304	0.97	0.86-1.10
Male parent	-2.00	0.94	4.51	0.0337	0.14	0.02-0.86
Parent's BMI	-0.07	0.24	0.09	0.7649	0.93	0.58-1.49
Parent affected	-0.17	0.89	0.04	0.8465	0.84	0.15-4.80
Constant	5.75	8.14	0.50	0.4797		

BMI: Body mass index; **CI:** Confidence interval; **OR:** Odds ratio; **P:** Probability; **REF:** Reference.

The impact of children disease on parental sleep has been studied for a variety of disorders, including sleep disturbances, malignancies, seizures, cystic fibrosis, respiratory disorders, or inherited metabolic diseases (42-46), among others. However, to the best of our knowledge, no study has specifically investigated the effect of dermatological disorders such as psoriasis or psoriatic arthritis on parental sleep quality.

A recent comprehensive literature review (47) has found that children and young adolescents suffering from chronic illnesses disrupt their parents' sleep in 15-86% of cases: in our study, we found that 80% of parents reported poor sleep quality. Recent findings suggest that parents of infants and toddlers with congenital heart disease report high parenting stress, poor sleep, and maladjustment similar to that reported by parents of children with cancer, obesity, and diabetes. Analyses indicate the stress-adjustment relationship is mediated by the quality of sleep. Poor sleep quality is associated with greater negative mood among those with high levels of parenting stressors. Conversely, high levels of parenting stressors were associated with larger increases in negative mood following a night of poor sleep (48).

Furthermore, sleep health has been rarely investigated from a gender perspective; most of the sample either consists of mothers only or the mothers correspond to the clear majority of the sample. A recent cross-sectional, descriptive study confirm that mothers of school-aged children with developmental disabilities experienced frequent sleep disruptions, short sleep duration, and poor sleep quality, with about one-third of these mothers' sleep interruptions being due to the sleep-related difficulties of their children with disabilities diseases (49). Existing scholarly studies show that females tend to report poorer sleep quality than males, even though polysomnography is not able to objectify this complaint (50). It is speculated that biological, physiological (hormone profile), clinical/para-physiological (pregnancy and menopause) factors may contribute, at least partially, to explain gender-related differences in sleep quality and behavior as well as sleep disturbances.

Parenting stress, with consequent sleep deprivation and circadian disruption, alters allostasis

and elevates allostatic load, affecting brain and body systems. Chronic circadian disturbance and shortened sleep time are linked to higher cortisol, obesity, and temporal lobe volume reduction. Circadian rhythmicity and sleep-wake rhythms are altered in mood disorders. (51); Thus, for these motivations and in line with precision medicine, therapies should be more and more respectful to both biological signature (52,53) and patient's environment (54,55) in order to guarantee the best response with the lowest risk of therapy-related side effects (56-59). However, despite its novelty, our study is not without any limitations. The major shortcoming is given by the study design, which, being cross-sectional, does not enable to make causal inferences in a statistically robust way. Further longitudinal investigations employing larger sample sizes are, therefore, urgently needed to replicate and corroborate our findings.

This study demonstrated that PsO, PsA, and AD also affected the sleep quality of the family, and in this specific case of parents, furthermore being a female parent with a child affected by psoriatic arthritis was a risk factor for poor sleep quality; thus, these data highlight the need to introduce the screening for sleep disorders among PsO, PsA and AD patients to assess in further detail the quality of life of patients and caregivers. Interventions aimed at improving sleep quality in parents of children with dermatological disorders and, in particular, psoriatic arthritis are expected to have important effects on parental psychological mental health and, more generally speaking, well-being. Sleep interventions for patients and parents may be helpful interventions to promote adjustment to chronic stress. However, given the above-mentioned limitations, further research is needed.

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Conflict of interests:

No conflict of interest.

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Integrated proteomics and transcriptomics identified novel potential prognostic biomarkers in ovarian cancer

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Ovarian cancer (OC) has been regarded as the most malignant gynecological neoplasm and leading mortality-causing cancer in females worldwide. It has a high fatality rate and is difficult to be detected early. The classification of OC has drawn the attention of numerous researchers, while the difference between layer classification is still unclear. This study explored the common genes between proteomics and transcriptomics and the potential mechanism related to layer classification. Our results provide several potential biomarkers in the early diagnosis of ovarian cancer. We identified 962 differentially expressed mRNA (DEGs) and 544 differentially expressed proteins (DEPs). The comprehensive analysis of the two omics found that the proteins in the MAPK pathway showed corresponding changes in ovarian cancer progression. Furthermore, CD14 and MECOM were up regulated in OC and higher in the advanced OC than in the early stage. In conclusion, our research provided 2 potential early prognostic biomarkers for OC.

Ovarian cancer (OC) is among the top ten cancers, leading to a large number of female deaths in females worldwide (1). In the early stage, OC usually has vague symptoms, such as loss of appetite, bloating, and abdominal swelling. Research showed that the lack of early diagnostic biomarkers and methods leads to the late detection of many OC patients (2). The long-term survival rates (>10 years) of the OC patients with early stage (stage I or II) are 80-95%, while patients with late stages (stage III or IV) are less than 30% (3, 4). As we know, tumor growth may lead tumor cells to metastasize to other organs such as the abdomen, lymph nodes, and lungs (5). As a result, OC's overall survival rate and progression-free survival rate have decreased; meanwhile, the

poor prognosis and recurrence rate are getting higher (6). Because of the high mortality of OC patients, more effective biomarkers and screening methods for early detection and prognostic surveillance of OC are urgently needed (7). Furthermore, protein profile differences between Late-stage surgical specimens and early-stage in ovarian cancer are not well known. Over the past decades, many biomolecules, including proteins, have been performed to identify novel OC biomarkers (8). The application and investigation of proteomics technologies have been widely used to the diagnosis and personalized treatment of OC (9).

Studies have shown that the genetic defect of cancer is transcribed and translated into proteins, meanwhile leading to abnormal phenotype (7).

Key words: ovarian cancer, transcriptomics, proteomics, CD14, MECOM

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So far, advancing proteomics technologies have been frequently applied to the early diagnosis and treatment of different cancers. Although several methodologies, including NGS and RNA-seq have been developed to characterize the genomes and transcriptomes of various cancers (10). RNA-Seq shows a comprehensive analysis of the whole transcriptome of the samples (11, 12). In ovarian cancer, RNA-Seq technology and single cell RNA sequence have enabled a more accurate detection in gene expression of mRNA and cell types, which helps identify novel diagnostic and prognostic biomarkers and discover the microenvironments in OC (13, 14).

Although the differential proteomics and transcriptomic profiling of OC have accumulated numerous achievements, the results of biomarkers are still scarce. There are two potential concerns for the DEPs and DEGs to be effective biomarkers. On the one hand, there may exist a vast gulf between transcripts and corresponding proteins (15). On the other hand, most biomarkers found by proteomics studies focused on blood samples, leading to the biomarkers not being specific to OC (16). Hence, it's necessary to analyze the molecular and cellular basis of OC subtypes and associate them with clinical characteristics, which improves the current diagnostic strategy.

This study aims to investigate proteome and transcriptome profiles of the human OC tissue and discover specific prognosis biomarkers during OC progress. Proteomics and RNA-seq technologies were employed to identify the alterations profiles of proteome and transcriptome between later-stage and early-stage OC, which might be associated with the development and progression of OC. In sum, the results from our research may help to supplement candidate biomarkers or key pathways of OC, which will provide a new insight into the development of OC.

MATERIALS AND METHODS

Clinical data of patients with ovarian cancer

A total of 9 surgical tissues from patients diagnosed with severe ovarian cancer (3 early-stage and 6 late-stage) were obtained from the China-Japan Union Hospital of Jilin University between June 2018 and July 2019. All the patients provided informed consent before surgery. All our study protocols were approved and supervised by the Ethics Committee of China-Japan Union Hospital of Jilin University. The clinicopathological features of the patients in the study are summarized in Table I. Patients were not subjected to any neoadjuvant therapy before surgery and patient information was obtained from medical charts and follow-up. Informed consent was obtained from each

Table I. The clinical characteristics of the OC patients

Characteristics	Variable	Number
Age	≥60	3
	<60	6
Sex	Male	0
	Female	9
Tumor Size	≥3 cm	6
	<3 cm	3
Tumor differentiation	Well and moderately	4
	Poorly	5
Tumor infiltration	T1 + T2	3
	T3 + T4	6
Local lymph node metastasis	Negative	7
	Positive	2

patient. Our experiments with human samples conformed to the standards set by the Declaration of Helsinki.

Hematoxylin and Eosin Staining

Paraffin-embedded tissues of ovarian cancer for Hematoxylin and eosin (HE) staining were obtained with informed consent from the OC patients. The slices of the OC tissues were placed in hematoxylin solution and eosin sequentially. Finally, the portions were sealed with neutral balsam and photographed with a microscope.

Transcriptome analysis

For transcriptome analyses, total RNAs were isolated from the OC samples using TRIzol reagent (Life Technologies) according to the manufacturer's instructions and RNA integrity was assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, USA). The RNA sequencing was performed using Illumina NovaSeq 6000 platform.

RNA-Seq data processing

The annotation information of mRNAs of OC in dataset were re-annotated by the latest transcript sequence of Ensembl database (GRCh38.p13) (17). The R package preprocessCore was used to perform quantile normalization on the data, $\log_2$ transformation was performed on the gene expression data. The average RNA expression was used when duplicate data was found, and low-abundance data were removed. Gene expression quantification was performed using RSEM (version 1.3.1). DESeq2 (version 1.24.0) was used to perform differential expressed gene evaluation of early OC and late OC. The raw data is uploaded in NCBI, and access number is GSE161015.

Protein extraction and digestion

All the samples were minced and lysed in 8M urea-containing protease and phosphatase inhibitors (ThermoFisher Scientific, MA, USA). The lysate was centrifuged at 15,000g for 10 min and the supernatant was collected. Protein concentration was determined by Bradford protein assay; 100 μ g proteins per sample were dissolved in 100 μ L 8 M urea. 2 μ L of 0.5 M TCEP was added and the sample was incubated at 37°C for 1 h, then 4 μ L of 1 M iodoacetamide was added to the sample and incubated for 30 min. Pre-chilled acetone was added to precipitate the proteins overnight at -20°C.

The precipitates were washed twice by 1 mL pre-chilled 90% aqueous acetone solution re-dissolved in 100 μ L 100 mM TEAB. Sequence grade modified 1:50 (enzyme: protein, weight: weight) trypsin (Promega, Madison, WI) was added to digest the proteins at 37°C. The peptide mixture was desalted by C18 ZipTip and quantified by Pierce quantitative colorimetric peptide assay (Thermo Scientific, MA, USA).

Tandem mass tags labeling

All peptide mixtures were labeled by using 10-plex TMT reagents (Thermo Scientific, USA) to quantify up to the ovarian cancer samples in the same LC-MS/MS analysis. The TMT sets was performed according to the manufacturer's instructions.

Proteomic data analysis

The mass spectrometry data was searched against the NCBI protein database (Homo_sapiens.GRCh38.pep.all) by Proteome Discoverer Software (version 2.2) (Thermo Scientific, USA). The search parameters: Carbamidomethylation (C) and TMT 6plex (K and N-terminus) were used as static modifications. Oxidation (M) and Acetyl (Protein N-Terminus) were used as dynamic modification. Peptide and fragment mass tolerance were set to 20ppm and 0.02 Da. The criteria that peptide spectrum match false discovery rate (FDR) <0.01 was used to filter results. The raw data is uploaded in <https://www.iprox.org/>, IPX0002579000.

Functional enrichment analysis

Enrichment analyses were performed by the Gene Ontology (GO) annotation database (18). Additionally, the KEGG database (Release 96.0) was used to analyze the pathways of DEGs and DEPs. Otherwise, the STRING database (version 11.0) was utilized to analyze the protein-protein interaction (PPI), and visualized by Cytoscape software (V 3.7.0).

TIMER database

TIMER (<http://timer.cistrome.org/>) is comprehensive database to analyze immune infiltrates of cancers (19). Various of immune cells in the OC microenvironment is estimated using a novel statistical method (20). TIMER2.0 was applied to estimate the abundance of immune infiltrates of OC tissues.

The human protein atlas database

The Human Protein Atlas database (version 20.0), which contains the spatial distribution of proteins in multiple human tissues and organs, was used to analyze the protein expression of CD14 or MECOM between the early stage and advanced stage ovarian cancer tissues (21, 22).

Single cell transcriptome integration analysis

A published HGSC single cell expression dataset (European Genome-Phenome Archive accession number: EGAS00001003452) was used for cell clustering and marker labeling by the CellAssign (version 0.99.11) and Seurat package (version 4.0), adapting the typical pipeline (14). In addition, the KEGG was used to explore the pathways related to highly active genes (top 2000) in ovarian cancer cells. The results of KEGG pathway and their associated genes were demonstrated with Goplot package (version 1.0.2).

Statistical analysis

In this study, the thresholds of $\log_2$ (Fold Change) >0.263 and $P\text{-adjust} < 0.05$ were used to identify significantly differentially expressed genes and proteins (DEGs/DEPs). Student's t-test was used to compare the significance of two groups. A p-value less than 0.05 is statistically significant.

RESULTS

Patient characteristics and pathological variable

A total of 9 patients diagnosed with ovarian cancer were included. The patient characteristics and pathological variables are summarized in Table I. Age of the patients ranged from 33 to 63 years. The characteristic microscopic appearances of tumor budding were showing on H&E staining. The early-stage tumor tissues limited to ovarian (Fig. 1A), while the advanced stage tumor tissues show large pleomorphic nuclei (Fig. 1B). According to the morphological characteristics, the tumors are divided into 2 types that 3 are belong to early stage (I/II), and 6 are advanced stage (III/IV).

Screening the differentially expressed genes and proteins

In order to screen significantly genes and proteins that can distinguish early and late stage of ovarian cancer. The RNA-seq and TMT proteomics technology were applied to identify the differentially expressed genes and proteins of these 9 tumor tissues, respectively. The DEGs and DEPs were visualized by volcano and heatmap. The volcano shows the distribution of the genes (Fig. 2A). We obtain 962 DEGs, which contain 790 up-regulated

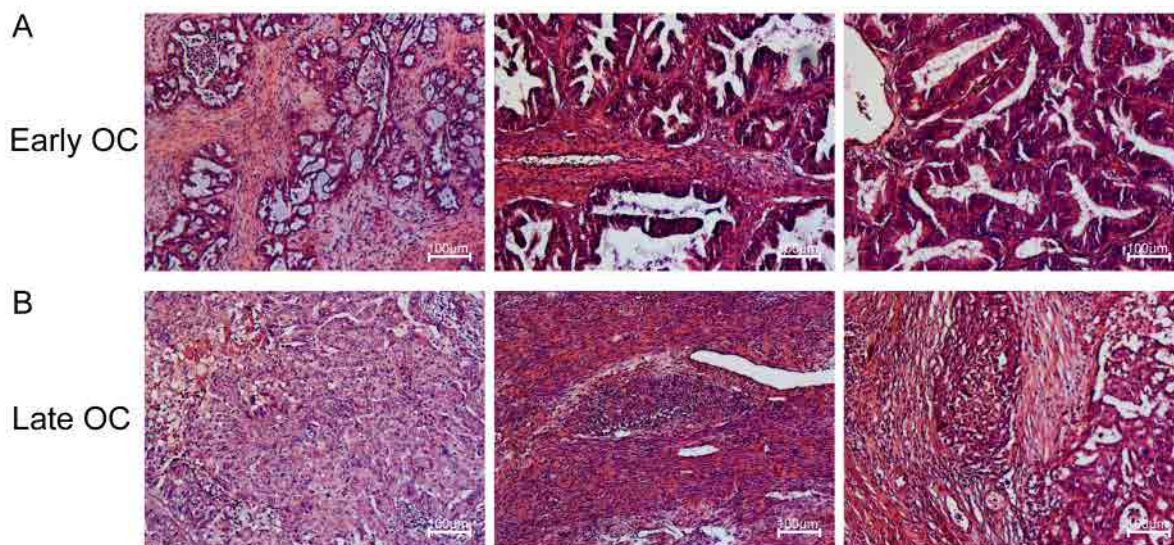


Fig. 1. The morphology of the patients' ovarian cancer tissues. The hematoxylin and eosin (HE) staining of ovarian cancer tissues of early stage (A) and late stage (B).

genes and 172 down-regulated genes in late-stage ovarian cancer tissue (Fig. 2B). This indicates that most genes were up regulated in advanced ovarian cancer patients. Moreover, protein abundance between early and late-stage ovarian cancer were also compared (Fig. 2C and 2D). Totally 544 DEPs were significantly dysregulated, of which, 455 and 89 proteins were up-regulated and down-regulated in the late-stage group, respectively.

Functional enrichment analysis of DEGs and DEPs

Functional enrichment analysis was implemented using GO biological process and KEGG pathway. The enrichment was performed for all significant DEGs and DEPs. The top biological process enrichment results of DEGs are visualized in Fig. 3A, which contains apoptotic process, positive regulation of NF- κ B transcription factor activity, T cell differentiation, inflammatory response and so

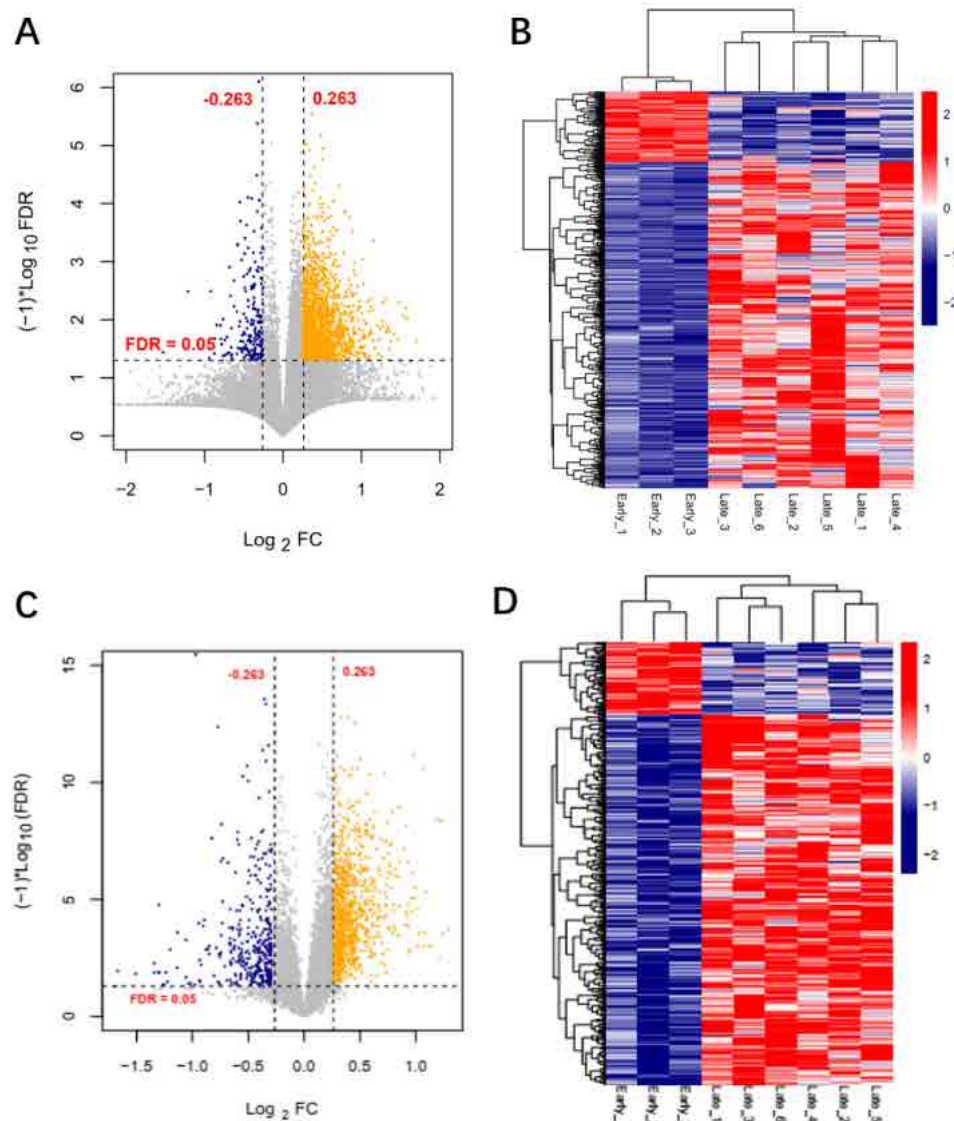


Fig. 2. The differentially expressed mRNAs and proteins between early stage and late-stage ovarian cancer patients' tissues. (A): Volcano plot of log₂ fold-changes differentially expressed mRNAs of early stage (I-II) and late stage (III-IV) ovarian cancer. mRNAs significantly elevated in late stage or early stage are colored in yellow and blue, respectively; (B): Heatmap of the significantly changed mRNAs between late stage and early-stage groups. Red represents up-regulated, and blue indicates the down-regulated mRNA in the late-stage patients; (C-D): Volcano plot and heatmap of the significantly changed proteins between late stage and early-stage groups.

on. The top enriched KEGG pathways of DEGs were shown in Fig. 3B, which contains MAPK signaling pathway, Oxidative phosphorylation, Pathways in cancer and Pathways in cancer. Otherwise, the biological processes of DEGs are mainly enriched in innate immune response, type I interferon signaling pathway, inflammatory response to antigenic stimulus and regulation of cell proliferation (Fig. 3C). These indicated both DEGs and DEPs are enriched in inflammatory and immune response. Furthermore, the enriched pathways are NF-kappa B signaling pathway, Transcriptional misregulation in cancer and metabolic pathways (Fig. 3D).

The common DEGs and DEPs between proteome and transcriptome of OC

The Venn map showed that there are 89 common DEGs between protein and mRNA level (Fig. 4A). Then, we performed a protein-protein interaction by Cytoscape software based on String database. There were 42 nodes and 47 lines (Fig. 4B) in

the network. Furthermore, we found that MAPK signaling pathway was significantly up regulated by enrichment analysis of the 89 DEGs (Fig. 4C).

CD14 and MECOM may be diagnostic biomarkers in early OC

MAPK signal pathway play a key role in the malignant transformation and proliferation of ovarian cancer in situ and secondary metastatic tumors (23, 24). Based on the genes of MAPK signal pathway, we successfully obtained the mRNA expression data of 2 DEGs from TCGA database by using GEPIA, which contained 426 cancer samples and 88 normal subjects. The mRNA expression levels of the two genes were significantly up regulated in OC, including CD14 ($P < 0.05$, Fig. 5A) and MECOM ($P < 0.05$, Fig. 5B). Moreover, based on the human protein atlas database, the immunohistochemical results of the two genes were acquired. As shown in Fig. 5C, 5D, the two proteins were up regulated in OC than normal controls.

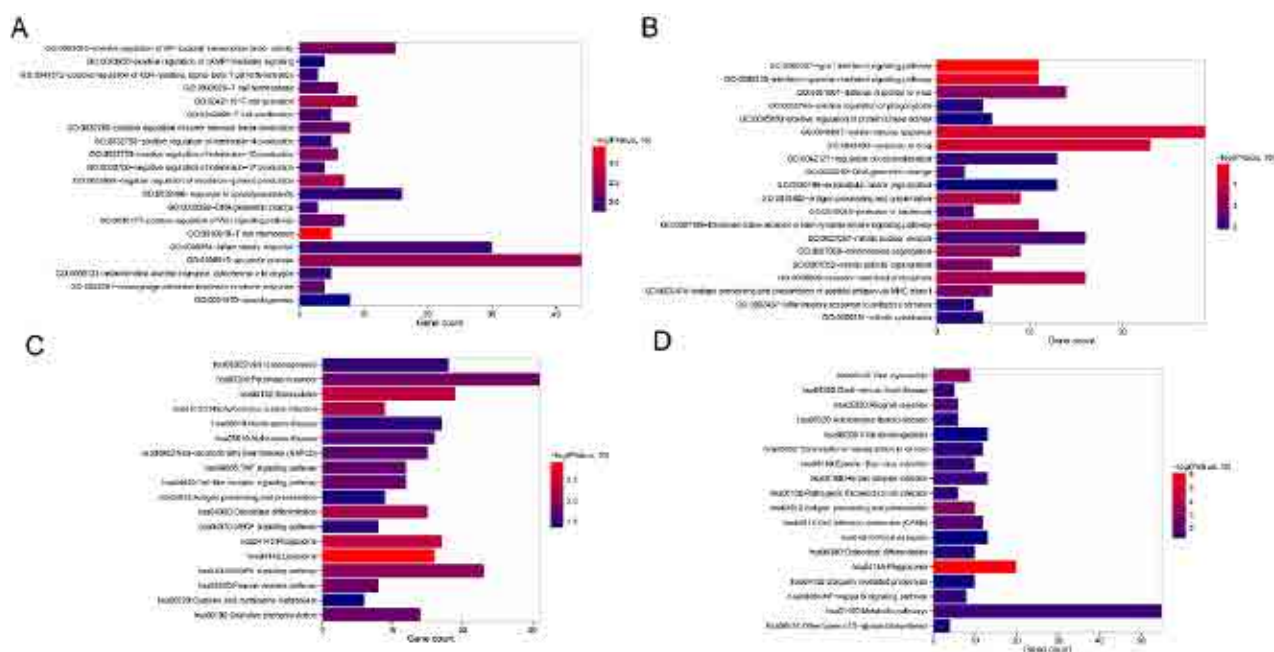


Fig. 3. Functional enrichment analysis between early stage and late-stage OC. (A-B): The top20 GO biological processes and top20 KEGG pathways of significantly changed mRNAs between advanced stage and early-stage patients; (C-D): The top20 GO biological processes and top20 KEGG pathways of significantly changed proteins between advanced stage and early-stage patients.

Subsequently, we assessed the prognostic value of CD14 and MECOM. The overall survival (OS) of 185 OC patients according to the CD14 (HR = 1.612, 95% CI = 1.232-2.108, $P = 4.437 \times 10^{-4}$) and MECOM (HR = 0.761, 95% CI = 0.586-0.987, $P = 3.945 \times 10^{-2}$) status by Kaplan-Meier Plotter analyses were shown in Fig. 5E-F. It is shown that CD14 was related to a worse prognosis but MECOM with better prognosis. Therefore, CD14 and MECOM can be used as surveillance biomarker for the prognosis of ovarian cancer.

As we know, tumor microenvironment is composed with different kinds of cells, especially tumor-infiltrating immune cells, including B cells, T cells, macrophages, neutrophil and dendritic cells (25). These immune cells can promote the occurrence and progression in ovarian cancer. The TIMER database was used to investigate the correlation between CD14, MECOM and the diverse

immune infiltrating cells. As shown in Fig. 5G, CD14 expression with relative content of tumor infiltrating cells, implying the significant role of the CD14 in tumor-infiltrating immune cells and tumor microenvironment ($P < 0.05$), while MECOM maybe not be related with the various immune cells ($P > 0.05$) (Fig. 5H).

Single-cell analysis of the distribution of CD14 and MECOM in OC

To confirm the distribution of CD14 and MECOM in ovarian cancer, the single-cell RNA sequencing dataset of ovarian cancer published by Zhang was included and analyzed in this study (14). Ovarian cancer cells are origin from the ovarian epithelial cell. The identification of ovarian cancer cells used R package of cell assigned and the epithelial cell markers KRT8, KRT18, KRT19 and MUC1. As shown in Fig. 6A, the ovarian cancer specimen

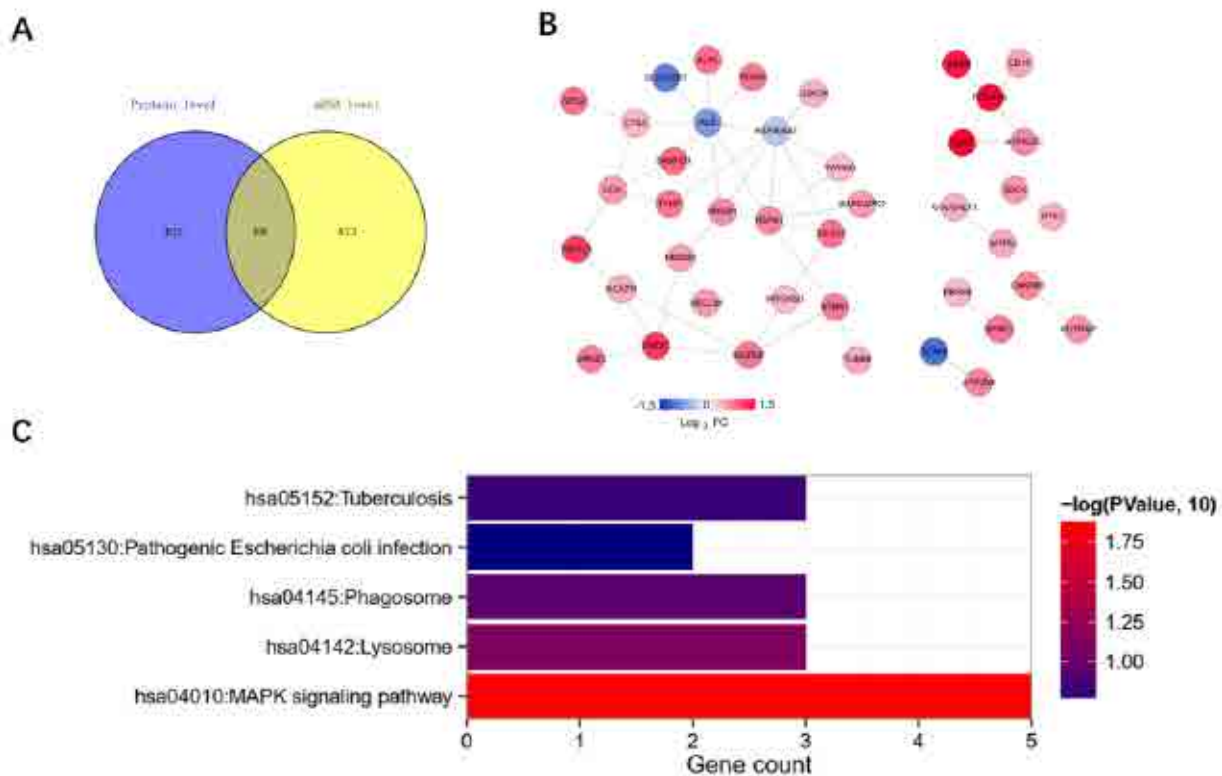


Fig. 4. The common significantly expressed genes between proteome and transcriptome. (A): Common significantly expressed genes between proteome and transcriptome of the two groups; (B): The protein-protein interaction analysis of the common genes between advanced stage and early-stage patients; (C): KEGG pathways of the common genes between advanced stage and early-stage patients. MAPK signal pathway is the most significantly.

mainly contains ovarian cancer cells, ovarian stromal and myofibroblast cells. What's more, immune cells such as monocyte cell, macrophage, B cell and T cell are also presented in ovarian cancer tissue. CD14 is clustered in the monocyte and macrophage cells mainly (Fig. 6B), while MECOM is clustered in the ovarian cancer cells (Figure 6D). The cancer related pathways such as MAPK and WNT signaling

pathway related genes was shown in Fig. 6C; notably, MECOM is one of the high active genes in ovarian cancer. In addition, we enriched the top 2000 expressed genes of the ovarian cancer cells with KEGG. In summary, CD14 and MECOM were up-regulated in ovarian cancer tissues, which may serve as a candidate of diagnostic and prognostic markers for ovarian cancer *in vivo*.

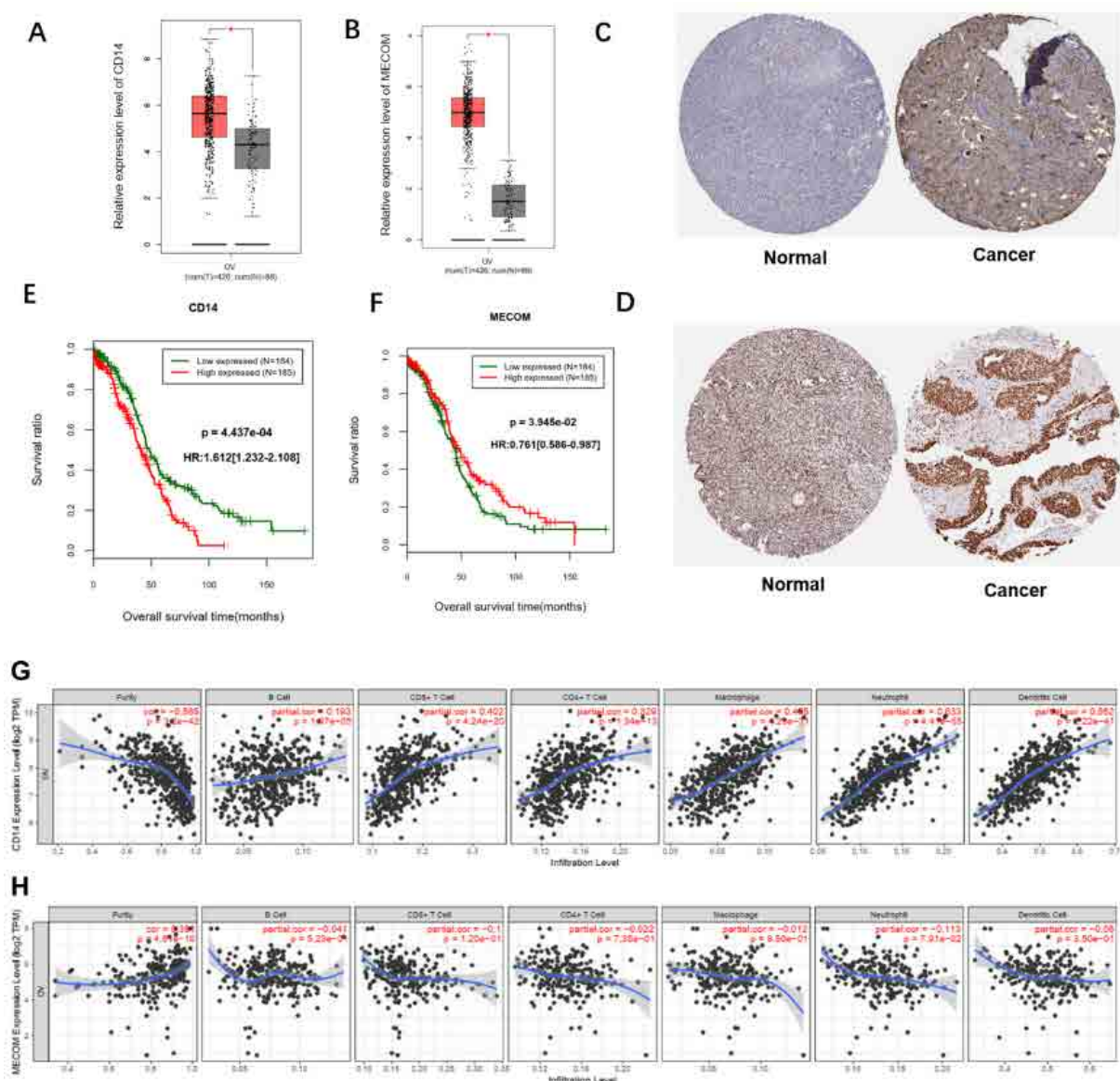


Fig. 5. The expression and overall survival of the CD14 and MECOM in OC. (A-B): The mRNA expression of CD14 and MECOM in TCGA dataset by GEPIA. Both CD14 and MECOM were up-regulated in ovarian cancer; (C-D) The proteins expression of CD14 and MECOM in HPA database. Both CD14 and MECOM were up regulated in ovarian cancer; (E-F): The OS of CD14 and MECOM by Kaplan-Meier Plotter. (G-H): The correlation of CD14 and MECOM relative expression with tumor purity and ovarian cancer infiltrating immune cells, like B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, neutrophils and dendritic cells were presented.

DISCUSSION

Clinically, the lack of accurate biomarkers of cancer limited the early diagnosis and prognostic monitoring of ovarian cancer. Ultra-high resolution mass spectrometers promote the development of quantitative proteomics. There is no doubt that quantitative proteomics is one of the most widely used approaches in biomarker discovery in recent

years (26, 27). Quantitative proteomics are generally used for the discovery of novel biomarker panels in cancers (28), cardiovascular disease and other diseases (29-31).

Also, proteomics technology was performed to investigate ovarian cancer. Throughput proteomics analysis of urine samples, Ni et al. found a urinary protein panel to classify benign and malignant ovarian tumors (32). In addition, extensive three-

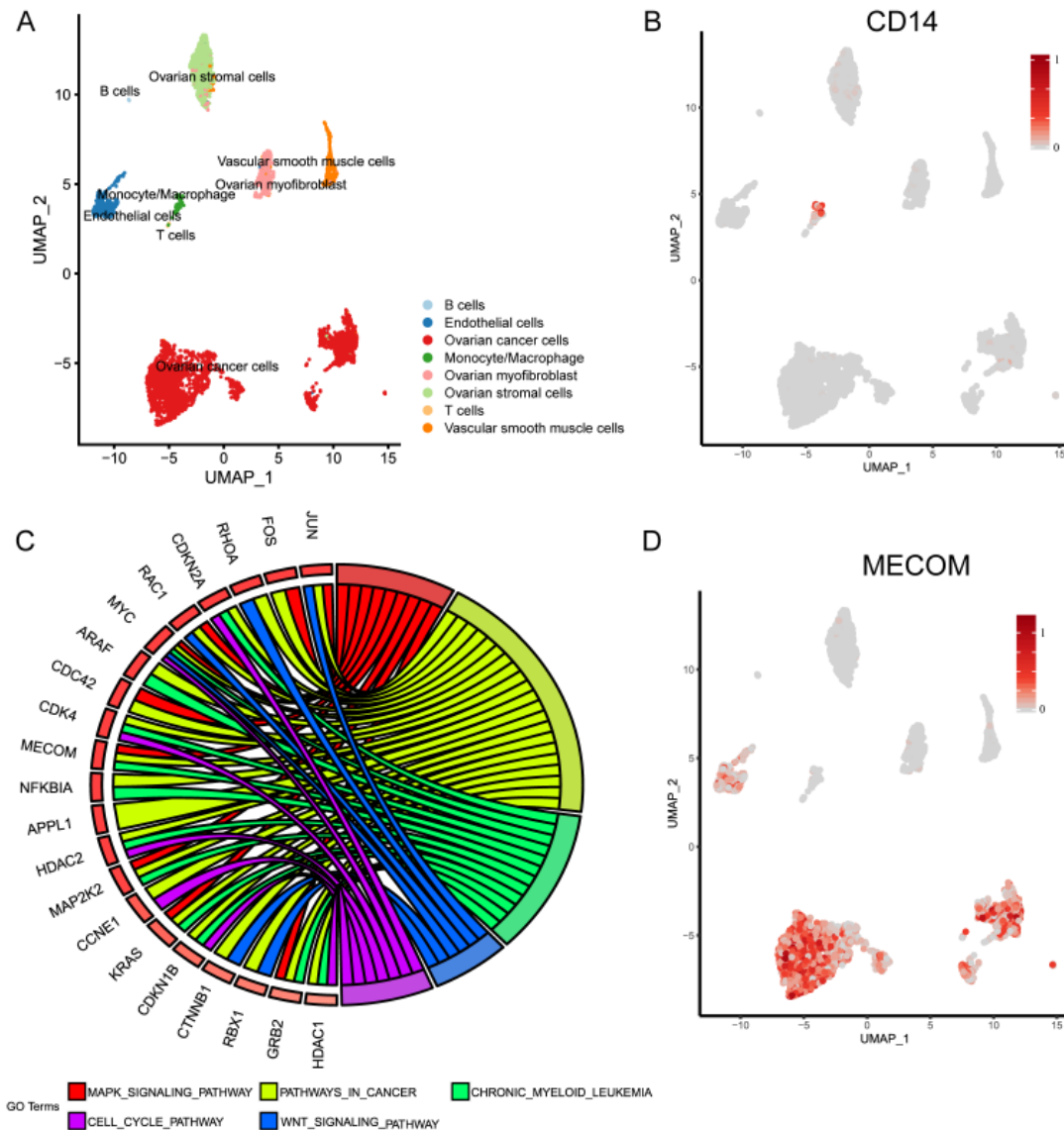


Fig. 6. Clustering and analysis of single cell expression data of OC. (A): UMAP plot indicated cell-types of ovarian cancer tissues; (B and D): Feature plots demonstrated the expression of selected genes on the UMAP plot (A); Scaled expression levels are depicted using a red gradient (grey denotes lack of expression); (C): GO Chord plot showed the KEGG pathway enriched from the top 2000 expressed genes in the ovarian cancer cells and some of their related genes. The red gradient indicates the level of gene expression.

dimensional intratumor proteomic heterogeneity revealed by multiregion sampling in high-grade serous ovarian tumor specimens (33). In this study, using TMT proteomics strategy, we found CD14 and MECOM were two key factors in ovarian cancer prognosis.

CD14 is on the surface of monocytes or mature macrophages and firstly identified as co-receptor of Toll-like Receptors (TLRs) and pattern recognition receptor (PRR) that binds directly to lipopolysaccharide (LPS) (34). CD14 proved to play a dual role in oncogenesis included activated TLR signaling pathway and pathways in tumor infiltrating immune cells (34). The microenvironment of ovarian cancer can promote the distant metastasis of tumor cells. Tumor microenvironment, including inflammatory cytokines, extracellular matrix, and various cells such as stromal cells and immune cells are shown in this study. These cells and extracellular components, especially the immune cell infiltration, establish a tumor autocrine-paracrine communication circuit and promote the invasion and metastasis of epithelial ovarian cancer cells. Otherwise, activation of CD14 in tumor infiltrating immune cells could promote cancer-related inflammation and induce cancer immunosurveillance and promote an immunosuppressive environment to facilitate tumor progression (34). Furthermore, CD14 is highly expressed in the serum of liver cancer and may be applied as a biomarker for the prognosis of liver cancer patients with HBV infection (35). These were consistent with our findings in clinical ovarian cancer tissue of this study.

MECOM is located at 3q26.2 and an oncogenic transcription factor in several kinds of cancers, such as glioblastoma (36), colorectal cancer (37) and leukemia (38). Otherwise, MECOM has been reported to be a potential therapeutic target in ovarian cancer (39). However, we confirm MECOM was up regulated in ovarian cancer and elevated in late stage than in early stage. Moreover, higher level of MECOM predicts better prognosis, this was different from what has been mentioned in other tumors above.

In conclusion, we performed a comprehensive analysis of proteomics and transcriptomics in early

and advanced ovarian cancer. CD14 and MECOM were found to be significantly up regulated in ovarian cancer tissues. The overall survival analyses indicated that the two proteins might be promising prognostic candidates for ovarian cancer. However, there are still some limits. The number of clinical samples in this study is too small, and subsequent verification need larger samples. In addition, the functions and regulatory mechanisms of candidate genes also need to be clarified.

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Long non-coding RNA ENTPD1-AS1 exerts pro-oncogenic activities in colorectal cancer by acting as a competing endogenous RNA for microRNA-432-5p to increase HDGF expression

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ENTPD1 antisense RNA 1 (ENTPD1-AS1), a long non-coding RNA, has been comprehensively studied in glioblastoma multiforme. This study aimed to measure ENTPD1-AS1 expression in patients with colorectal cancer (CRC) and identify its function in the behavior of CRC cells. The expression of ENTPD1-AS1 was measured in a panel of tissue specimens obtained from CRC patients via quantitative real-time polymerase chain reaction. Proliferation, migration, and invasion of CRC cells were evaluated using Cell Counting Kit-8, Transwell cell migration, and invasion assays. Moreover, tumor xenografts were established to evaluate the growth of CRC cells in vivo, and bioinformatics analysis was performed to identify putative complementary miRNAs that interact with ENTPD1-AS1, which was further corroborated in a series of mechanistic studies. We found that ENTPD1-AS1 was overexpressed in CRC based on our sample cohort's Cancer Genome Atlas database. Interference of ENTPD1-AS1 impeded CRC cell proliferation. In addition, in vitro migration and invasion as well as in vivo tumor growth were inhibited after ENTPD1-AS1 knockdown. Mechanistically, ENTPD1-AS1 predominantly functioned as a competing endogenous RNA to sponge microRNA-432-5p (miR-432-5p), resulting in the upregulation of hepatoma-derived growth factor (HDGF) expression in CRC cells. Finally, rescue function tests revealed that miR-432-5p inhibition or HDGF restoration could counteract the inhibitory effects of ENTPD1-AS1 knockdown in CRC cells. Overall, ENTPD1-AS1 may increase HDGF expression in CRC cells by sequestering miR-432-5p, thereby boosting oncogenicity. Thus, the ENTPD1-AS1/miR-432-5p/HDGF regulatory pathway may provide a theoretical basis for the identification of novel treatment strategies for CRC.

Colorectal cancer (CRC) ranks third in terms of morbidity and is the second leading cause of cancer-associated deaths globally(1). In the past decade, China has experienced a significant increase in CRC-associated morbidity and mortality; this disease is

now the fastest-growing cancer in China (2). Recent developments in the diagnosis and multidisciplinary treatments, including surgery, chemotherapy, and radiotherapy, have improved the clinical outcome of patients with CRC(3); however, effective therapy

Key words: *ENTPD1 antisense RNA 1; ceRNA; molecular sponge; microRNA-432-5p; hepatoma-derived growth factor*

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for patients with advanced-stage CRC remains a significant challenge, and the prognosis for these patients is largely unfavorable and very poor (4, 5). Tumor recurrence, distant metastasis, and drug resistance all contribute to the poor clinical efficacy of CRC(6). Although significant efforts have been made to reveal the complete mechanism underlying CRC pathogenesis, the detailed mechanisms are yet to be thoroughly elucidated (7, 8). Therefore, deciphering these pathogenesis mechanisms is essential to improve the diagnosis, prognosis, and treatment of CRC.

Long non-coding RNAs (lncRNAs) represent transcribed RNA molecules with >200 nucleotides(9). Although they lack protein-coding ability, they play a significant role in various normal physiological processes and disease pathogenesises(10). Numerous studies have revealed that lncRNAs are an important driving force for carcinogenesis and cancer progression(11-13). Recently, numerous lncRNAs have been reported to be aberrantly expressed in CRC and are implicated in the modulation of CRC behavior through the action of homologous tumor-promoting or -inhibiting genes(14-16).

MicroRNAs (miRNAs) are a group of highly conserved, non-coding RNA transcripts, that are 17–24 nucleotides in length(17). As gene regulators, miRNAs can directly bind to the 3'-untranslated regions (UTRs) of their target genes through base-pairing, resulting in mRNA degradation and/or translation inhibition(18). The expression of miRNAs in CRC is generally dysregulated and closely related with the genesis and progression of CRC through the disruption of normal cellular processes(19, 20). Recently, an increasing number of studies have affirmed that the competing endogenous RNA (ceRNA) theory is a plausible explanation for the tumorigenesis and development regulation by lncRNAs(21-23). This theory contends that lncRNAs function as ceRNAs by sequestering certain miRNAs, thus promoting the expression of the target mRNA(24). Therefore, investigating the detailed role of lncRNAs in CRC may lead to the identification of novel biomarkers and therapeutic targets.

ENTPD1-AS1 has been comprehensively studied

in glioblastoma multiforme(25). Nevertheless, its specific role and molecular mechanism in CRC require further investigation. Therefore, the purpose of this study was to detect the expression status of ENTPD1-AS1 in CRC and explore the detailed functions of ENTPD1-AS1 on the behavior of CRC cells. Importantly, the putative mechanisms responsible for the regulatory effects of ENTPD1-AS1 in CRC were unveiled by bioinformatics analysis and experimental verification.

MATERIALS AND METHODS

Clinical tissues

The study was approved by the Research Ethics Committee of The First People's Hospital of Chongqing Liang Jiang New Area. Written informed consent was provided by all participants. A total of 51 pairs of CRC tissues and matched adjacent normal tissues were obtained from patients with CRC admitted to our Hospital between October 2014 to May 2016. All participants had not been treated with preoperative chemotherapy or radiotherapy. In addition, none of the patients had been previously diagnosed with another type of cancer. All tissues were immersed in RNALater™ RNA Stabilization Reagent (Beyotime; Shanghai, China) and stored in liquid nitrogen until use. The clinicopathological parameters of all patients with CRC are described in Table I.

Cell lines

The CRC cell lines HCT116 and HT29 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 ng/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). Two other CRC cell lines, SW480 and SW620, were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and maintained in L-15 medium (Gibco; Thermo Fisher Scientific Inc.) containing 10% FBS, 100 U/ml penicillin, and 100 ng/ml streptomycin. The regular human colon epithelial cell line FHC was purchased from ATCC and cultured in DMEM:F12 medium (Gibco; Thermo Fisher Scientific Inc.) containing 10% FBS, 25 mM HEPES,

10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone, and 20 ng/mL recombinant human epidermal growth factor. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Cell transfection

Small interfering RNAs (siRNAs) specific to ENTPD1-AS1 (si-ENTPD1-AS1) and negative control (NC) siRNA (si-NC) were synthesized by GenePharma Co., Ltd (Shanghai, China). MiR-432-5p mimic, NC miRNA mimic (miR-NC), miR-432-5p inhibitor, and NC inhibitor were purchased from Ribobio Co., Ltd (Guangzhou, China). For HDGF overexpression, the full-length HDGF open reading frame was inserted into the pcDNA3.1 plasmid to create the pcDNA3.1-HDGF plasmid. The empty plasmid pcDNA3.1 served as the control of pcDNA3.1-HDGF plasmid. Cells were seeded into 6-well plates and cultured to approximately 70%–80% confluence before transfecting with the above constructs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

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

TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was applied in total RNA extraction, which was quantified with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). In order to detect miR-432-5p expression, total RNA was reverse-transcribed into cDNA using the miScript RT kit (Qiagen GmbH, Hilden, Germany). Next, quantitative PCR was performed using the miScript SYBR Green PCR Kit (Qiagen GmbH). U6 small nuclear RNA was used as an internal control for miR-432-5p expression.

For the quantification of ENTPD1-AS1 and HDGF, the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China) and TB Green® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.) were used for reverse transcription and quantitative PCR, respectively. GAPDH was used as a reference for ENTPD1-AS1 and HDGF. Each sample was analyzed in triplicates, and all qRT-PCR reactions were performed on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of each gene was analyzed using the 2^{−ΔΔC_q} method.

The sequences of the primers were: ENTPD1-AS1 forward, 5'-GGACACTTAAGAGCCTTCGGCT-3' and

reverse, 5'-TTAGCGGCAAGTTAACGGGAA-3'; HDGF forward, 5'-GATCGAGAACAACCCTACTGTCAA-3' and reverse, 5'-GCATTCCCCTTCTTATACCCG-3'; GAPDH forward, 5'-ACCTGACCTGCCGTCTAGAAAA-3' and reverse, 5'-TTGAAGTCAGAGGAGACCACCTG-3'; miR-432-5p forward, 5'-TCGGCAGGUCUUGGAGUAGG-3' and reverse, 5'-CACTCAACTGGTGTCTGTGGA-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3';

Cell Counting Kit-8 (CCK-8) assay

After a 24-h incubation period, the transfected cells were collected by incubation with 0.25% (w/v) trypsin–0.53 mM EDTA solution and resuspended in complete culture medium to obtain a single cell suspension. The cell suspension (100 µl) containing 2 × 10³ cells was seeded into each well of a 96-well plate. Cellular proliferation was monitored every 24 h for a total of 72 h. At each time point, 10 µl of CCK-8 reagent was added into each well and the cells were cultured under the above conditions for another 2 h. The absorbance at 450 nm was measured using a microplate reader, and growth curves were plotted accordingly.

Transwell cell migration and invasion assays

Cell migration was measured using Transwell chambers (size, 8 µM diameter; BD Biosciences). Briefly, transfected cells were washed twice with PBS and resuspended in basal medium without FBS. A total of 200 µl cell suspension containing 5 × 10⁴ cells was added to the upper compartment of the Transwell chamber, whereas the lower compartment was filled with 500 µl culture medium containing 20% FBS. After culturing for 24 h, the migrated cells were fixed and stained with 4% formaldehyde and 0.05% crystal violet, respectively, followed by imaging and counting under an inverted light microscope. To detect cell invasion, the Transwell chambers were precoated with Matrigel matrix (BD Biosciences), and the remaining experimental steps were performed as described above.

Tumor xenograft experiments

The animal procedures were approved by the Animal Care and Use Committee of The First People's Hospital of Chongqing Liang Jiang New Area. In order to develop a stable ENTPD1-AS1 knockdown cell line, short hairpin

RNA (shRNA) specific to ENTPD1-AS1 (sh-ENTPD1-AS1) and NC shRNA (sh-NC) were cloned into the pLKO.1 vector (Addgene Inc.). Then, 293T cells (Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences) were transfected with the generated vectors along with psPAX2 and pMD2.G utilizing Lipofectamine® 2000. After 2 days, the lentiviruses carrying either sh-ENTPD1-AS1 or sh-NC were harvested and transfected into HCT116 cells. ENTPD1-AS1 stable knockdown cells were selected with puromycin.

Four-week-old BALB/c nude mice were obtained from HFK Bioscience (Beijing, China), and the mice were subcutaneously injected with HCT116 cells stably overexpressing sh-ENTPD1-AS1 or sh-NC. After 1 week, the size of the tumor xenografts was measured every 3 days. Tumor volume was analyzed using the following formula: volume = length × width²/2. Thirty-one days after injection, the mice were euthanized by cervical dislocation and the tumor xenografts were removed for weighing and further analysis.

Bioinformatics analysis

The expression of ENTPD1-AS1 in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) was determined using the Cancer Genome Atlas (TCGA) database, and we obtained the data from online database GEPIA (<http://gepia.cancer-pku.cn/>). The log2FC Cutoff value is 1.0.

lncLocator (26) (<http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/>) was used to predict the subcellular localization of ENTPD1-AS1. Two publicly available algorithms, Starbase 3.0(27) (<http://starbase.sysu.edu.cn/>) and LncBase Experimental version 2.0(28) (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2findex-experimental), were used to identify putative miRNAs targeting ENTPD1-AS1. Starbase 3.0 and LncBase Experimental version 2.0 predicted a total of 88 and 17 miRNAs targeting ENTPD1-AS1, respectively. The levels of five overlapping miRNAs, including miR-26a-5p, miR-27a-3p, miR-27b-3p, miR-365a-3p, and miR-432-5p, in CRC were examined applying TCGA database. miR-365a-3p and miR-432-5p were downregulated in CRC. At last, qRT-PCR analysis revealed that miR-432-5p was notably overexpressed in CRC cells after si-ENTPD1-AS1 transfection. Thus, miR-432-5p was selected for further experimental identification.

The potential target of miR-432-5p was predicted using the TargetScan (<http://www.targetscan.org>) and Starbase 3.0.

Subcellular fractionation

Nuclear and cytoplasmic fractions of CRC cells were separately isolated using the Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada). The expression ratio of ENTPD1-AS1 in the nuclear and cytoplasmic fractions was determined using qRT-PCR. GAPDH and U6 were considered as the cytoplasmic and nuclear controls, respectively.

RNA Immunoprecipitation (RIP) assay

The RIP assay was performed using the EZ-Magna RIP™ RNA Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) based on the procedure recommended by the manufacturer. CRC cells were lysed in complete RIP lysis buffer. Overall, 100 µl of cell extract was obtained and cultured with RIP buffer supplemented with magnetic beads conjugated with anti-Argonaute 2 (Ago2) antibody or normal mouse IgG (Millipore). After an overnight incubation at 4°C, the magnetic beads were collected, rinsed with wash buffer, and treated with Proteinase K for protein dissolution. Finally, the immunoprecipitated RNA was subjected to qRT-PCR analysis. The input containing 10% of the cell lysate was used as the positive control, whereas IgG was used as the NC.

Luciferase reporter assay

The sequences of ENTPD1-AS1 and HDGF containing the predicted wild-type (wt) binding site of miR-432-5p were synthesized and inserted into the psiCHECK™-2 luciferase reporter vector (Promega Corporation, Madison, WI, USA). The resulting luciferase reporter vectors were labeled as ENTPD1-AS1-wt and HDGF-wt, respectively. The mutant (mut) luciferase reporter vectors, namely ENTPD1-AS1-mut and HDGF-mut, were produced in a similar manner. For the reporter assay, CRC cells were seeded into 24-well plates prior to being transfected with the synthesized reporter vectors in parallel with miR-432-5p mimic or miR-NC using Lipofectamine® 2000. The transfected cells were harvested at 48 h post-transfection and luciferase activity was examined using the Dual-luciferase Reporter Assay Kit (Promega Corporation).

Western blot analysis

Cell lysis buffer containing phosphatase and protease

inhibitors (KeyGEN BioTECH; Nanjing, China) and the BCA Protein Assay Kit (KeyGEN BioTECH) were used for total protein isolation and quantification, respectively. Equal amounts of protein were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel followed by transferring onto polyvinylidene fluoride membranes. The membranes were incubated with 5% skim milk at room temperature for 2 h to block the non-specific binding sites. The primary antibodies targeting HDGF (ab128921) or GAPDH (ab181602; Abcam, Cambridge, MA, USA) were incubated with the membranes overnight. After washing three times with western blot wash buffer (KeyGEN BioTECH), the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (ab205718; Abcam). The protein signals were visualized using Immobilon® ECL Ultra Western HRP Substrate (Millipore). GAPDH served as an internal control for normalizing HDGF expression.

Statistical analysis

All experimental data was analyzed using SPSS software 19.0 (SPSS, Chicago, IL, USA). The overall survival curves were plotted utilizing the Kaplan-Meier method, and survival was compared with a log-rank test. Differences between two groups were determined by Student's *t* test. One-way analysis of variance with Tukey's test was used to compare the differences among multiple groups. Spearman correlation analysis was used for evaluating relative expression among ENTPD1-AS1, miR-432-5p, and HDGF. All results are expressed as the mean $\pm$ standard error and *P* values less than 0.05 were considered statistically significant.

RESULTS

ENTPD1-AS1 deletion inhibits cell proliferation, migration, and invasion in CRC

ENTPD1-AS1 expression in COAD and READ was determined using the TCGA database. The results indicated that ENTPD1-AS1 was significantly upregulated in COAD and READ compared with that in normal tissues (Fig. 1A). ENTPD1-AS1 expression was also measured using qRT-PCR in 51 pairs of CRC tissues and matched adjacent normal tissues. Consistent with the results from the TCGA database, the expression of ENTPD1-AS1

was higher in the CRC tissues than that in matched adjacent normal tissues (Fig. 1B). Furthermore, all four CRC cell lines exhibited higher ENTPD1-AS1 expression than the normal human colon epithelial cell line FHC (Fig. 1C). Moreover, patients with CRC and high ENTPD1-AS1 levels showed poor clinical outcomes compared with those with low ENTPD1-AS1 levels (Fig. 1D). The number of death in the high and low ENTPD1-AS1 group is, respectively, 16 and 10. Additionally, the average survival time of high and low ENTPD1-AS1 group is 34.79 and 47.58 months, respectively.

To investigate the functional role of ENTPD1-AS1 in various CRC processes, specific siRNAs targeting ENTPD1-AS1 were generated to silence ENTPD1-AS1 expression in HCT116 and SW480 cells. si-ENTPD1-AS1#1 exhibited the highest silencing efficiency among the three siRNAs and was therefore used for functional assays (Fig. 1E). Silencing ENTPD1-AS1 suppressed the proliferative ability (Fig. 1F) of CRC cells. The migration and invasion abilities of CRC cells after ENTPD1-AS1 incorporation was determined by Transwell cell migration and invasion assays. Additionally, interference of ENTPD1-AS1 significantly inhibited the migration and invasion of CRC cells (Fig. 1G). Collectively, the data suggested that ENTPD1-AS1 exerts tumor-promoting effects during CRC progression.

ENTPD1-AS1 functions as a ceRNA by sequestering miR-432-5p in CRC cells

The subcellular localization of lncRNAs determine their activity and underlying mechanism of action (29). Accordingly, lncLocator was used to predict the subcellular localization of ENTPD1-AS1. ENTPD1-AS1 was predicted to be primarily distributed in the cytoplasm (Fig. 2A), which was further confirmed by subcellular fractionation assay (Fig. 2B). The cytoplasmic lncRNAs can function as ceRNAs or miRNA sponges, thereby regulating genes at the post-transcriptional level (30). Using bioinformatics analysis, Starbase 3.0 and LncBase Experimental version 2.0 predicted a total of 88 and 17 miRNAs targeting ENTPD1-AS1, respectively. As shown in Fig. 2C, miR-26a-5p, miR-27a-

3p, miR-27b-3p, miR-365a-3p, and miR-432-5p were predicted as ENTPD1-AS1's downstream miRNAs by both online databases for lncRNA target prediction. Next, the expression of these miRNAs in CRC was analyzed using the TCGA database. MiR-26a-5p (Fig. 2D), miR-27a-3p (Fig. 2E), and miR-27b-3p (Fig. 2F) were highly expressed and other two miRNAs, miR-365a-3p (Fig. 2G) and miR-432-5p (Fig. 2H), were weakly expressed. Therefore, miR-365a-3p and miR-432-5p were selected for further analysis.

qRT-PCR was performed to measure the effect of ENTPD1-AS1 on miR-365a-3p and miR-432-5p expressions in CRC cells. The expression level of miR-432-5p was significantly overexpressed in CRC cells following ENTPD1-AS1 downregulation, whereas the expression of miR-365a-3p was unchanged upon si-ENTPD1-AS1 transfection (Fig. 2I). Additionally, miR-432-5p expression was measured in 51 pairs of CRC tissues and matched adjacent normal tissues by qRT-PCR. The expression of miR-432-5p was downregulated in CRC tissues (Fig. 2J) and inversely

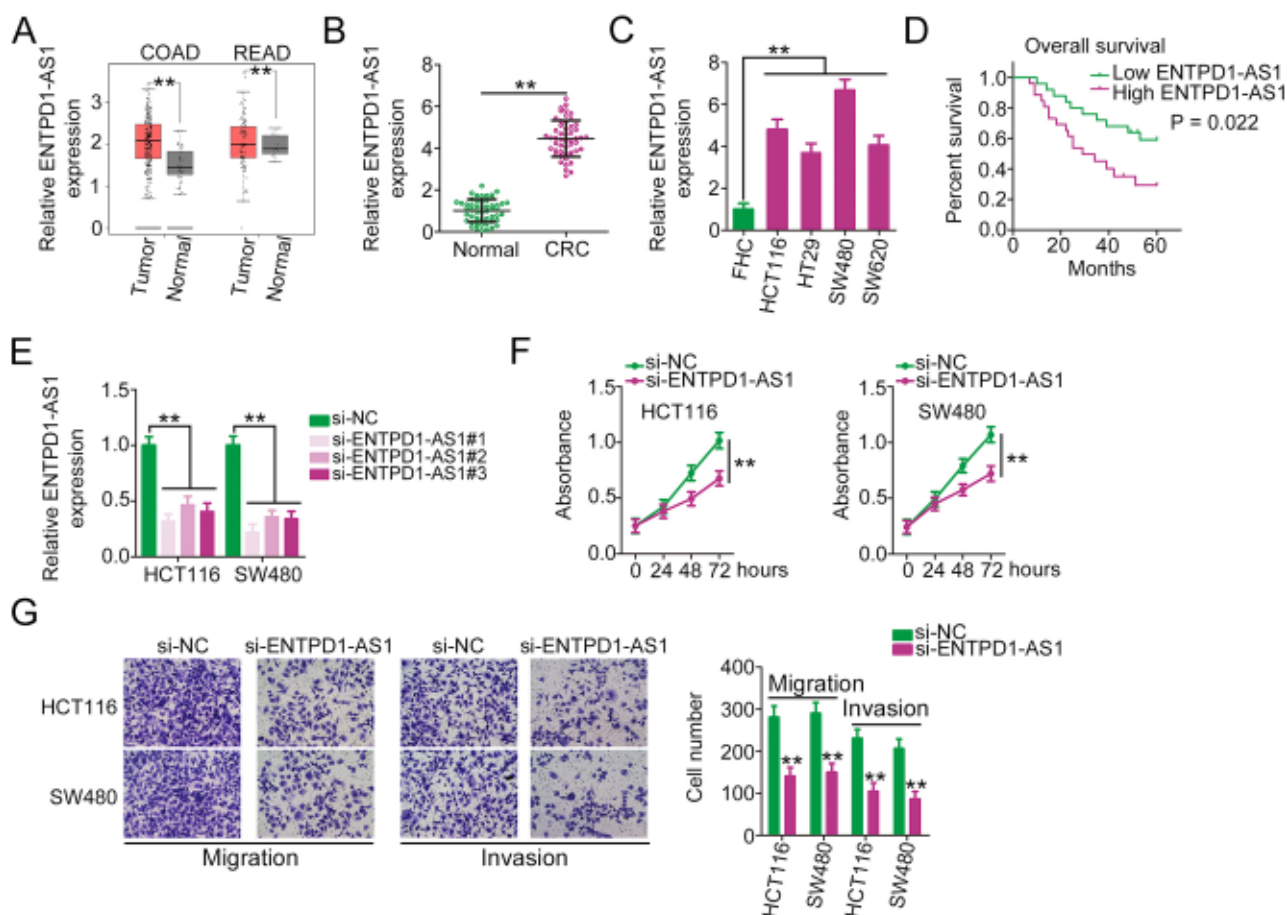


Fig. 1. ENTPD1-AS1 is upregulated in CRC and plays pro-oncogenic roles in cancer progression. (A): ENTPD1-AS1 expression in COAD ($P = 0.0000065$); and READ ($P = 0.048$); was analyzed using TCGA database; (B): qRT-PCR was performed to detect ENTPD1-AS1 expression in 51 pairs of CRC tissues and matched adjacent normal tissues; (C): Expression of ENTPD1-AS1 in four CRC cell lines and the normal human colon epithelial cell line FHC was measured using qRT-PCR; (D): The correlation between ENTPD1-AS1 expression and overall survival in CRC was analyzed; (E): ENTPD1-AS1 expression was measured using qRT-PCR in CRC cells after transfection with specific siRNAs targeting ENTPD1-AS1 or si-NC; (F): The proliferation of CRC cells transfected with si-ENTPD1-AS1 or si-NC was tested by the CCK-8 assay; (G): The migratory and invasive abilities of si-ENTPD1-AS1-transfected or si-NC-transfected CRC cells were investigated by Transwell cell migration and invasion assays, respectively. ** $P < 0.01$.

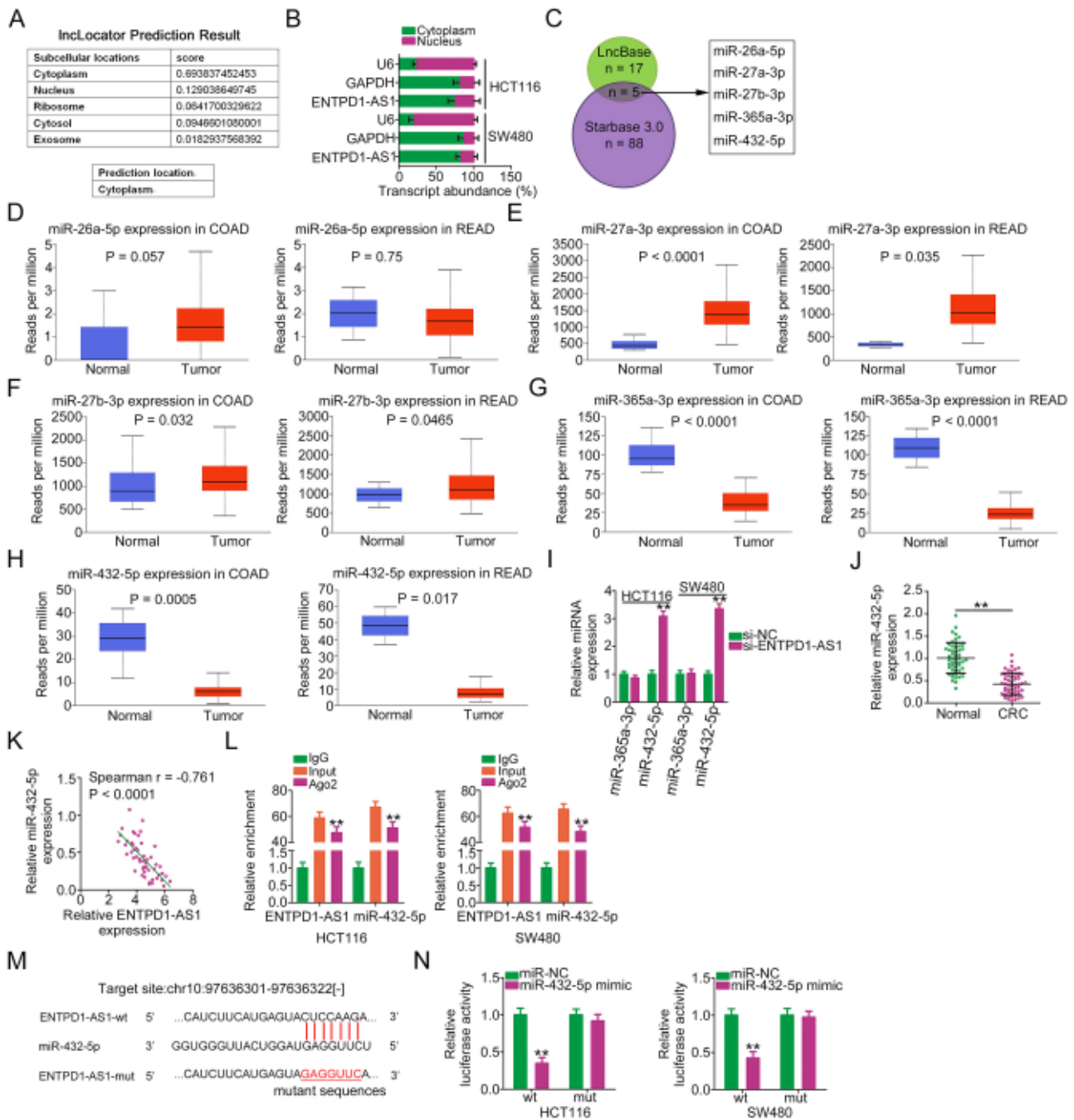


Fig. 2. ENTPD1-AS1 functions as a molecular sponge for miR-432-5p in CRC. (A): The subcellular localization of ENTPD1-AS1 was predicted using lncLocator (a lncRNA subcellular localization predictor); (B): Subcellular fractionation assay was performed to establish the distribution of ENTPD1-AS1 in CRC cells; (C): The putative miRNAs that target ENTPD1-AS1 were obtained from Starbase 3.0 and LncBase Experimental version 2.0; (D-H): The expression of miR-26a-5p, miR-27a-3p, miR-27b-3p, miR-365a-3p, and miR-432-5p in CRC was analyzed using TCGA database; (I): qRT-PCR was used to determine the changes in expression of miR-365a-3p and miR-432-5p in CRC cells after ENTPD1-AS1 knockdown; (J): Expression of miR-432-5p in 51 pairs of CRC tissues and matched adjacent normal tissues was assessed by qRT-PCR; (K): Spearman correlation analysis was used to evaluate the expression correlation between ENTPD1-AS1 and miR-432-5p in 51 CRC tissues (Spearman $r = -0.761$); (L): The enrichments of ENTPD1-AS1 and miR-432-5p in the immunoprecipitated RNA were examined by RIP assay; (M): The sequences of ENTPD1-AS1 containing miR-432-5p binding sites or mutant binding sites are shown; (N): Luciferase reporter assay was performed to verify the binding between miR-432-5p and ENTPD1-AS1 in CRC cells. ** $P < 0.01$.

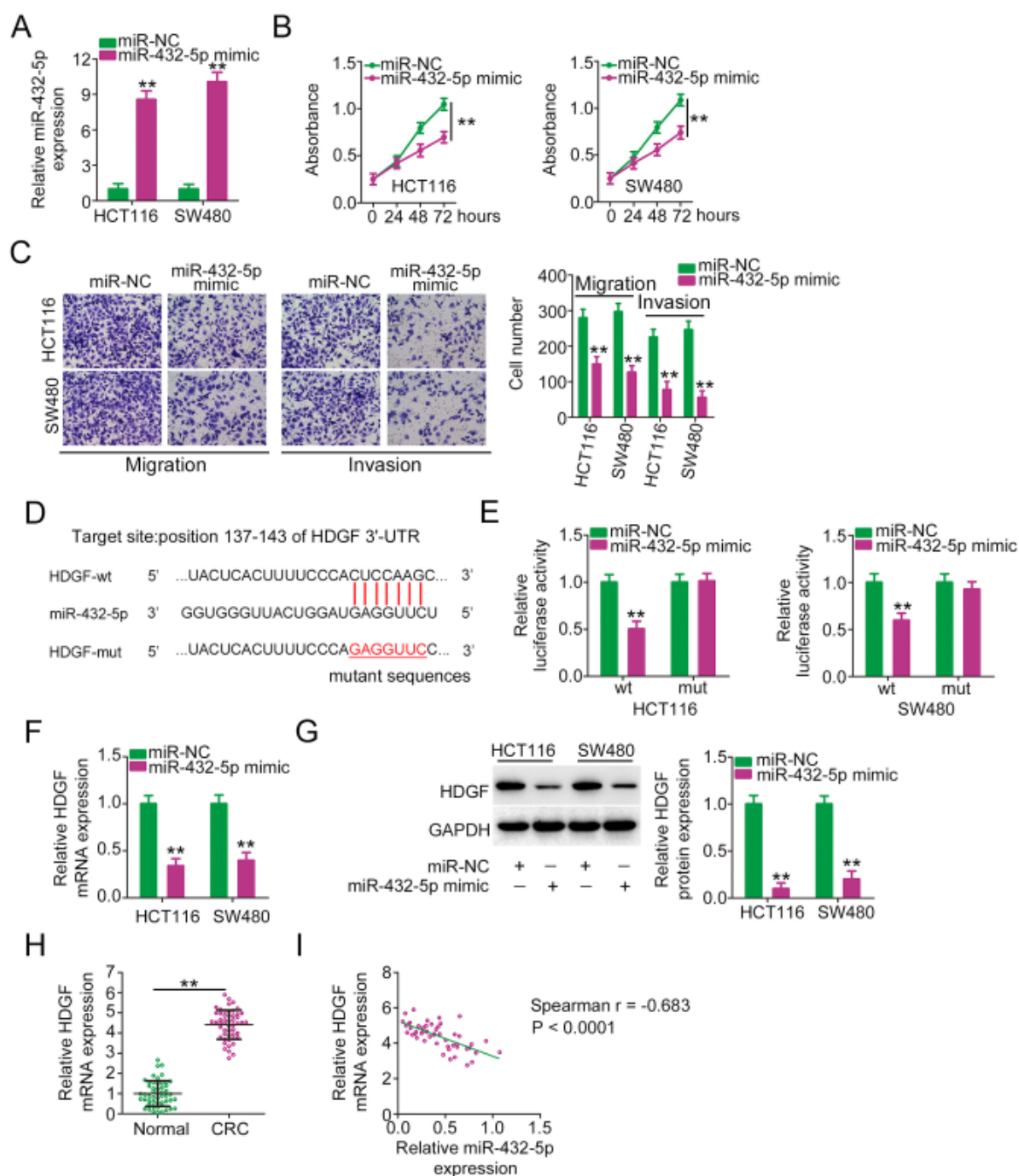


Fig. 3. HDGF is a direct target of miR-432-5p in CRC. (A): CRC cells were transfected with miR-432-5p mimic or miR-NC. Transfected cells were subjected to qRT-PCR to evaluate transfection efficiency; (B): CCK-8 assay was performed to test the proliferation of miR-432-5p overexpressed-CRC cells; (C): The migration and invasion of CRC cells transfected with miR-432-5p mimic or miR-NC was determined by Transwell cell migration and invasion assays; (D): The wild-type and mutant binding sequences of miR-432-5p within the sequences of HDGF 3'-UTR are presented; (E): Luciferase reporter assay was used to measure the luciferase activity of CRC cells after cotransfection with miR-432-5p mimic or miR-NC and HDGF-wt or HDGF-mut; (F, G): qRT-PCR and western blot analysis were conducted to measure the mRNA and protein expression of HDGF in CRC cells after miR-432-5p overexpression; (H): HDGF expression in 51 pairs of CRC tissues and matched adjacent normal tissues was determined via qRT-PCR; (I): The correlation between miR-432-5p and HDGF levels in the 51 CRC tissues was explored via Spearman correlation analysis (Spearman $r = -0.683$). ** $P < 0.01$.

correlated with that of ENTPD1-AS1 (Fig. 2K). The RIP assay confirmed that ENTPD1-AS1 and miR-432-5p were significantly enriched in Ago2 immunoprecipitation, indicating the coexistence of ENTPD1-AS1 and miR-432-5p in the same RNA-induced silencing complex (RISC) (Fig. 2L). The binding interaction between ENTPD1-AS1 and miR-432-5p was verified using luciferase reporter assay. Fig. 2M shows the binding site of miR-432-5p within ENTPD1-AS1. The results of luciferase reporter assay indicated that overexpression of miR-432-5p reduced the luciferase activity of ENTPD1-AS1-wt in CRC cells, but exerted no effect on the luciferase activity of the ENTPD1-AS1-mut (Fig. 2N). Cumulatively, the above data suggested that ENTPD1-AS1 functions as a miR-432-5p sponge in CRC cells.

MiR-432-5p upregulation inhibits the malignant characteristics of CRC cells and directly targets HDGF

Given that miR-432-5p was downregulated in CRC, the function of miR-432-5p was evaluated in more detail. MiR-432-5p mimic was introduced into

CRC cells to overexpress miR-432-5p expression (Fig. 3A). Transfection with miR-432-5p mimic significantly decreased the proliferation of CRC cells (Fig. 3B). Furthermore, the migration and invasion of CRC cells was inhibited by miR-432-5p overexpression (Fig. 3C).

Bioinformatics analysis was conducted to identify potential targets of miR-432-5p. As presented in Fig. 3D, the HDGF 3'-UTR possesses complementary sequences with miR-432-5p. Luciferase reporter assay was used to verify this prediction and the results indicated that the luciferase activity of HDGF-wt in CRC cells was suppressed by miR-432-5p overexpression. In contrast, the luciferase activity of HDGF-mut remained unchanged in response to miR-432-5p mimic co-transfection (Fig. 3E). Furthermore, the mRNA and protein (Fig. 3F, G) levels of HDGF were significantly reduced by miR-432-5p upregulation in CRC cells. Measuring HDGF expression revealed that HDGF was strikingly elevated in CRC tissues compared with that in adjacent normal tissues (Fig. 3H). Besides,

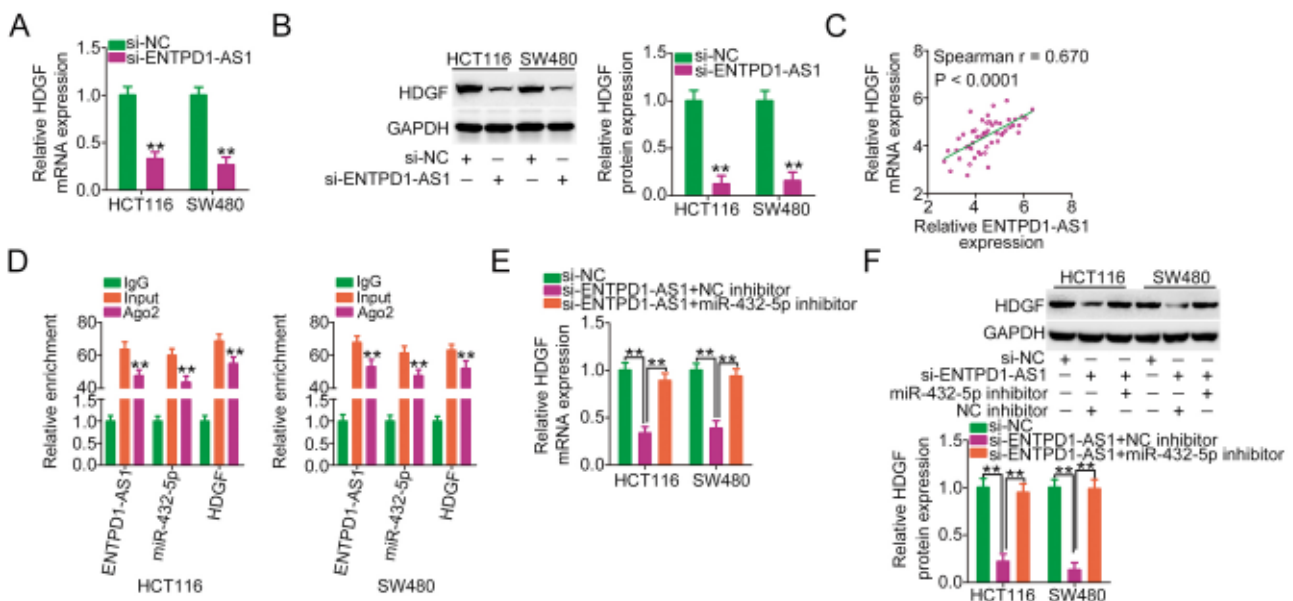


Fig. 4. ENTPD1-AS1 enhances HDGF expression in CRC cells by decoying miR-432-5p. (A, B): CRC cells were transfected with si-ENTPD1-AS1 or si-NC. Transfected cells were harvested and subjected to qRT-PCR and western blot analysis for the detection of HDGF mRNA and protein expression, respectively; (C): The correlation between ENTPD1-AS1 and HDGF expression in 51 CRC tissues was evaluated by Spearman correlation analysis (Spearman $r = 0.670$); (D): RIP assay was applied to determine the enrichment of ENTPD1-AS1, miR-432-5p, and HDGF in the immunoprecipitated RNA; (E, F): si-ENTPD1-AS1 together with miR-432-5p inhibitor or NC inhibitor was introduced into CRC cells. HDGF mRNA and protein expression in different groups was detected by qRT-PCR and western blot analysis, respectively. ** $P < 0.01$.

Spearman correlation analysis identified an inverse correlation between HDGF and miR-432-5p in the 51 CRC tissues (Fig. 3I). Therefore, miR-432-5p may be an anti-oncogenic miRNA and HDGF is its direct target in CRC.

ENTPD1-AS1 positively modulates HDGF expression in CRC cells by decoying miR-432-5p

To further explore the association between ENTPD1-AS1, miR-432-5p, and HDGF in CRC, the level of HDGF in CRC cells with ENTPD1-AS1 knockdown was determined. The results indicated that depletion of ENTPD1-AS1 significantly reduced HDGF expression in CRC cells (Figs

4A and B). The correlation between ENTPD1-AS1 and HDGF expression in the 51 CRC tissues was assessed by Spearman correlation analysis. HDGF mRNA expression manifested a positive association with ENTPD1-AS1 expression in the CRC tissues (Fig. 4C). Furthermore, RIP assay validated the interactions of ENTPD1-AS1, miR-432-5p, and HDGF in the same RISC (Fig. 4D). Rescue experiments were carried out to determine whether miR-432-5p was essential for the regulatory activity of ENTPD1-AS1 on HDGF expression in CRC cells. ENTPD1-AS1-deficient CRC cells were co-transfected with miR-432-5p inhibitor or NC inhibitor, and HDGF mRNA and protein expression

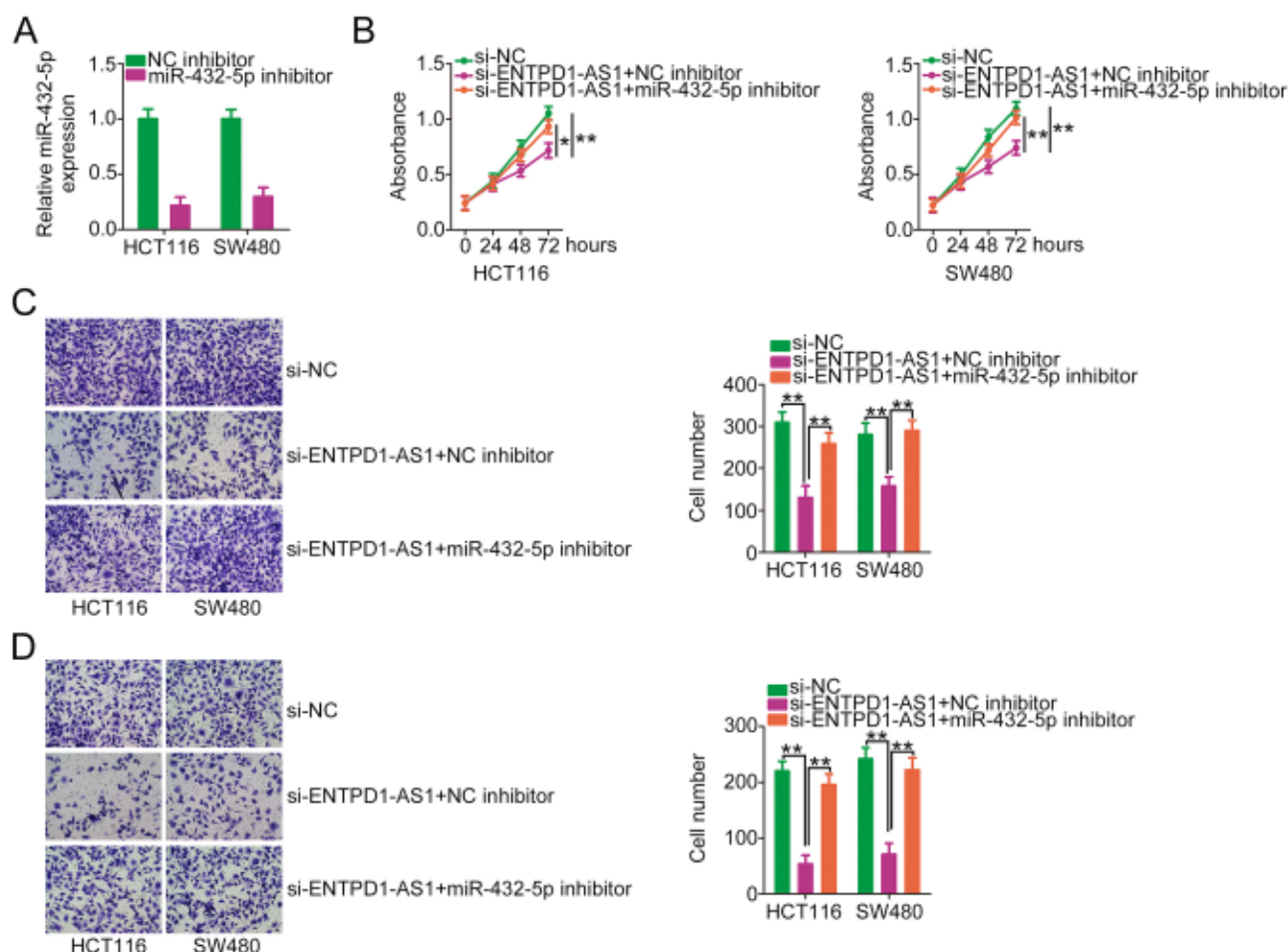


Fig. 5. Inhibition of miR-432-5p neutralizes the functions of ENTPD1-AS1 knockdown in CRC cells. (A): MiR-432-5p expression was measured using qRT-PCR after transfection with miR-432-5p inhibitor or NC inhibitor into CRC cells; (B): CRC cells were transfected with miR-432-5p inhibitor or NC inhibitor in the presence of si-ENTPD1-AS1. CCK-8 assay was performed to assess the proliferation ability; (C, D): Transwell cell migration and invasion assays were used to measure the number of migrating and invading CRC cells that were cotransfected with si-ENTPD1-AS1 and miR-432-5p inhibitor or NC inhibitor. * $P < 0.05$ and ** $P < 0.01$.

was measured. Decreased expression of ENTPD1-AS1 triggered a significant reduction in HDGF mRNA and protein levels (Figs 4E and F), which were restored by cotransfection with the miR-432-5p inhibitor. Collectively, ENTPD1-AS1 functioned as a ceRNA by sequestering miR-432-5p in CRC cells, thereby increasing HDGF expression.

Increasing the output of miR-432-5p/HDGF axis counteracts the cancer-inhibiting activities of ENTPD1-AS1 knockdown in CRC cells

Rescue experiments were performed to explore whether ENTPD1-AS1 functions by regulating the miR-432-5p/HDGF axis. First, the efficiency of miR-432-5p inhibitor in CRC cells was assessed

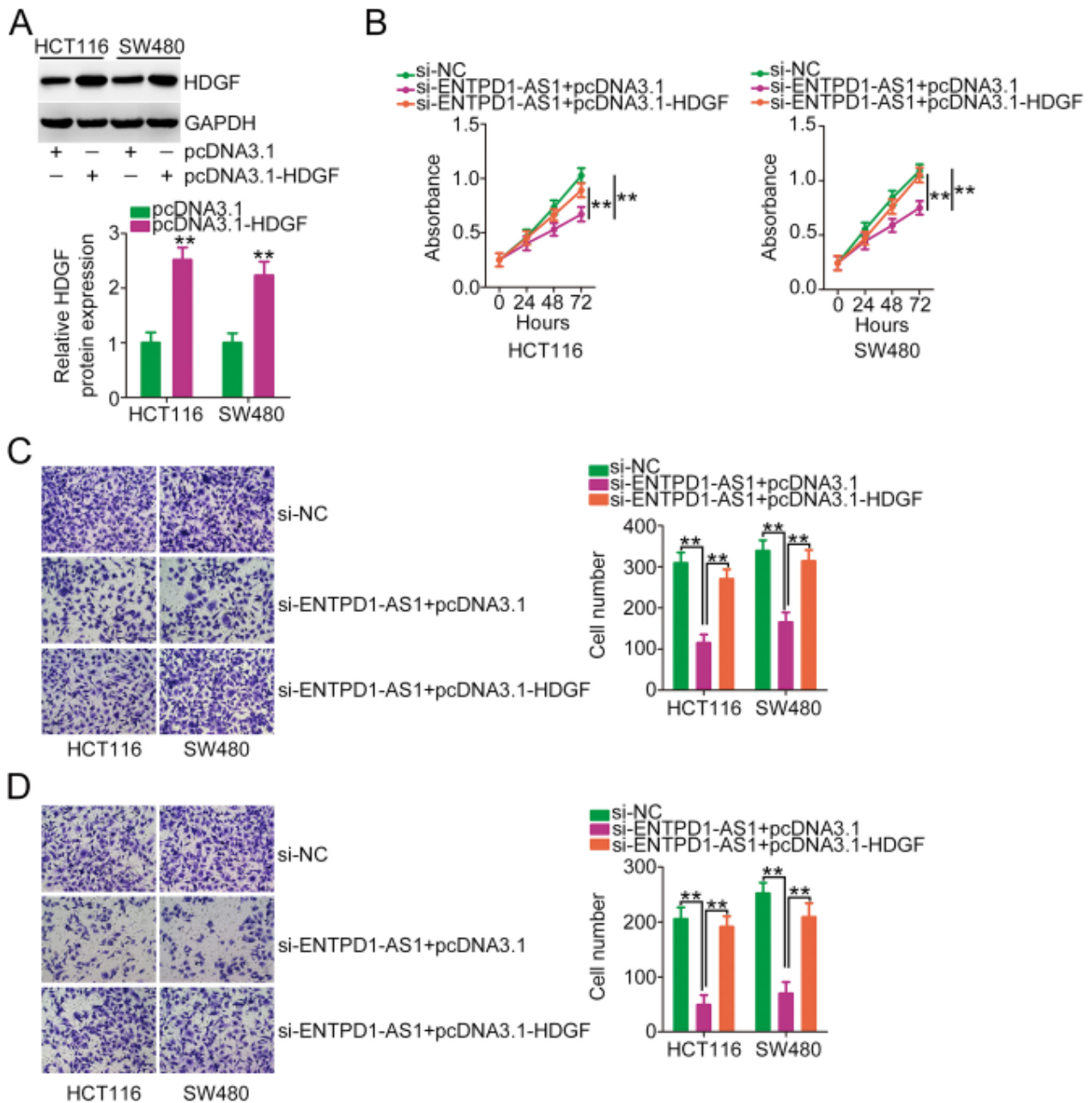


Fig. 6. The inhibitory actions of si-ENTPD1-AS1 in CRC cells are counteracted after HDGF restoration. (A): Western blot analysis was used to detect HDGF protein expression in CRC cells after introducing pcDNA3.1-HDGF or pcDNA3.1; (B-D): The pcDNA3.1-HDGF or pcDNA3.1 alongside si-ENTPD1-AS1 were transfected into CRC cells. Cell proliferation, migration, and invasion were assessed by CCK-8, and Transwell cell migration, and invasion assays, respectively. ** $P < 0.01$.

by qRT-PCR. Decreased expression of miR-432-5p in CRC cells indicated that the transfection efficiency of miR-432-5p inhibitor was sufficient (Fig. 5A). MiR-432-5p inhibitor or NC inhibitor in combination with si-ENTPD1-AS1 were transfected into CRC cells, and their effects on cell proliferation, migration, and invasion were studied. Downregulation of ENTPD1-AS1 significantly inhibited the proliferation of CRC cells, whereas miR-432-5p inhibition partially abrogated the effect (Fig. 5B). In addition, inhibition of miR-432-5p counteracted the inhibition effects of ENTPD1-AS1 silencing on the migration and invasion (Figs 5C and D) capacities of CRC cells.

Similarly, transfection with the HDGF overexpression plasmid pcDNA3.1-HDGF significantly increased endogenous HDGF expression in CRC cells (Fig. 6A). For rescue experiments, CRC cells were transfected with the pcDNA3.1-HDGF or empty pcDNA3.1 plasmid alongside si-ENTPD1-AS1. Knockdown of ENTPD1-AS1 resulted in a marked reduction of cell proliferation, whereas the addition of HDGF neutralized these inhibitory effects (Fig. 6B).

Furthermore, overexpressed HDGF reversed the si-ENTPD1-AS1-mediated effects on CRC cell migration and invasion (Fig. 6C, D). Therefore, ENTPD1-AS1 contributed to the oncogenicity of CRC cells by controlling the activity of the miR-432-5p/HDGF axis.

Interference of ENTPD1-AS1 hinders CRC growth in vivo

Tumor xenografts were established to investigate the functional role of ENTPD1-AS1 in CRC growth in vivo. The volume (Fig. 7A, B) and weight (Fig. 7C) of the resulting tumors were smaller in the sh-ENTPD1-AS1 group than those in the sh-NC group. The tumor xenografts were resected and subjected to molecular assays. The ENTPD1-AS1 stably depleted-tumor xenografts exhibited decreased ENTPD1-AS1 expression (Fig. 7D) and increased miR-432-5p expression (Fig. 7E) compared with the tumor xenografts from the sh-NC group. Additionally, the HDGF mRNA (Fig. 7F) and protein (Fig. 7G) levels were downregulated in the tumor xenografts of sh-ENTPD1-AS1 group. These results suggested that ENTPD1-AS1 silencing impairs CRC growth in vivo.

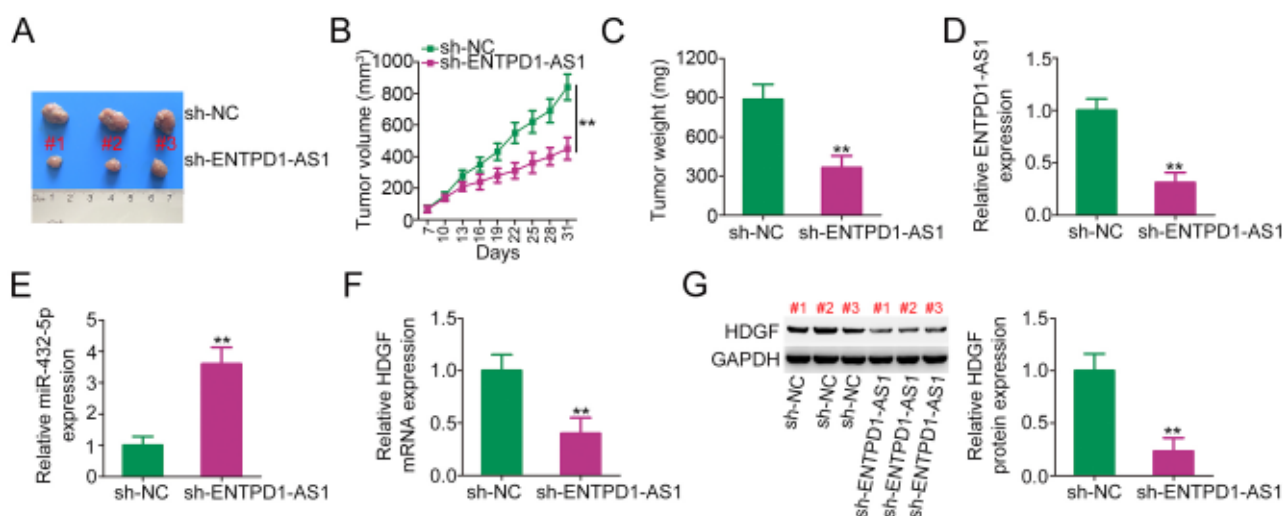


Fig. 7. ENTPD1-AS1 depletion inhibits CRC growth in vivo. (A): Representative photographs of the tumors obtained from the sh-ENTPD1-AS1 and sh-NC groups; (B): The volume of tumors was recorded every 3 days up to 30 days following cell injection; (C): All mice were euthanized at 31 days after cell inoculation, and tumors were excised and weighed; (D, E): Total RNA of tumor xenografts was isolated, and ENTPD1-AS1 and miR-432-5p expression was measured using qRT-PCR; (F, G): The expression levels of HDGF mRNA and protein were measured using western blot analysis in tumor xenografts collected from the sh-ENTPD1-AS1 and sh-NC groups. ** $P < 0.01$.

DISCUSSION

Emerging studies have shown that lncRNAs contribute to carcinogenesis and cancer progression (31-33). LncRNAs are differentially expressed in CRC, and their dysregulation results in the progressive, unlimited growth and metastasis of tumors (13, 34, 35). Consequently, the identification of lncRNAs involved in CRC progression is of utmost importance to find new promising targets for guiding diagnosis, prognosis, and treatment. Despite the recent identification of various lncRNAs, the detailed roles of these molecules in CRC and their associated mechanisms continue to evolve. In the present study, we focused on the expression and detailed functions of ENTPD1-AS1 in CRC. The molecular events underlying the pro-oncogenic role of ENTPD1-AS1 in CRC were revealed in detail.

ENTPD1-AS1 is an independent prognostic marker for glioblastoma multiforme (25). However, to the best of our knowledge, the expression profile and specific role of ENTPD1-AS1 in CRC has not been fully explored. We analyzed the expression of lncRNAs in CRC using TCGA database. ENTPD1-AS1 was found to be one of the most aberrantly expressed lncRNAs in CRC. Consistently, we demonstrated that ENTPD1-AS1 was overexpressed in CRC tissues and cell lines. With respect to its function, the loss of ENTPD1-AS1 inhibited CRC cell proliferation, migration, and invasion *in vitro*. Moreover, ENTPD1-AS1 interference inhibited the tumor growth of CRC cells *in vivo*. These results suggest that ENTPD1-AS1 has an important role during CRC progression.

LncRNAs are believed to function as endogenous molecular sponges for miRNAs, which contain miRNA response elements that competitively target miRNAs (36, 37). By sequestering miRNAs, lncRNAs are implicated in the modulation of gene expression, thereby affecting multiple biological processes (38). The mechanisms underlying the activity of ENTPD1-AS1 to promote the aggressive capability of CRC cells are unclear and warrant further investigation. Herein, ENTPD1-AS1 was predicted as a cytoplasmic lncRNA, which is consistent with the results of subcellular fractionation assays. The results

indicated that this molecule may interact with certain miRNAs in the cytoplasm of CRC cells and regulate gene expression at the post-transcriptional level.

By searching Starbase and LncBase Experimental, miR-432-5p was predicted to be a putative target of ENTPD1-AS1. We hypothesized that the interplay between ENTPD1-AS1 and miR-432-5p can explain the cancer-promoting activity of ENTPD1-AS1 in CRC. Our results confirmed that ENTPD1-AS1 silencing promotes the expression of miR-432-5p in CRC cells. In addition, ENTPD1-AS1 and miR-432-5p exhibited an inverse correlation in CRC tissues. Furthermore, the results of RIP and luciferase reporter assays collectively demonstrated that ENTPD1-AS1 is capable of specifically binding and interacting with miR-432-5p. Therefore, ENTPD1-AS1 acts as a miR-432-5p sponge in CRC.

MiR-432-5p is aberrantly expressed in various human cancer types (39-42). Functionally, miR-432-5p plays an anti-oncogenic role by inhibiting the aggressive characteristics of human cancers (39-42). However, the precise function of miR-432-5p in CRC remains uncertain. In this study, our results indicated that miR-432-5p was weakly expressed in CRC based on both the TCGA database and our sample cohort. Through a series of functional experiments, overexpression of miR-432-5p inhibited the oncogenicity of CRC cells. Our work further revealed that miR-432-5p directly targeted HDGF in CRC cells and its expression was positively regulated by ENTPD1-AS1. To identify an association among ENTPD1-AS1, miR-432-5p, and HDGF, subsequent mechanistic analyses were conducted. These experiments demonstrated a regulatory effect of ENTPD1-AS1 on HDGF expression in CRC cells through sequestration of miR-432-5p and indicated that the 3'UTR of mRNAs plays an important role in the mRNA binding to non-coding RNAs and its effect on gene expression (43). These observations collectively suggest that ENTPD1-AS1 functions predominantly as a ceRNA to decoy competitive binding to miR-432-5p, consequently relieving the inhibitory actions of miR-432-5p on its target, HDGF.

HDGF was first isolated from conditioned culture medium collected from the hepatoma cell line HuH7(44). It is a heparin-binding growth factor that

is upregulated in CRC (45). High HDGF expression exhibited a significant correlation with histological differentiation and lymph node metastasis in CRC (45). Additionally, HDGF was demonstrated to be an independent prognostic indicator for predicting the prognosis of patients with CRC (45). HDGF exerts cancer-promoting effects during colorectal carcinogenesis and progression (45-48). In our study, rescue function assays were performed, which showed that the inhibitory effects of ENTPD1-AS1 silencing on the malignant characteristics of CRC cells were reversed by increasing the output of the miR-432-5p/HDGF axis. Thus, the miR-432-5p/HDGF axis is the downstream mediator of ENTPD1-AS1 in CRC.

In conclusion, this study revealed that ENTPD1-AS1 functions as a ceRNA to sponge miR-432-5p and decreases the suppressive effects of miR-432-5p on HDGF expression, thereby promoting the malignancy of CRC cells. The identification of the ENTPD1-AS1/miR-432-5p/HDGF regulatory pathway offers a theoretical basis to design new treatments for CRC.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

Xiaokai Bi, Qianyu Huang, and Huan Deng designed the current research. Zihan Qi conducted the tumor xenograft experiments. Other experiments were carried out by Huan Deng, Qian Li, Xiaokai Bi, and Qianyu Huang. The manuscript was drafted by Xiaokai Bi and Qianyu Huang.

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of The First People's Hospital of

Chongqing Liang Jiang New Area. Written informed consent was provided by all participants. The animal procedures were approved by the Animal Care and Use Committee of The First People's Hospital of Chongqing Liang Jiang New Area.

Competing interests

No conflict of interest.

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Effects of a training protocol for the prevention of shoulder overuse syndrome in wheelchair rugby athletes: a pilot study

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The growing popularity of paralympic sports and the improvement of infrastructure accessibility has led to an increase of disabled people regularly practicing sports. However, besides the beneficial effects of physical activity, these athletes have a higher risk of upper limb musculoskeletal overuse disorders. Despite this, the literature is still inconclusive about shoulder overuse injury etiology and prevention strategies. Therefore, this study aimed to evaluate the effect of a training protocol to prevent shoulder overuse syndrome in wheelchair rugby (WR) athletes. A total of 12 athletes, affected by tetraplegia (50%), paraplegia (26%), paraparesis due to cerebral palsy (8%), transfemoral amputation (8%), and osteogenesis imperfecta (8%) were evaluated at baseline (T0) and after 4 weeks (T1) of a self-administrated exercise program. In addition, each subject underwent a dynamometric test of the biceps brachii, deltoid and pectoralis muscles. A significant increase of dynamometric values was evident at T1 in all tested muscles.

The growing popularity of paralympic sports and improved social conditions and infrastructure accessibility have increased the number of disabled people regularly taking part in sports practice worldwide. The latest Tokyo 2020 Paralympics games featured over 4400 competing athletes from 161 countries, setting the record for the most athletes ever participating in Paralympic games (1). Wheelchair sports, including wheelchair rugby (WR), are among the most practiced sports by people with physical impairment. Besides the beneficial effects of physical activity, high competition and

intense and frequent training sessions increase the risk of upper limb musculoskeletal overuse disorders in this category of athletes.

Epidemiological studies have found that the shoulder is the most common complaint site among manual wheelchair athletes (2) with an overall incidence ranging between 16% and 76% (3). The most common diseases reported in the literature are bicep brachii tendinitis, humeral acromion impingement, and shoulder instability (2, 3). Despite the lack of clear evidence on the etiology of shoulder disorders in wheelchair players, some

Key words: rehabilitation, wheelchair rugby, shoulder overuse

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risk factors seem to be related to the type of sport activity practiced (e.g., repetitive overhead gestures, intensity of joint load, intensity and frequency of training sessions etc.) (4, 5) and to daily wheelchair use (e.g., self-propulsion, body lifting and transfer, incorrect posture etc.) (6, 7). Shoulder injury-related factors include muscle imbalance, abnormal scapular biomechanics, age, and body weight (3).

Lin et al. analyzed the muscle recruitment of healthy subjects during wheelchair propulsion. They showed that the supraspinatus, infraspinatus, anterior deltoid, pectoralis major and biceps long head are the most involved muscles during the propulsion phase, while the middle and posterior deltoid and supraspinatus muscles are responsible for the recovery phase (8). Compared to healthy subjects, overhead wheelchair athletes tend to have overdeveloped flexors, internal rotators and adductors, but poorly developed external rotators and scapulothoracic muscles (4,5). This muscular imbalance and the repetitive pushing of the wheelchair predispose them to shoulder impingement and poor stability of the humerus (5).

Franchin et al. performed a kinematic analysis of the propulsion technique in spinal cord injured (SCI) wheelchair rugby athletes and found that most players (especially low SCI players) used a semi-circular pattern of push, which is considered the safest technique for upper limb disorders. Conversely, high SCI players tended to use an arcing pattern that promotes a faster speed but with a higher risk of injury (6).

As the participation of disabled people in sport increases, strategies to reduce the rate of injuries and improve performances are required. Regarding shoulder overuse syndrome prevention in wheelchair rugby players, no specific training protocols are present in the literature. This study aims to evaluate the effects of a training protocol for the prevention of shoulder overuse syndrome in athletes practicing wheelchair rugby.

MATERIALS AND METHODS

The study was conducted between October 2-29th, 2019,

Table I. *Demographic and clinical characteristics of the wheelchair athletes*

Rugby athletes (N.12)	
Age (mean and ds)	39.7 ± 5.41
Male/Female (n.)	12/0
Diagnosis (%):	
Tetraparesis	6 (50%)
Paraplegia	3 (26%)
Paraparesis cerebral palsy	1 (8%)
Transfemoral amputation	1 (8%)
Osteogenesis imperfecta	1 (8%)
Occupation (n.):	
Unemployed	6/12
Employed	6/12
Years of sport practice (mean and ds)	7.79 ± 8.60
Years of wheelchair use (mean and ds)	15 ± 5.60

in the Physical Medicine and Rehabilitation ward of the University Hospital “Agostino Gemelli” of Rome. Twelve athletes regularly practicing wheelchair rugby were recruited from local wheelchair rugby clubs.

Inclusion criteria required wheelchair rugby practice on a regular basis (at least 2 times/week) for at least 1 year prior to inclusion in the study, age 18 years and over, and the acceptance of the informed consent. Athletes presenting with shoulder pain exceeding 5/10 on the numeric pain rating scale (NPRS), surgery or luxation in the past three months, spasticity or contractures of the upper extremity were excluded. Demographic data of the subjects are summarized in Table I.

After signing an informed consent, each athlete underwent a physiatry screening followed by an arm dynamometric test using a dynamometer (CITEC HHD CT3002/30) with an arm applicator. Both the physical exam and the dynamometric tests were performed in our ward. The maximal standardized isometric force of the biceps brachii, deltoid, and pectoralis major muscles were bilaterally measured at baseline (T0) and after 4 weeks (T1) of a standardized training protocol for the rebalancing of the shoulder girdle muscles. To set the individualized exercise program, the patient's workload was assessed via the OMNI-resistance exercise scale, which is a validated ten-point scale that allows a qualitative assessment of the maximal fatigue threshold that is subjectively perceived by an individual during physical exercise (9).

Training protocol

The training protocol included a period of 4 weeks of twice a week self-administrated strengthening exercises for the biceps, deltoid and, pectoralis major muscles with one-minute recovery time between the series. Details about the exercise protocol are summarized in Table II. The

training program was first explained by an experienced physiotherapist with the help of some demonstration videos to facilitate comprehension. The program was started only after the candidates demonstrated sufficient proficiency in performing the proposed exercises. In order to verify the patients' adherence to the program, each subject was asked to fill a daily workout diary which was then checked at T1. All procedures were performed in accordance with the Declaration of Helsinki.

RESULTS

A total of 12 male athletes with a mean age of 39.7 ± 5.41 years, accepted to participate in this study. The most prevalent disability type was tetraparesis (50%), followed by paraplegia (26%), paraparesis due to cerebral palsy (8%), transfemoral amputation (8%) and osteogenesis imperfecta (8%). The disability onset was 18.16 ± 12.04 years; range 2-42 years. The characteristics of athletes are described in Table I. The daily workout diary analysis showed a 100% adherence of the participants to the training protocol provided. Table III shows the absolute dynamometric values of tested muscles at baseline and after 4 weeks of training, as well as the percentage of muscle strength gain after the exercises program. All candidates showed an increase in the mean dynamometric values from baseline in all the tested muscles at T1. Specifically, a mean increase of 26.3 N (± 4.2) was observed in the right biceps brachii, 22.3 N (± 0.3) in the left biceps brachii, 8.6 N (± 5.2) in the right deltoid, 6.5 N (± 2.1) in the left deltoid, 25.3 N (± 4.1) in the right pectoralis major and 18,4

Table II. *Exercise program*

Exercise type	Series x Repetition	Resting time	Muscles or joint
Shoulder circling	3x20	20s	Scapulothoracic joint
Handlebars curl	3x20	1min	Biceps brachii
Rubber bands	3x20	1min	Pectoralis major
Handlebars raises	3x20	1min	Deltoid

N (± 0.9) in the left pectoralis major. No adverse events or shoulder trauma were reported during our four weeks exercise protocol.

DISCUSSION

Pain and shoulder discomfort are frequent complaints in people who depend on their upper limbs for wheelchair mobility, transfers, and daily life activities (ADL). In addition to this, wheelchair athletes sustain increased external load and repetitive stress due to sports activity. The amplitude, direction, frequency, and duration of the external load on a joint are important factors that can promote stress and degenerative soft tissue changes (10). Studies based on human biomechanical models have demonstrated that even everyday low-intensity wheelchair use generates large amounts of stress in the scapula-humero-thoracic musculature (11).

It is well-known that sport activity brings physical benefits and improvement of the psychological condition and quality of life in disabled people (5). Despite this, muscle imbalance and abnormal scapular biomechanics may increase the risk of shoulder musculoskeletal disorders in athletic manual wheelchair users (5, 12). Arguably, a study by Fullerton et al. (13) found that, compared to wheelchair athletes, the odds of having shoulder pain were twice as high among nonathletes wheelchair users. Hence, according to the authors, the promotion

of active exercise for wheelchair users should be encouraged to decrease shoulder pain, increase functionality, and live more pain-free years. Overall, prophylactic measures to avoid shoulder injuries in wheelchair athletes are not yet fully explored in the literature. For this reason, education on appropriate prevention exercise and the use of proper equipment that promotes better biomechanics is desirable.

Modern wheelchairs for WR are designed with particular emphasis on players' safety and comfort. However, high-quality equipment-related costs, together with the lack of sufficient financial resources, enforce the use of wheelchairs of poorer quality standards (14). Regarding training modalities to prevent shoulder injury, all evidence available comes from studies conducted on basketball wheelchair athletes. A study by Burnham et al. only briefly mentions shoulder adduction strengthening exercises as a potential modality to prevent shoulder rotator cuff impingement syndrome (12). Other studies only give indistinct recommendations on muscle stretching, general flexibility and strength training to prevent muscular imbalance and impingement (10, 15).

Our pilot study shows that the application of a simple self-administrated exercise protocol was effective at improving the recruitment of the shoulder muscles most responsible for wheelchair mobility. In particular, an increase in overall muscle strength was observed in the pectoralis major and biceps brachii

Table III. Mean dynamometric values (Newton) at T0 and T1 and percentage of muscle strength gain

Evaluated muscle	T0 dynamometry (Mean and SD)	T1 dynamometry (Mean and SD)	% of muscle strength increase
Right Biceps Brachii	163.5 $\pm$ 7.6	189.8 $\pm$ 4.2	16.1%
Left Brachialis Biceps	176.1 $\pm$ 2.1	198.4 $\pm$ 0.3	12.7%
Right Deltoid	86.5 $\pm$ 6.8	95.1 $\pm$ 5.2	9.9%
Left Deltoid	87.1 $\pm$ 1.7	93.6 $\pm$ 2.1	7.5%
Right Pectoralis Major	100.2 $\pm$ 6.9	125.5 $\pm$ 4.1	25.2%
Left Pectoralis Major	90.8 $\pm$ 3.1	109.2 $\pm$ 0.9	20.3%

SD = standard deviation

muscles, which are some of the most involved muscles during the propulsion phase (8). The heterogeneity and the small size of our sample do not allow us to make any generalization of the results. However, based on these preliminary observations, we believe that the proposed training protocol based on self-administered exercises could help rebalance the shoulder girdle and prevent shoulder overuse pathologies in wheelchair rugby players. Further prospective studies conducted on a larger sample of patients are needed to verify this hypothesis.

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Alternative splicing of immune-related genes predicts the outcome in lung adenocarcinoma

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Lung adenocarcinoma (LUAD) is one of the most fatal cancers, and more studies are needed for a better understanding of this disease. Studies have reported that alternative splicing (AS) in cancers has important prognostic value. Therefore, this study aimed to determine the correlation between the AS events of immune-related genes and LUAD prognosis. The gene expression matrix and clinical information of patients with LUAD were downloaded from The Cancer Genome Atlas database. Univariate and multivariate Cox regression analyses were used to screen the differentially expressed AS (DEAS) events of immune-related genes related to prognosis. A protein-protein interaction network was constructed using the abovementioned genes, and enrichment analysis was performed. Finally, we analyzed the regulatory relationship between DEAS events and AS factors and analyzed the prognostic subtypes via cluster analysis. A total of 188 out of 2,227 immune-related AS events (111 of 373 immune-related genes) were significantly ($p < 0.05$) associated with the overall survival (OS) of patients with LUAD. Three AS types, namely alternate promoter, retained intron, and exon skip, significantly distinguished high-risk patients from low-risk patients. Further analysis revealed that the alternatively spliced immune genes were involved in the activation of the immune response as well as in cytokine production and signaling and that the AS events were highly correlated with splicing factors. Finally, the immune-related genes were subgrouped into four clusters, with each cluster differently predicting the OS of patients with LUAD. In summary, AS events in immune genes are novel prognostic factors for LUAD and imply dysregulated AS in the immune system and abnormal antitumoral immune responses.

Lung adenocarcinoma (LUAD) accounts for approximately 40% of all lung cancer cases and is the leading cause of cancer-related mortality (1, 2). The overall five-year survival rate of LUAD is nearly 15%, primarily due to late-diagnosed LUAD in the

advanced stages (3). Identification of prognostic factors for cancers improves the understanding of tumor development and may help improve clinical practices. Several studies have identified different prognostic factors for LUAD (4-6), but further

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research is needed to improve our understanding of the pathogenesis and outcome of LUAD.

In recent years, immune system-based cancer therapies have shown great promise in LUAD (7). The immune system effectively eliminates most pathological microorganisms and toxic substances for the host while retaining healthy cells and maintaining self-tolerance (8). Programmed cell death protein 1 (PD-1) plays an indispensable role in fine-tuning T cell function and maintaining immune homeostasis (9). Studies have reported that cancer patients with high PD-L1 expression often show better results in receiving anti-PD-1 antibody treatment (10). However, no correlation between PD-L1 expression and overall survival (OS) was found in other studies (11). Therefore, the correlation between immune-related genes and the prognosis of LUAD needs further investigation. Alternative splicing (AS) produces multiple mRNA variants from a single transcript and is a tightly regulated process and variable in order to fulfill the different demands of cells (12). Dysregulated AS is associated with multiple diseases, including myotonic dystrophy, Alzheimer's disease, and spinal muscular atrophy (13, 14). Abnormal AS also occurs in cancers, such as leukemias, sarcomas, hepatocellular adenocarcinoma, breast cancer, and LUAD (14-16). A lack of the RAS-binding domain of BRAF V600E resulting from AS in melanoma leads to resistance to RAF inhibitors (17). AS is emerging as a new hallmark of cancers (18, 19) and contributes to tumor development, drug resistance, immune evasion, and metastasis (20, 21). AS is critically involved in forming the tumor immune microenvironment, which includes tumor cells, tumor-associated fibroblasts, immune cells, stromal tissue, and other components (22). Low levels of SOD2-78301-AT in patients with LUAD have been reported to always be accompanied by better OS. In addition, there is a significant correlation between the expression of SOD2 in LUAD and the presence of immune infiltration (23).

AS also has prognostic value in many types of cancers (24-27). Several studies have revealed its prognostic function in LUAD. Cai et al. showed that AS can evaluate lymph node metastasis status and

predict the OS probability of patients with LUAD (28). Abnormal AS of *BCL2L1*, *MDM2*, *MDM4*, *MET*, and *NUMB* was reported in lung cancer (29). Another study also demonstrated the association of different AS types with the OS of patients with LUAD (30). It was also found that genes that exhibit different AS patterns during T cell activation are usually closely related to cell cycle regulation (31). However, these studies focused on the global AS in LUAD but did not examine AS events in genes with specific functions and prognostic roles.

This study tried to determine the AS events of immune-related genes and explored their association with LUAD prognosis. The global differential AS events were analyzed by comparing LUAD and healthy samples, followed by overlapping with a list of immune-related genes. The OS-associated immune AS events were identified via univariate Cox regression, and the association of AS types with OS was further analyzed via multivariate Cox regression. The OS-related immune genes were examined by protein-protein interaction (PPI) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and clustered into different subgroups. This study provides novel insights into the AS of immune genes and reveals its prognostic value and dysregulated antitumor responses in LUAD.

MATERIALS AND METHODS

Data collection and processing

The RNA sequencing (RNA-seq) data and corresponding clinical information of 585 patients with LUAD (526 tumor and 59 paracancerous samples) were downloaded from The Cancer Genome Atlas (TCGA) University of California Santa Cruz Xena database (32). All transcriptomic data were standardized by log2 (count + 1), and the Ensembl_ID was converted to Symbol_ID in Python based on gene annotation information from GENCODE (33). The mean value was designated to the corresponding transcript in Python for transcripts with multiple values. Genes with an expression value of 0 in more than 50% of the samples were excluded from the study. A total of 58,388 genes passed the criteria and were normalized using the "normalize between arrays"

function in the 'limma' R package (version 3.9.19) (34).

AS was recognized by SpliceSeq (35), and the percent spliced in (PSI) value was calculated to quantify AS events. A total of 493 tumors and 59 paracancerous samples were included in this study, and the PSI matrix contained 42,655 AS events (percentage of samples with PSI value ≥ 75 , an average of PSI value ≈ 0.05). The distribution and frequency of AS in LUAD were analyzed, and the corresponding gene number was recorded. The clinical parameters [(age, gender, tumor stage, primary tumor (T), regional lymph nodes (N), and distant metastasis (M)] were included in this study after excluding those containing missing values in more than 50% of samples (36). Details of the study design are illustrated in Fig. 1 as a flowchart.

Analysis of differentially expressed AS (DEAS)

To explore the DEAS in the samples, a *t*-test using the 'limma' package (version 3.9.19) was applied to examine the statistical significance of the PSI value of each AS event between tumor and paracancerous samples

(34). The adjusted $p < 0.05$ represents upregulation or downregulation of corresponding AS events.

Analysis of immune genes

The list of 1,697 immune-related genes was obtained from the Immunology Database and Analysis Portal (ImmPort) in InnateDB (37). This list was generated using automatic searches of Entrez Gene and Gene Ontology records using immunology-related keywords and was then manually curated by immunology experts examining various literature sources. Combined with AS analysis in LUAD, the differential immune genes were selected with a cutoff of $p < 0.05$. Venn analysis was used to demonstrate the intersection set of immune-related genes and DEASs.

Univariate and multivariate Cox survival analysis of AS events

To screen for prognosis-related immune-related DEASs, univariate Cox regression analysis was used to analyze the correlation between survival and immune-related DEASs in 493 patients using the 'survival' package in R (38). Statistical significance was set at $P < 0.05$.

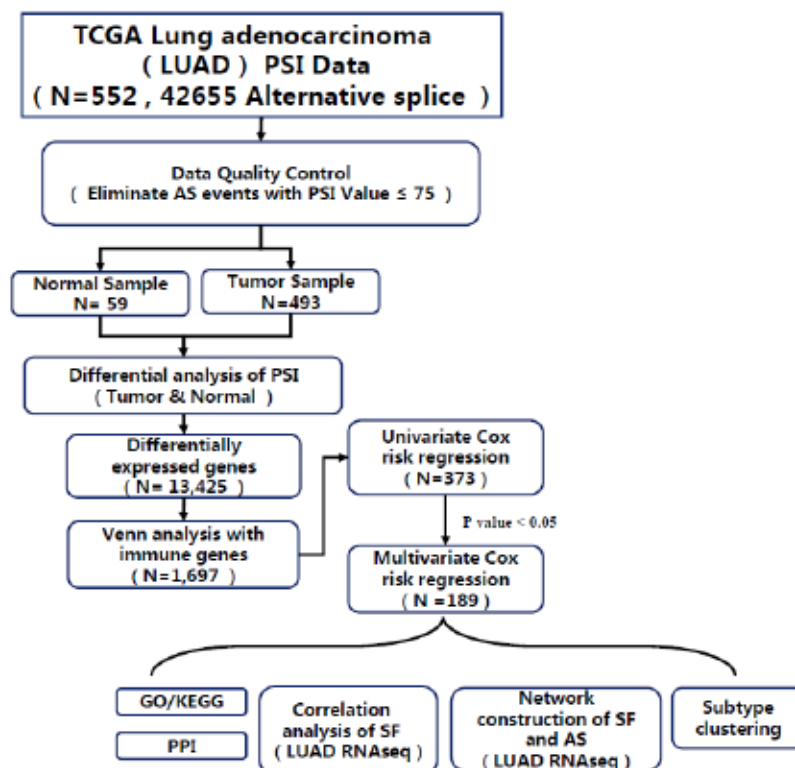


Fig. 1. Workflow for the selection and analysis of the overall survival-associated alternative splicing events in immune genes.

R software was used to perform univariate Cox regression followed by multivariate Cox regression analyses based on OS to determine independent prognostic factors (38). The seven AS subtypes were exon skip (ES), retained intron (RI), alternate donor site (AD), alternate acceptor site (AA), alternate promoter (AP), alternate terminator (AT), and mutually exclusive exons (ME), and the total PSI value was calculated for each subtype. The significant AS events ($p < 0.05$) were selected for multivariate Cox analysis, and the patients with LUAD were divided into high- and low-risk groups according to the median value of PSI. Kaplan-Meier analysis was used to test the model's ability to distinguish patient survival. The OS of patients in each group was compared with the log-rank test. Statistical significance was set at $P < 0.05$.

Clustering of prognosis-related molecular subtypes of LUAD

We performed unsupervised grouping of survival-related immune DEASs in LUAD followed by k-means clustering using 'NbClust' in R (39) to explore the different AS patterns. Then, the difference in survival between groups was analyzed. Finally, principal component analysis (PCA) and linkage clustering were performed using the 'prcomp' and 'pheatmap' packages in R, respectively (40, 41).

Enrichment analysis of DEASs related to prognosis

Gene ID was converted based on the *Homo sapiens* Entrez gene IDs using the online tool Metascape (42). Enrichment analysis for gene ontology, biological process (BP), KEGG, Reactome gene sets, and Wiki pathways were performed separately with cutoffs of similarity score > 0.3 , $p < 0.01$, and enrichment factor > 1.5 . The similarity of genes in each term was further clustered based on the κ coefficient (κ score > 0.3). The term with the smallest p-value represented each cluster.

Construction of the PPI network

The PPI network of the immune-related DEASs were constructed using the 'STRINGdb' package in R (score threshold = 400) (43) and was visualized using Cytoscape software (version 3.6.1; <https://cytoscape.org/>).

DEAS- and splicing factor (SF)-based construction of the regulatory network

SpliceAid-F is an exhaustive repository of SFs,

including information on their expression, functional domains, protein and chemical interactors, and RNA-binding and nonbinding sites (44). Through extensive database and literature screening, information on 71 SFs were obtained, and all of these spliced proteins were investigated. The expression values of the 71 SFs in LUAD were extracted from RNA-seq data after quality control, and 52 SF expression values were included in the follow-up study. Spearman's rank correlation coefficient evaluated the correlation of survival-related immune DEASs and SFs. A co-expression network of the correlated genes (cutoff $p < 0.01$) was constructed using Cytoscape_v3.8.2 (45).

RESULTS

Characterizing AS in immune-related genes in tumor and paracancerous samples

A total of 42,655 AS events were identified from the RNA-seq data of 493 LUAD tumor samples and were categorized into seven subtypes (ES, RI, APAT, AD, AA, and ME) (Fig. 2A). The frequency of the AS and gene counts in each AS type were different, and the gene count was correlated with the AS count. The number of ES events was significantly higher than that of all other AS types, whereas ME occurred least frequently in LUAD (Fig. 2B).

We compared the differences in AS events in tumor and paracancerous samples and found that 13,425 DEASs out of 42,655 AS events in 6,419 genes were significantly different between paracancerous and tumor samples ($p < 0.05$) (Fig. 2C). Of the 13,425 DEASs, 6,336 were upregulated (3,978 corresponding genes), and 7,089 were downregulated (4,830 corresponding genes). PCA revealed apparent clustering corresponding to sample types (tumor or paracancerous) and remarkable separation of tumor samples from paracancerous samples (Fig. 2D). These results suggest the existence of different splicing events between paracancerous and tumor tissues.

Venn analysis was performed between 1,697 immune-related genes and 6,419 DEASs. Among them, 373 immune-related genes and 2,227 corresponding AS events were identified (Supplementary Table I). Our results demonstrate

that AS occurs in the immune system and may be relevant to immune responses in tumors.

Univariate Cox survival analysis of AS events of immune-related genes

The association of the 373 immune genes (2,227 corresponding AS events) with the survival of patients with LUAD was analyzed using univariate Cox regression. A total of 188 AS events in 111 genes were significantly associated with OS, and there were 20, 13, 48, 12, 80, and 15 AS events in AA, AD, AP, AT, ES, and RI types, respectively. Of note, AS events from six out of seven AS types were associated with OS, except ME, which occurred with the lowest frequency in LUAD.

Multivariate Cox analysis of the prognostic power of immune-related AS events

Multivariate Cox regression was performed to analyze the independent prognostic factors from the six AS subtypes (111 immune genes and 188 corresponding AS events) that are associated with the OS of patients with LUAD with a cutoff of $p < 0.05$. Three AS types, namely AP, RI, and ES, were significantly distinguished as the high-risk group from the low-risk group (Fig. 3A-D). Patients in the high AP, ES, or RI group had a worse prognosis than those in the low AP, ES, or RI group. AP showed the highest area under the curve (AUC) value of 0.684 (Fig. 3E), while the AUC values of AA, ES, and RI were 0.532, 0.635, and 0.583, respectively.

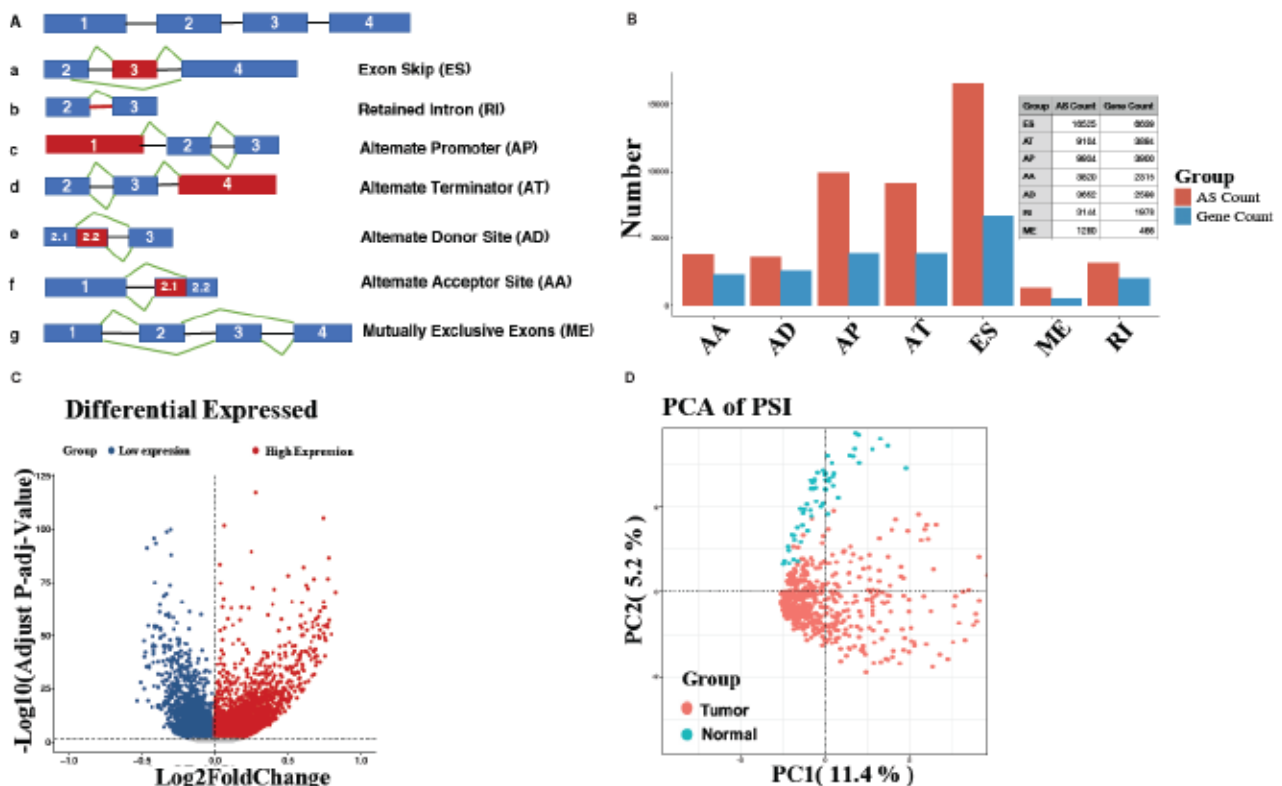


Fig. 2. Characterization of alternative splicing (AS) in immune-related genes in tumor and paracancerous samples. **A)** Schematic diagram of different AS types identified in lung adenocarcinoma (LUAD). **B)** Distribution of AS types in LUAD. **C)** Fold changes of AS in LUAD samples compared with normal samples. The red spot represents upregulation, the blue spot represents downregulation, and grey represents no significant difference ($p \geq 0.05$). **D)** Principal component analysis of AS events in LUAD and normal samples.

These results demonstrate that immune-related AS events (AP, RI, and ES) can predict the prognosis of patients with LUAD.

Clustering of prognosis-related molecular subtypes of LUAD

Unsupervised clustering was performed to examine the internal association of the 188 AS events with the 111 survival-related immune genes. Grouping into four clusters was optimal for these AS events (Fig. 4A). PCA revealed good separation between different clusters (Fig. 4B), and the similarity matrix also showed high independence between different clusters and associations within clusters (Fig. 4C). Finally, we tried to determine the associations of these clusters with patient survival and found that each cluster showed significantly variable associations with OS ($p = 0.035$), implying that they may independently

contribute to the OS of patients with LUAD through different mechanisms (Fig. 4D).

Enrichment analysis of DEASs

To investigate potential BPs that are associated with immune-related AS events, the 111 genes were clustered, and KEGG enrichment analysis was performed. As a result, cytokine production and signaling, immune response activation, and Toll-like receptor cascades were significantly enriched (Fig. 5A and B), which suggests that the important components of the immune system are reshaped by AS in LUAD and may lead to the global change of antitumoral immune responses.

Construction of the PPI network

In total, 111 prognosis-related immune genes

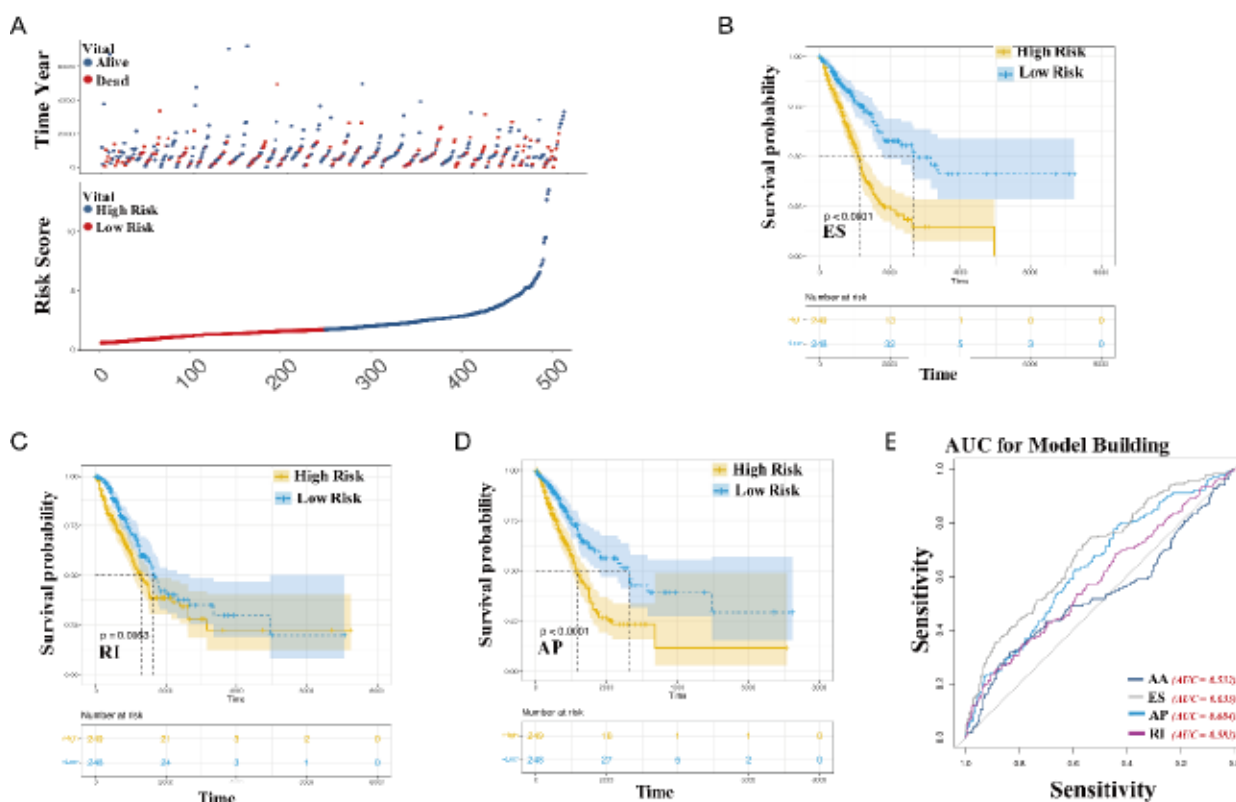


Fig. 3. Immune-related AS types predict the overall survival of LUAD. **A)** Risk scores and living status based on immune-related AS types. **B-D)** Kaplan-Meier survival curve of AS types: alternate acceptor site (AA) (**B**), exon skip (ES) (**C**), retained intron (RI) (**D**), alternate promoter (AP) (**E**). Area under the curve of AA, AP, ES, and RI.

were used to construct the PPI network, which included 100 genes and 379 edges (Fig. 6). The genes with the top 10 degrees were *VEGFA*, *CASP8*, *IRF3*, *IRF7*, *CTNNB1*, *CASP1*, *IKBKG*, *MAP2K1*, *ITGB1*, and *SMAD3*.

Regulatory network of DEASs and SFs

SFs are major contributors of AS and play important roles in oncogenesis (46-48). We found 64 negative and 58 positive correlations of 11 SFs with 111 DEASs ($p < 0.01$; (Fig. 7A and B). We further examined the correlation of representative SFs and immune genes and found that *CASP8* was positively correlated with *TIA.1* and *nPTB*, whereas *ATG9A* and *IKBKG* were negatively correlated with *hnRNPD0* and *hnRNPK*, respectively (Fig. 7C and D).

DISCUSSION

LUAD is a common cancer that causes high mortality annually (49). Evaluation and prediction of the clinical outcome of LUAD are critical for patient care and disease management. Our current study revealed that the AS events in immune-related genes were good prognostic biomarkers for the evaluation of the OS of patients with LUAD and may be associated with altered immune responses and compromised antitumoral immunity in LUAD. Further study is required to clarify these findings and expand our understanding of LUAD and AS in the immune system and their roles in regulating immune responses.

AS is a conservative and tightly controlled process to ensure flexible adaptation to different demands

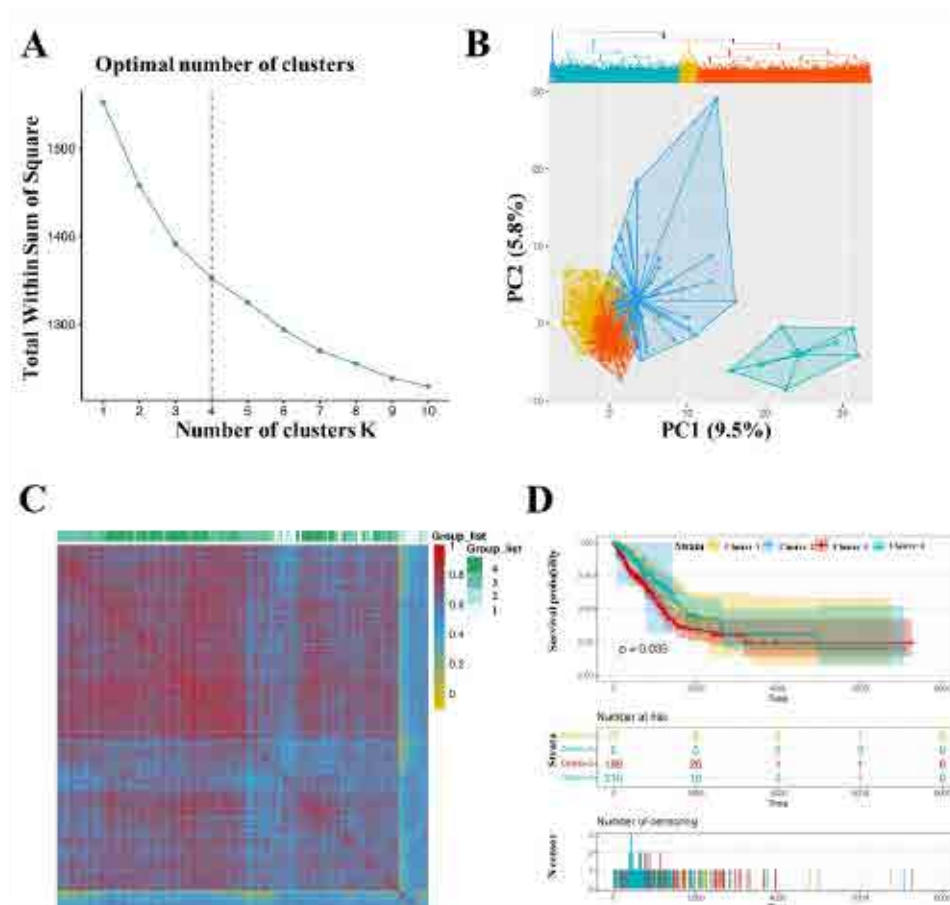


Fig. 4. Clustering of prognosis-related molecular subtypes of LUAD. **A)** Optimized clustering parameters based on the NbClust algorithm. **B)** Principal component analysis of four optimized clusters. **C)** Similarity matrix between four gene clusters. **D)** Kaplan-Meier survival curve of each cluster.

of cells in response to variable environments by multiplying the diversity of gene products without substantially increasing the genome. AS, as part of evolution, is variable among individuals and participates in the pathogenesis of many diseases (50). AS alteration is common in cancers and is emerging as a novel hallmark of cancers (14-16, 18, 19). Previous studies have demonstrated the prognostic power of AS events in multiple cancers (24-27), but these studies in the context of LUAD are limited (28, 30); moreover, most studies have focused on global AS events in tumors rather than on a set of genes with a specific function. This study established a prognostic model for LUAD based

on RNA-seq data from the TCGA database. This model mainly focused on immune-related genes, which are critical for keeping tumors in check but may also promote their progression when hijacked by the tumor. We identified 111 immune genes and 188 corresponding AS events associated with the OS of LUAD. These AS events belonged to seven AS types, six of which (ES, RI, AP, AT, AD, and AA) were significantly associated with OS. Of note, three AS types, namely AP, RI, and ES, were able to stratify the high-risk group from the low-risk group with an AUC value of 0.58 to 0.69. These results demonstrate that AS in immune-related genes is a prognostic factor of LUAD.

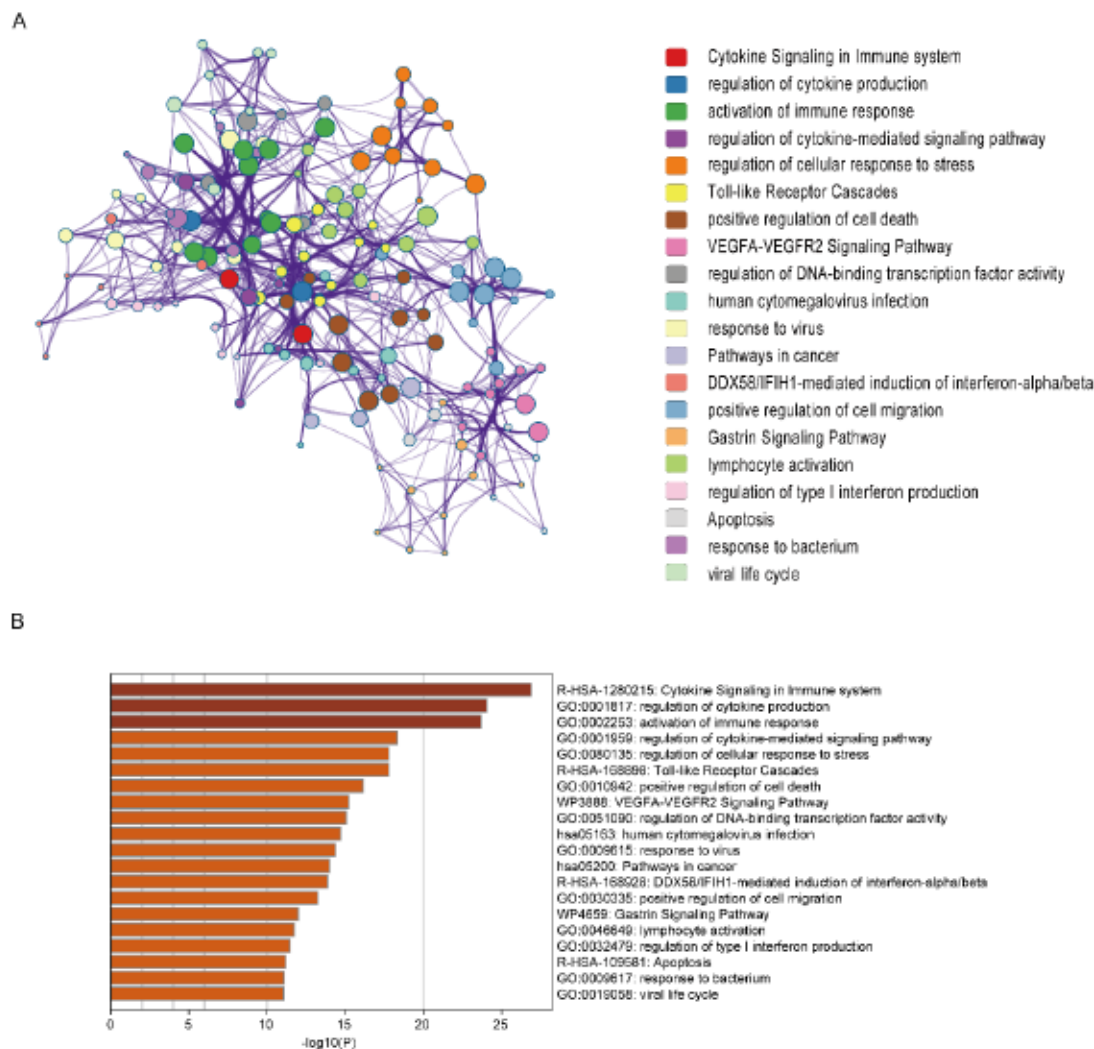


Fig. 5. Pathway enrichment analysis of overall survival-related immune genes. **A)** κ coefficient-based gene cluster. **B)** Pathway enrichment analysis.

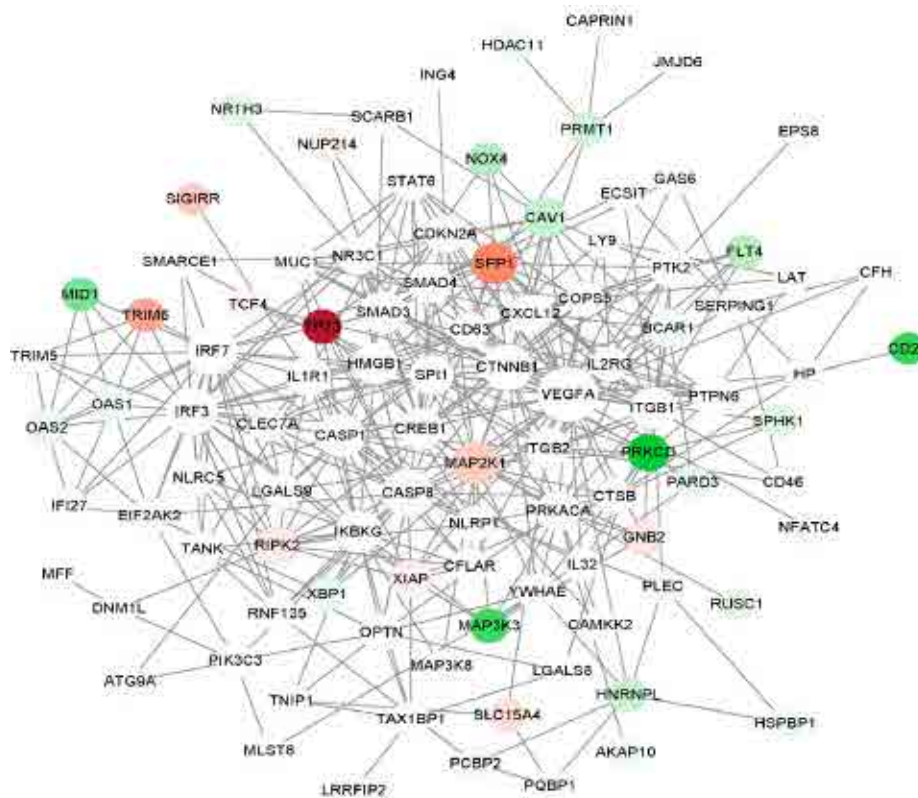


Fig. 6. Protein-protein interaction network of overall survival-associated immune genes. Red represents upregulation, while green represents downregulation. A darker color represents a higher $|\log_2FC|$ value.

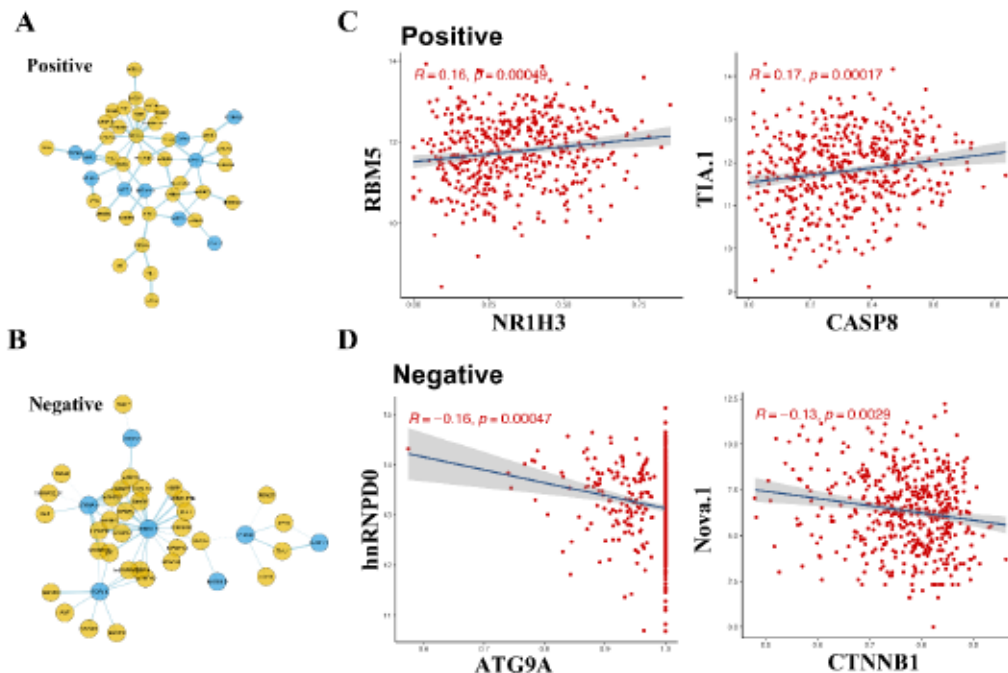


Fig. 7. Regulatory network of AS and splicing factors (SFs). **A-B**) Network of positively (**A**) and negatively associated (**B**) AS with SFs. The yellow circle represents the immune gene, and the blue circle represents the SF. **C-D**) Pearson correlation of representative SFs with immune genes. (**C**) Positive correlation of CAP8 with TIA.1 and nPTB. (**D**) Negative correlation of ATG9A and IKGKG with hnRNPD0 and hnRNPK, respectively.

Due to the biological heterogeneity of LUAD, the use of anatomic sites or TNM stage to stratify high-risk patients has limited clinical utility. Cluster analysis can facilitate the categorization of heterogeneous disorders into disease subtypes (51). Several studies have found that LUAD can be classified into subgroups based on gene expression patterns, which provides powerful conditions for future targeted therapy and immunotherapy (52). In this study, Kaplan-Meier analysis of the association of clusters with prognosis revealed distinct patterns of survival. This result indicates that the four clusters may independently contribute to the OS of patients with LUAD through different mechanisms and may also serve as an effective biomarker for prognosis prediction. With the critical functions of the immune system in tumors, functional enrichment analysis was performed to evaluate the pathways that were affected by AS. The top three enriched pathways were cytokine signaling in the immune response, regulation of cytokine production, and activation of the immune response. Cytokines play vital roles in regulating the immune response, including activation or suppression of immune responses, transduction of survival or death signaling, and maintenance of microenvironment homeostasis (53). For example, as a novel member of the IL-1 cytokine family, IL-38 has been reported to act as an antagonist and has been further reported to affect host immunity or the tumor microenvironment and contribute to the progression of lung adenocarcinoma (54).

Immunotherapy has emerged as a promising approach to treat cancer, and one of the most favorable strategies to activate the immune response is immune checkpoint blocking therapy (55). Anti-CTLA4 and anti-PD-1 were used to block inhibitory molecules on immune cells to initiate antitumor immunity (56). Our results imply that the immune responses may be altered during LUAD progression by modifying the AS of immune genes. The interactions of these immune genes centralized on some important immune regulators, including IRF3, IRF7, SMAD3, SMAD4, and STAT6. IRF3, and IRF7, that regulate the production of type I interferons (IFN- α and IFN- β) (53), which play

complex pro- and antitumoral roles (57, 58). In addition, IRF3 serves as a proapoptotic factor in the late phase of viral infection that triggers the RLR-induced IRF3-mediated pathway of apoptosis and eliminates the infected cells and invading microbes (59). SMADs are regulated by TGF- β 1, which are involved in fibrosis (60). A previous study has reported that inhibiting the TGF- β /Smad signaling pathway could inhibit liver fibrosis progression (61). Interleukin-9 induction requires cooperation between the Notch and TGF- β -SMAD3 signaling pathways (62). STAT6 mediates the activation of T helper type 2 (Th2) immune responses (63), but the effect of the Th2 response in tumors is also complex and context-dependent.

Whether these changes may promote or suppress antitumoral immune responses remains unknown, and further study is needed to elucidate the underlying mechanism, which may be helpful for the manipulation of antitumor immune responses.

AS events are mainly regulated by splicing factors (64). This study assessed the correlation between SFs and DEASs and revealed 64 significantly negative and 58 significantly positive correlations of 11 SFs with 111 DEASs. For example, CASP8 was positively correlated with the RNA-binding protein TIA.1, which functions as a posttranscriptional regulator of gene expression and aggregates to form stress granules following cellular damage. TIA.1 has been reported to participate in the regulation of alternative pre-mRNA splicing of bound mRNAs (65). A previous study has confirmed that when TIA.1 has interfered, the RT-PCR amplification of the target transcript encoding CASP8 is significantly reduced (66). Indeed, the correlation between the various DEASs and SFs identified in this study needs to be further experimentally verified in the future. The present study established a novel AS-based prognostic model for LUAD and identified the OS-associated AS in immune genes. These results provide a better understanding of LUAD and indicate the importance of immune responses in the disease. Further study is required to elucidate the role of AS of immune gene and explore novel antitumor therapies by manipulating immune responses in LUAD.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

Conception and design of the research: LJ; acquisition of data: LJ, YZ, HL; analysis and interpretation of data: DZ, JS-W; statistical analysis: JW, YT; drafting the manuscript: LJ, GY; revision of manuscript for important intellectual content: GY, DZ. All authors read and approved the final manuscript.

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The impact of trastuzumab therapy and cardiovascular risk factors on cardiac function in HER2- positive breast cancer patients

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Background: Aside from the clinical benefits of trastuzumab in treating HER2- positive breast cancer, cardiotoxicity is identified as a major side effect. Cardiac function should be closely monitored before, during, and after trastuzumab therapy.

Aim: To assess cardiac function during trastuzumab therapy and the effect of risk factors (RF) for cardiotoxicity and cardiovascular (CV) diseases in HER2-positive breast cancer patients.

Materials and Methods: Ninety-six HER2-positive female breast cancer patients (mean age, 59.57±9.6 years) were enrolled in the study. At the time, they were on sequential therapy with anthracyclines (IV to VI cycles) within the FAC regimen (fluorouracil 500 mg/m², doxorubicin 50 mg/m², cyclophosphamide 500 mg/m²) applied per 21 days, and after that, trastuzumab therapy (6 mg/kg of body weight, per 21 days for one year). In all patients, blood pressure (BP), heart rate (HR), electrocardiographic and echocardiographic parameters (left ventricular ejection fraction – LVEF (%), fractional shortening – FS (%), end-diastolic diameter – EDD (mm), and left ventricular mass – LVM (g)) were assessed at the beginning and after the therapy with trastuzumab.

Results: There were no significant changes in arterial BP before and after trastuzumab in the examined patients. Ectopic beats (EB) were registered during the therapy in 11 patients, supraventricular in 7, and ventricular extrasystoles in 4 patients on the electrocardiogram (ECG). In most of the patients with EB in the middle of the treatment period with trastuzumab, sinus tachycardia was also recorded. At the end of trastuzumab therapy in the examined group, LVEF decreased by 1.73 %, FS by 0.86 %, EDD increased by 1.26 mm, and LVM increased by 9.6 g. Cardiotoxicity was registered in 4 (4.17%) patients. Greater left ventricular dysfunction was observed in older patients, patients with prior radiation therapy, and those with three or more cardiovascular risk factors (CVRF).

Conclusion: This study shows that the treatment with trastuzumab does not significantly affect BP and ECG changes. The rate of cardiotoxicity after trastuzumab therapy was low and was expressed through reduction of LVEF, an increase in EDD, and LVM. In patients with three or more CVRFs, there was a higher decrease of LVEF than in patients without any cardiovascular risk factors.

Trastuzumab (Herceptin®, Roche) is the first humanized monoclonal antibody directed against the human epidermal growth factor receptors 2 (HER2) and represents the milestone in the treatment of HER2-

positive breast cancer (1). In addition, the use of trastuzumab in adjuvant, neoadjuvant, and metastatic treatment settings reduces the risk of relapse and fatal outcomes and increases the survival of these patients (2).

Key words: cardiotoxicity, echocardiographic parameters, risk factors, trastuzumab

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Around 20-30% of breast cancers have overexpression of HER2 receptors, and these are considered to be high-grade tumors with worse prognoses (3). HER2 transmembrane tyrosine kinase receptor is a member of the epidermal growth factor receptor (EGFR) family. It is involved in regulating many important cell-type-specific functions, especially in cell growth, proliferation, and survival. HER2 receptors are found in most cell types to a greater or lesser extent, including cardiomyocytes. By binding to the extracellular domain of HER2 receptors, trastuzumab suppresses the growth, proliferation, and survival of cancer cells (4).

Aside from the clinical benefits of trastuzumab in treating HER2-positive breast cancer, cardiotoxicity is identified as the major side effect, occurring as both symptomatic and asymptomatic cardiac dysfunction (5). Additionally, trastuzumab has the potential for causing cardiotoxicity in patients receiving anthracyclines, as either concurrent or prior therapy (6). In patients on trastuzumab therapy, the incidence of heart failure has been 1-4%; 10% have asymptomatic left ventricular (LV) dysfunction, while as high as 34% experience trastuzumab cardiotoxicity when combined with anthracyclines (7). Trastuzumab does not cause any ultrastructural change of myocardiocytes as anthracyclines do, but myocardial dysfunction under cardiac stress is nevertheless present. Cardiotoxicity induced by trastuzumab is reversible and is not dose-dependent. It increases when trastuzumab is combined with anthracyclines. After adequate management of heart failure, normal cardiac function can be recovered (8). Clinical evaluation and assessment of cardiovascular risk factors (CVRFs) and comorbidities should be performed in all patients before the initiation of trastuzumab therapy. In addition, LV function must be assessed before treatment and at 3-month intervals during the administration of trastuzumab. The gold standard for detecting cardiac dysfunction before, during, and after trastuzumab therapy is echocardiography, and the most commonly used indicator of systolic function is ejection fraction. Therefore, this study aimed to assess cardiac function during trastuzumab therapy and the effect of risk factors (RF) for cardiotoxicity and

cardiovascular (CV) diseases in HER2-positive breast cancer patients. Early identification of patients at risk for cardiotoxicity is a major challenge for both cardiologists and oncologists to define more individually tailored antineoplastic therapeutic interventions (9).

MATERIALS AND METHODS

Ninety-six HER2-positive female breast cancer patients (mean age, 59.57 ± 9.6 years) were examined, and 59 patients were on adjuvant, while 37 patients were on neoadjuvant trastuzumab therapy. Patients who underwent adjuvant trastuzumab therapy after surgery received anthracycline therapy (IV to VI cycles) within the FAC regimen (fluorouracil 500 mg/m², doxorubicin 50 mg/m², cyclophosphamide 500 mg/m²) every 21 days, followed by trastuzumab therapy (6 mg/kg of body weight every 21 days) for one year.

In all patients, blood pressure (BP), heart rate (HR), electrocardiographic, and echocardiographic parameters (left ventricular ejection fraction – LVEF (%), fractional shortening – FS (%), end-diastolic diameter – EDD (mm), and left ventricular mass – LVM (g) were assessed at the beginning and after the therapy with trastuzumab.

The impact of risk factors for cardiotoxicity in all 96 patients was examined, such as age (older than 50 years), radiation therapy (50 patients with prior radiation therapy had 25 daily sessions of 2 Gy dose each, up to the total dose of 50 Gy), and prior anthracycline therapy as well as the presence of risk factors for cardiovascular diseases.

All subjects gave informed, signed consent to participate in the study, and both institutional ethics committees approved the study: The Faculty of Medicine, Niš, University of Niš, number 12-1250/11 and the Institute for Treatment and Rehabilitation “Niška Banja”, number 03-14190/1.

Statistical analyses

Statistical significance of the differences between mean values of the monitored parameters at the beginning and the end was tested using the paired samples t-test. In addition, Wilcoxon rank test was used in cases where the hypothesis of normality distribution was not fulfilled. The significance level in all tests was set at $p < 0.05$. Data analysis was performed using the Statistical Package Social Sciences Version 20.0 (SPSS).

RESULTS

There were no significant changes in arterial BP before and after trastuzumab in the examined patients (systolic BP 119.90 ± 16.04 mmHg vs 121.30 ± 13.06 mmHg; diastolic BP 75.89 ± 7.25 mmHg vs 76.30 ± 7.65 mmHg) and HR (68.78 ± 7.69 /min vs 67.92 ± 7.90 /min). At the beginning and after the last therapy with trastuzumab, all patients were in sinus rhythm on electrocardiography (ECG), one patient had left bundle branch block, and one had right bundle branch block. During therapy, ectopic beats were registered in 11 (11.45%), SVES in 7 (7.29%), and VES in 4 (4.16%) patients. In most of these patients with ectopic beats in the middle of the treatment period with trastuzumab, sinus tachycardia was also recorded. Cardiotoxicity was registered in 4 (4.17%) patients, according to the criteria of the ASE (10).

At the end of trastuzumab therapy in the examined group ($n=96$), LVEF decreased by 1.73% (from $65.49 \pm 5.82\%$ to $63.76 \pm 6.43\%$; $p=0.007$), FS by 0.86% (from $36.18 \pm 5.10\%$ to $35.32 \pm 4.91\%$; $p=0.123$), EDD increased by 1.26 mm (from 47.53 ± 4.48 mm to 48.80 ± 5.19 mm; $p=0.005$), and LVM increased by 9.60 g (from 161.55 ± 43.76 g to 171.15 ± 47.88 g; $p=0.031$), as shown in Table I.

The group of patients older than 65 years (33 pts) showed more conspicuous changes in the values of echocardiographic parameters (LVEF

reduced by 4.56% ($p=0.002$); EDD increased by 1.70 mm ($p=0.037$), and LVM increased by 13.15 g ($p=0.104$), compared to those younger than 65 (EF reduced by 0.25% ($p=0.650$); FS was reduced by 1% ($p=0.157$), EDD increased by 1.04 mm ($p=0.057$), and LVM increased by 7.73 g ($p=0.148$) at the end of trastuzumab therapy (Table II).

Of 96 examined patients, 50 patients (52.1%) received IV, and 46 patients (47.9%) received VI cycles of anthracyclines. LVEF reduction (1.77%; from 65.58 ± 5.20 to 63.81 ± 5.59 ; $p=0.048$) was greater in patients with VI cycles of anthracyclines, while patients with IV cycles showed greater increases in EDD by 1.68 mm (from 47.19 ± 4.20 to 48.88 ± 5.50 ; $p=0.006$), and LVM by 19.89 g (from 153.76 ± 43.31 mm to 173.65 ± 51.06 mm; $p=0.003$) at the end of trastuzumab therapy. The results are shown in Table III.

Radiation as the risk factor for cardiotoxicity was also studied. Sixty-eight patients (71.8%) had radiation therapy, 35 patients (51.47%) to the left breast, and 33 patients (48.53%) to the right breast, while 28 patients received no radiation therapy. At the end of trastuzumab therapy, the reduction of LVEF (2.08%; from 66.16 ± 4.96 to 64.07 ± 6.58 ; $p=0.005$) and increase of EDD (1.3 mm; from 47.45 ± 4.43 to 48.76 ± 4.10 ; $p=0.013$) were greater in patients who had prior radiation compared to those who had not. In addition, the patients who had left

Table I. Differences between the average values of echocardiographic parameters at the beginning and at the end of trastuzumab therapy.

	B	E	D	p ¹
LVEF(%)	65.49 ± 5.82	63.76 ± 6.43	1.73	p = 0.007
FS (%)	36.18 ± 5.10	35.32 ± 4.91	0.86	p = 0.123
EDD (mm)	47.53 ± 4.48	48.80 ± 5.19	1.26	p = 0.005
LVM (g)	161.55 ± 43.76	171.15 ± 47.88	9.60	p = 0.031

B: value at the beginning of therapy with trastuzumab; **E:** value at the end of treatment with trastuzumab; **D:** difference between B and E; **LVEF:** left ventricular ejection fraction; **EDD:** end-diastolic diameter; **FS:** fractional shortening; **LVM:** left ventricular mass. ¹Paired samples t-test.

Table II. Differences between the average values of echocardiographic parameters at the beginning and at the end of trastuzumab therapy depending on patient age.

	N	B	E	D	p ¹
Younger than 65					
LVEF (%)	63	64.92 ± 5.09	64.67 ± 5.81	0.25	0.650
FS (%)	63	36.02 ± 5.20	35.02 ± 4.13	1.00	0.157
EDD (mm)	63	47.18 ± 4.44	48.22 ± 4.38	1.04	0.057
LVM (g)	63	150.79 ± 39.12	158.53 ± 42.92	7.73	0.148
Older than 65					
LVEF (%)	33	66.60 ± 5.75	62.04 ± 1.43	4.56	0.002
FS (%)	33	36.49 ± 4.91	35.90 ± 1.07	0.59	0.520
EDD (mm)	33	48.21 ± 4.56	49.91 ± 6.43	1.70	0.037
LVM (g)	33	182.08 ± 45.34	195.24 ± 48.23	13.15	0.104

N: number of pts; *B*: value at the beginning of therapy with trastuzumab; *E*: value at the end of treatment with trastuzumab; *D*: difference between *B* and *E*; **LVEF**: left ventricular ejection fraction; **EDD**: end-diastolic diameter; **FS**: fractional shortening; **LVM**: left ventricular mass. ¹Paired samples *t*-test.

Table III. Differences between the average values of echocardiographic parameters at the beginning and at the end of trastuzumab therapy, depending on anthracycline cycles.

	N	B	E	D	p ¹
IV cycles					
LVEF (%)	50	65.41 ± 6.38	63.72 ± 7.17	1.69	0.650
FS (%)	50	36.13 ± 5.31	35.04 ± 5.88	1.09	0.465
EDD (mm)	50	47.19 ± 4.20	48.88 ± 5.50	1.68	0.006
LVM (g)	50	153.76 ± 43.31	173.65 ± 51.06	19.89	0.003
VI cycles					
LVEF (%)	46	65.58 ± 5.20	63.81 ± 5.59	1.77	0.048
FS (%)	46	36.24 ± 4.92	35.63 ± 3.60	0.61	0.465
EDD (mm)	46	47.91 ± 4.70	48.72 ± 4.90	0.08	0.235
LVM (g)	46	170.02 ± 43.11	168.43 ± 44.57	1.58	0.783

N: number of patients; *B*: value at the beginning of therapy with trastuzumab; *E*: value at the end of treatment with trastuzumab; *D*: difference between *B* and *E*; **LVEF**: left ventricular ejection fraction; **EDD**: end-diastolic diameter; **FS**: fractional shortening; **LVM**: left ventricular mass. ¹Paired samples *t*-test.

breast radiation before trastuzumab therapy showed a greater decrease in LVEF (2.53%; from 65.56 ± 5.25 to 63.03 ± 6.43 ; $p=0.020$) and increase in EDD (1.72 mm; from 47.80 ± 4.10 to 49.52 ± 6.42 ; $p=0.032$), compared to those who had radiation to the right breast (Table IV).

According to the presence of CVRF, the studied

patients were divided into two groups: a group of patients with CVRF – 70 pts (73%), and a group without CVRF – 26 pts (27%); the baseline patient CVRF characteristics are shown in Table V.

A greater decrease in LVEF (2.58%, $p=0.001$) after the last trastuzumab therapy was recorded in the group of pts with CVRF, compared to pts without

Table IV. Differences between the average values of echocardiographic parameters in pts with and without prior radiation therapy.

	N	B	E	D	p
Without radiation					
LVEF (%)	28	63.89 ± 7.38	63.01 ± 6.11	0.88	0.477 ¹
FS (%)	28	36.00 ± 5.43	34.31 ± 3.62	1.69	0.055 ¹
EDD (mm)	28	47.75 ± 4.43	48.91 ± 4.10	1.15	0.301 ¹
LVM (g)	28	143.24 ± 37.83	164.27 ± 44.75	21.02	0.301 ¹
With radiation					
LVEF (%)	68	66.16 ± 4.96	64.07 ± 6.58	2.08	0.005 ²
FS (%)	68	36.25 ± 5.00	35.73 ± 5.32	0.51	0.450 ²
EDD (mm)	68	47.45 ± 4.53	48.76 ± 5.16	1.30	0.013 ²
LVM (g)	68	169.09 ± 44.04	173.98 ± 49.15	4.89	0.337 ²
Radiation to the right breast					
LVEF (%)	33	66.78 ± 4.62	65.18 ± 6.65	1.60	0.125 ²
FS (%)	33	36.49 ± 5.21	35.19 ± 5.05	1.30	0.225 ²
EDD (mm)	33	47.07 ± 4.99	47.95 ± 4.56	0.86	0.204 ²
LVM (g)	33	165.90 ± 49.95	167.04 ± 47.57	1.14	0.336 ²
Radiation to the left breast					
LVEF (%)	35	65.56 ± 5.25	63.03 ± 6.43	2.53	0.020 ²
FS (%)	35	36.03 ± 4.86	36.25 ± 5.58	0.22	0.801 ²
EDD (mm)	35	47.80 ± 4.10	49.52 ± 6.42	1.72	0.032 ²
LVM (g)	35	172.10 ± 38.15	180.53 ± 50.3	8.43	0.182 ²

N: number of patients; B: value at the beginning of therapy with trastuzumab; E: value at the end of treatment with trastuzumab; D: difference between B and E; LVEF: left ventricular ejection fraction; EDD: end-diastolic diameter; FS: fractional shortening; LVM: left ventricular mass. ¹Wilcoxon rank test. ²Paired samples t-test.

CVRF. In addition, in the group of pts who had CVRF, EDD increased by 1.09 mm ($p=0.031$) (Table VI).

Pts with diabetes mellitus had the highest decrease in LVEF (by 9.96%) at the end of trastuzumab therapy,

and the reduction was lowest in the group of patients who smoked, by 2.07%, which is given in Table 7.

The greatest reduction in FS was observed in patients with diabetes mellitus (4.36%) and the

Table V. Baseline characteristics of the risk factors for cardiovascular disease in the examined pts.

Risk factors	N	Percentage
ARTERIAL HYPERTENSION	44	47.31
HYPERLIPIDAEMIA	23	23.95
SMOKING	23	23.95
DIABETES MELLITUS	5	5.2
OBESITY	28	29.16
HEREDITY	45	46.87

N: number of pts

Table VI. Differences between the average values of echocardiographic parameters in pts with and without risk factors.

	N	B	E	D	P
Without CVRF					
LVEF (%)	26	64.30 ± 5.63	64.85 ± 5.48	0.55	0.647 ¹
FS (%)	26	35.21 ± 4.94	34.42 ± 4.19	0.78	0.522 ¹
EDD (mm)	26	46.29 ± 4.14	48.00 ± 4.40	1.71	0.169 ¹
LVM (g)	26	148.75 ± 41.86	159.65 ± 46.85	10.89	0.183 ¹
With CVRF					
LVEF (%)	70	65.94 ± 5.86	63.36 ± 6.70	2.58	0.001 ²
FS (%)	70	36.54 ± 5.15	35.65 ± 5.14	0.89	0.180 ²
EDD (mm)	70	48.01 ± 4.54	49.10 ± 5.46	1.09	0.031 ²
LVM (g)	70	166.30 ± 43.78	175.42 ± 47.88	9.12	0.097 ²

N: number of patients; *B*: value at the beginning of therapy with trastuzumab; *E*: value at the end of treatment with trastuzumab; *D*: difference between *B* and *E*; *LVEF*: left ventricular ejection fraction; *EDD*: end-diastolic diameter; *FS*: fractional shortening; *LVM*: left ventricular mass.

¹Wilcoxon rank test. ²Paired samples *t*-test.

lowest in patients with hyperlipidemia (0.38%). A statistically significant decrease in FS (2.42%, $p=0.048$) was noticed in the group of obese patients (28 pts) (Table VIII).

The highest increase in EDD after therapy was observed in the group of patients with diabetes mellitus (5.38 mm; $p=0.042$) and the group of patients with hypertension, 1.25 mm ($p=0.054$) (Table IX).

At the end of trastuzumab therapy, LVM was increased most in diabetics (22.94 g, $p=0.078$). In patients with hypertension and hyperlipidemia,

the increase was 14.96 g ($p=0.039$) and 14.57 g ($p=0.050$), respectively (Table X).

The group of patients with CVRF was divided into three subgroups: the subgroup of patients with one CVRF (23 pts); the subgroup of patients with two CVRFs (18 pts); and the subgroup of patients with three and more CVRF (29 pts).

Compared to the baseline values, at the end of trastuzumab treatment, the reduction of LVEF (4.12%) and FS (1.51%) was greatest in the subgroup of patients with three and more CVRFs. The greatest

Table VII. Difference between the average EFLK values in pts with CVRF.

	N	N (%)	EF1 (%)	EF2 (%)	D	p
HTA	44	47.31	65.72 ± 5.90	62.64 ± 7.84	3.08	0.009 ²
HLP	23	23.95	64.68 ± 5.41	61.13 ± 7.71	3.58	0.061 ¹
SMOKING	23	23.95	65.91 ± 5.65	63.84 ± 4.63	2.07	0.038 ¹
DIABETES MELLITUS	5	5.2	62.16 ± 4.75	52.20 ± 9.96	9.96	0.223 ¹
OBESITY	28	29.16	66.02 ± 6.53	62.00 ± 9.21	4.02	0.009 ¹
HEREDITY	45	46.87	66.37 ± 5.18	63.49 ± 6.07	2.88	0.001 ²

N: number of patients; *EF1*: LVEF at the beginning of trastuzumab therapy; *EF2*: LVEF at the end of trastuzumab therapy; *D*: difference between EF1 and EF2; *HTA*: arterial hypertension, *HLP*: hyperlipidaemia. ¹Wilcoxon rank test. ²Paired samples *t*-test.

Table VIII. Difference between the average FSLK values in pts with the CVRF.

	N	N (%)	FS1 (%)	FS2 (%)	D	p
HTA	44	47.31	36.63 ± 5.31	35.34 ± 5.90	1.29	0.111 ²
HLP	23	23.95	34.74 ± 5.65	34.36 ± 6.10	0.38	0.740 ¹
SMOKING	23	23.95	37.19 ± 5.51	36.28 ± 3.80	0.91	0.159 ¹
DIABETES MELLITUS	5	5.2	32.76 ± 1.55	28.40 ± 4.92	4.36	0.276 ¹
OBESITY	28	29.16	37.18 ± 6.02	34.76 ± 6.53	2.42	0.048¹
HEREDITY	45	46.87	36.65 ± 5.31	35.19 ± 5.23	1.46	0.151 ²

N: number of patients; *FS1*: FS at the beginning of trastuzumab therapy; *FS2*: FS at the end of trastuzumab therapy; *D*: difference between FS1 and FS2; *HTA*: arterial hypertension, *HLP*: hyperlipidaemia. ¹Wilcoxon rank test. ²Paired samples *t*-test.

Table IX. Difference between the average EDD values in patients with the CVRF.

	N	N (%)	EDD1 (mm)	EDD2 (mm)	D	p
HTA	44	47.31	48.60 ± 4.52	49.85 ± 5.43	1.25	0.054 ¹
HLP	23	23.95	48.92 ± 4.16	51.10 ± 5.21	2.18	0.021 ²
SMOKING	23	23.95	47.47 ± 3.80	48.80 ± 4.52	1.32	0.250 ²
DIABETES MELLITUS	5	5.2	48.90 ± 5.40	54.28 ± 7.81	5.38	0.042 ²
OBESITY	28	29.16	48.77 ± 4.97	50.47 ± 5.80	1.70	0.068 ²
HEREDITY	45	46.87	48.10 ± 4.29	48.54 ± 5.78	0.44	0.493 ¹

N: number of pts; **EDD1**: EDD at the beginning of trastuzumab therapy; **EDD2**: EDD at the end of trastuzumab therapy; **D**: difference between EDD1 and EDD2; HTA: arterial hypertension, HLP: hyperlipidaemia. ¹Wilcoxon rank test. ²Paired samples t-test.

Table X. Difference between the average LVM values in pts with CVRF.

	N	N (%)	LVM1 (g)	LVM2 (g)	D	P
HTA	44	47.31	169.74 ± 44.32	184.70 ± 46.50	14.96	0.039 ¹
HLP	23	23.95	177.50 ± 46.96	192.07 ± 51.81	14.57	0.050 ²
SMOKING	23	23.95	156.30 ± 46.05	164.09 ± 47.79	7.79	0.279 ²
DIABETES MELLITUS	5	5.2	187.44 ± 49.73	210.39 ± 63.82	22.95	0.078 ²
OBESITY	28	29.16	177.61 ± 52.32	184.29 ± 46.73	6.68	0.189 ²
HEREDITY	45	46.87	166.51 ± 40.79	173.08 ± 48.38	6.57	0.346 ¹

N: number of patients; **LVM1**: LVM at the beginning of trastuzumab therapy; **LVM2**: LVM at the end of trastuzumab therapy; **D**: difference between LVM1 and LVM2; HTA: arterial hypertension, HLP: hyperlipidaemia. ¹Wilcoxon rank test. ²Paired samples t-test.

increase in EDD, by 1.93 mm, was seen in patients with two CVRFs, while LVM increased significantly by 13.31 g in patients with three and more than three CVRFs after the last trastuzumab therapy.

DISCUSSION

Over the past years, advances in therapy and screening have led to reducing mortality, delay of disease progression, and improvement in the quality of life of breast cancer patients. Unfortunately, the

consequence of the increased survival rates is an increasing number of patients who may experience late side effects of antineoplastic therapy.

Cardiac function should be closely monitored because of the risk of cardiac dysfunction associated with trastuzumab therapy. Evaluation of cardiac status should be done based on medical history, detailed history of earlier and present diseases and problems, physical examination, electrocardiogram, and echocardiographic examination. The main strategy is the detection of high-risk patients and their treatment (11).

Table XI. Difference between the average EF values depending on the number of CVRF.

Risk factors	N	B	E	D	p ¹
One					
EF (%)	23	66.00 ± 5.81	64.00 ± 4.13	2.00	0.037
FS (%)	23	36.54 ± 4.16	35.66 ± 3.45	0.88	0.170
EDD (mm)	23	47.95 ± 4.54	48.11 ± 5.38	0.16	0.861
LVM (g)	23	163.94 ± 41.73	160.64 ± 43.43	3.30	0.590
Two					
EF (%)	18	66.22 ± 6.37	65.56 ± 7.92	0.66	0.875
FS (%)	18	36.63 ± 5.59	35.34 ± 5.36	1.29	0.782
EDD (mm)	18	46.76 ± 5.08	48.71 ± 5.56	1.93	0.045
LVM (g)	18	168.28 ± 45.66	185.62 ± 49.05	17.34	0.158
Three and more					
EF (%)	29	65.57 ± 5.75	61.46 ± 7.03	4.12	0.004
FS (%)	29	35.75 ± 5.97	34.24 ± 5.81	1.51	0.211
EDD (mm)	29	48.81 ± 4.16	49.89 ± 5.54	1.06	0.252
LVM (g)	29	167.70 ± 45.59	181.01 ± 48.99	13.31	0.027

N: number of patients; *B*: value at the beginning of therapy with trastuzumab; *E*: value at the end of treatment with trastuzumab; *D*: difference between *B* and *E*; *EF*: left ventricular ejection fraction; *EDD*: end-diastolic diameter; *FS*: fractional shortening; *LVM*: left ventricular mass. ¹Wilcoxon rank test.

Hypertension (or hypotension) occurs in 2-28% of cases as a side effect during and after trastuzumab therapy. The most common pathogenetic mechanism of these side effects is inhibiting azote oxide synthase activity and reducing azote oxide production. In addition, there is a significant increase in vasoconstriction, peripheral vascular resistance, and blood pressure (12). Since hypertension is a potent and modifying risk factor for the development of cardiac dysfunction, it is monitored before and after each treatment cycle. In our study, mean blood pressure values before treatment were 119.9 mmHg for systolic BP and 75.89 mmHg for diastolic BP, and after the therapy, 121.30 mmHg for systolic

BP and 76.30 mmHg for diastolic BP. In 7.2% of patients, hypertension occurred with maximum values of systolic BP of 160 mmHg and diastolic BP of 90 mmHg. During antineoplastic therapy (especially with anthracyclines and taxanes), a wide range of cardiac arrhythmias can occur in the treated patients. Sinus tachycardia, bradyarrhythmias, and disorders of the conduction system are the ones most commonly reported. They can cause severe symptoms, become life-threatening, or impose a change in the patient's treatment plan. In 16-36% of cases, they occur at the beginning or during therapy (13, 14). In 10.4% of patients, single ventricular and supraventricular extrasystoles and tachycardia were

registered during therapy, which is a rate lower than that observed by Tamargo et al. (16-36%) (14). In our study, in all patients sinus rhythm was detected on electrocardiogram (ECG) at the beginning and after the last therapy with trastuzumab; one patient had left bundle branch block, and one had right bundle branch block. During the therapy, ectopic beats were registered in 11 (11.45%), supraventricular in 7 (7.29%), and ventricular extrasystoles in 4 (4.16%) patients. There were no changes at the end of therapy.

In addition to ejection fraction, the parameters for assessing systolic function are left ventricular volume, shortening fraction, stroke and minute volume, and left ventricular mass. A common feature of trastuzumab-induced cardiotoxicity is increased left ventricular volume, decreased ejection fraction, and decreased myocardium contractility (15).

Due to cardiotoxicity data in adjuvant trastuzumab phase II and phase III studies, despite a remarkable tumor response to this therapy, a retrospective analysis of the studies was performed by CREC. According to the results from seven studies (NCCTG; N9831, NSABP, B-31, HERA, Fin Her, BCIRG 006), which included 1,219 patients, cardiotoxicity was found in 112 patients (9.2%) (16, 17). The rate of cardiac dysfunction ranged from 8% to 30%. In our study, there was a statistically significant decrease in LVEF after trastuzumab therapy, by 1.73% ($p=0.007$), which was less than that in the reported CREC analysis.

According to the criteria of the ASE (18), asymptomatic reduction of LVEF was reported in 4 (4.17%) patients. In these patients, trastuzumab therapy was discontinued. However, when the increase in LVEF occurred, the therapy was reintroduced after one month, which indicated the reversibility of trastuzumab-induced cardiotoxicity. Compared to a meta-analysis published in 2015 that reported serious cardiotoxicity in 3% of patients receiving trastuzumab therapy, this study showed a higher proportion - 4.17% (19).

FS is the echocardiographic parameter widely used as an indicator of global systolic function. It is especially useful because the shortening of the circumferential fibers of the left ventricle largely contributes to the impact volume magnitude. In this sense, the shortening fraction is similar to the ejection

fraction. In the absence of regional wall kinetics anomalies, the two indicators closely correlate, although the absolute values of the shortening fraction are lower than EF. In 2012, Lang et al. reported early changes in myocardial function and morphology after three and six months of trastuzumab therapy. An increase in the left ventricular end-diastolic and end-systolic diameters, as well as a decrease in the LVEF and FS, were observed (20). In our study, FS decreased by 0.86%, and EDD increased by 1.26 mm.

One of the first adaptation mechanisms of LV to pressure or volume loading is the increase of muscle mass or left ventricular weight. When the LV is loaded with pressure, its mass increases more due to the thickening of the walls and less due to volume increases. In conditions where the left ventricle is loaded with volume, the mass of the left ventricle increases most at the expense of enlargement of the heart cavities (21). In addition, trastuzumab alters mitochondrial integrity, leading to a decrease in adenosine triphosphate and contractile dysfunction in cardiac muscle cells. Contractile dysfunction occurs as a result of ventricular dilation, thinning of the chamber walls, and decreased contraction efficiency, thus leading to dilated cardiomyopathy (22).

The incidence of chemotherapy toxicity increases with age. In the NSABP B-31 study, only 16% of 1,600 subjects were over 60 years of age. In these studies, age was found to predispose to trastuzumab cardiotoxicity (23). In the Italian register, 32.6% of patients were 60 years old or older. The cumulative risk of cardiotoxicity associated with trastuzumab for young people or those above 70 years was 1.3% versus 6.4% after one year, 2% versus 9.8% after two years, and 2.2% versus 9.8% after three years. In the US registry, for metastatic HER2 breast cancer treated with trastuzumab, the incidence of left ventricular dysfunction was 4.8%, 2.8%, and 1.5% for subjects ≥ 75 , 65-74, or < 65 years old, respectively (24). In our study, 65.6% of patients were older than 65, and after one year of trastuzumab therapy, a greater LVEF reduction, by 4.56% ($p=0.002$), was shown, and EDD increased by 1.70 mm ($p=0.032$), compared to younger patients.

Anthracyclins are potentially cardiotoxic. Their use in combination with trastuzumab leads to a

synergistic increase in the incidence of cardiac events (which is 27%). Since it causes cardiomyocyte apoptosis by free radical production, administration of trastuzumab may exacerbate sublethal damage by inhibiting HER2 signaling and cardiomyocyte repair, which can explain the so-called “dual hit” hypothesis. In a recent study by Cardinale et al., involving 2,625 patients (with the mean follow-up of 5.2 years), the incidence of cardiotoxicity after anthracycline treatment was 9% (in 98% of cases, it occurred in the first year and was asymptomatic) (25). In our study, the administration of VI cycles of anthracycline-containing chemotherapy before trastuzumab therapy resulted in greater LVEF reductions cumulative and dose-dependent.

The incidence of radiotherapy-induced heart disease is 10 - 30% higher 5 - 10 years after the last treatment. In a retrospective analysis of the MD Anderson Oncology Centre, cardiovascular events occurred in 7% of patients undergoing radiation of the right breast and 26% of those who had left breast radiation (26). The significance of radiotherapy was not shown in the N9831 study – the incidence of cardiovascular events in radiation patients was 2.2%, compared to 2.9% in those who did not have radiotherapy. In a prospective study at the Curie Institute in Paris, after the completion of adjuvant treatment with trastuzumab and concomitant radiotherapy, symptomatic heart failure occurred in 4% of patients, 2% of whom had serious cardiovascular complications (27). In this study, patients who had undergone previous radiotherapy had a significantly greater decrease in EF, by 6.58% ($p=0.005$), as well as an increase in EDD, by 1.3 mm ($p=0.013$), after trastuzumab therapy, compared to those without radiotherapy. Higher reductions in EF and increase in EDD at the end of trastuzumab were seen in patients with left breast radiation therapy (2.53%; 1.72 mm), compared to those who had right breast radiation therapy (1.60%; 0.86 mm).

In the study of Mantarro et al. in 2016, cardiotoxicity of trastuzumab was higher in smokers (5.3%), patients with dyslipidemia (3.9%), those with BMI ≥ 25 (6.5%), diabetes (6.2%), hypertension (5.5%) or a positive history of cardiovascular disease (19.1%) (28).

We showed that a greater decrease in LVEF

(0.85%, $p=0.001$) after the last trastuzumab therapy was encountered in the group of patients with CVRF – 70 patients (72.1%), compared to those without CVRF. In the group of patients who had CVRF, EDD increased by 1.09 mm ($p=0.031$), and LVM increased by 9.12 g ($p=0.097$). The reduction of LVEF after trastuzumab therapy was greatest in the group of obese patients (4.02%, $p=0.009$); in smokers, it was 2.07% ($p=0.038$); in those with heredity factor, it was 2.88% ($p=0.009$); and in those with hypertension 3.08% ($p=0.009$). The highest increase in EDD after trastuzumab therapy occurred in patients with diabetes, 5.38 mm ($p=0.042$), while the highest LVM increase was in arterial hypertension, 14.96 g. The patients with three or more CVRF showed the greatest LVEF reduction (4.12%) and the greatest LVM increase (13.31 g).

CONCLUSION

This study shows that the treatment with trastuzumab does not significantly affect BP and ECG changes. The rate of cardiotoxicity after trastuzumab therapy was low and presented as reducing LVEF, increased EDD, and increased LVM. The risk factors for cardiotoxicity, such as age over sixty-five and prior anthracycline therapy, had a significant impact on reducing LVEF and increased EDD. Previous administration of anthracyclines led to reduced LVEF, and it was cumulative and dose-dependent. Patients with CVRF had a greater LVEF decrease than those without CVRF. The presence of three or more CVRF had a considerable impact on the greatest LVEF reduction and the greatest LVM increase.

Conflicts of interest

All authors report no conflicts of interest relevant to this article.

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AATF knockdown reduces cell proliferation and promotes cell apoptosis in LPS-induced sepsisY. Li[#], L. Feng[#], Y. Zhao, Y. Cong, and W.L. Tang^{*}*Department of Emergency, Huadong Hospital Fudan University, Shanghai, China**Received July 8, 2021 – Accepted December 6, 2021*[#]These Authors contributed equally to this work

The authors investigated the function of the apoptotic antagonistic transcription factor (AATF) in lipopolysaccharide (LPS)-induced H9C2 cell apoptosis and proliferation. After transfecting H9C2 cells with short hairpin RNA (shRNA), a shAATF-162 gene interference model was established. Cells were divided into mock, shRNA, and shNC groups and were treated with LPS. The cell counting kit-8 (CCK-8) assay and annexin V PE/7-AAD staining were used to assess cell viability and apoptosis, respectively. The protein levels of p53 upregulated modulator of apoptosis (PUMA) and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) were determined using western blotting. Interleukin 6 and tumor necrosis factor α (TNF- α) secretion was determined using enzyme-linked immunosorbent assay (ELISA) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Results of the CCK-8 assay showed that AATF knockdown decreased proliferation and potentiated apoptosis in LPS-induced H9C2 cells compared with that in the shRNA and mock groups. The western blotting results indicated that AATF knockdown increased PUMA and NOXA protein levels in LPS-induced H9C2 cells compared to that in the shNC and mock groups. ELISA and RT-qPCR results showed that AATF knockdown significantly decreased the LPS-induced secretion of IL-6 and TNF- α in H9C2 cells compared to that in the shNC and mock groups. This study showed that AATF inhibition attenuated cell viability, potentiated apoptosis, and decreased the LPS-induced secretion of IL-6 and TNF- α in H9C2 cells.

Sepsis remains a significant health issue worldwide (1, 2) and is caused by infection from gram bacteria, viruses, fungi, and parasites, resulting in a dysregulated host response (3, 4). Myocardial injury is a common complication of sepsis, resulting in a high incidence of organ damage (5). Cardiac dysfunction is present in > 40% of sepsis patients and is related to mortality rates of up to 70% (6). Sepsis remains a commonly fatal disease, with early diagnosis limited by the lack of related biomarkers and risk factors. Thus, sepsis biomarkers with sufficient discriminatory power must be identified

to increase early detection rates. Additionally, the elucidation of the regulatory mechanism of the overwhelming inflammatory response in sepsis has become a critical topic for its treatment.

The apoptotic antagonistic transcription factor (AATF/Che-1) is a nuclear protein that interacts with RNA polymerase II (7, 8). *AATF* is an evolutionarily conserved gene in eukaryotes that is involved in multiple biological processes, including regulating cellular viability via eliciting cell cycle progression, autophagy, DNA damage and repair, and apoptosis suppression (9-12). AATF is a critical inducer of

Key words: sepsis; AATF; LPS; cell viability; cell apoptosis

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the neural tissue and diverse tumors (13-16). AATF regulates cell apoptosis in the nucleus by inhibiting pro-apoptotic genes, including *PUMA*, *BAX*, and *BAK* (17). Our previous study showed that AATF might be involved in the pathogenesis of sepsis (18); however, the mechanism remains unclear.

Lipopolysaccharide (LPS), also known as bacterial endotoxin, is a component of the cell wall of gram-negative bacteria that encounters endothelial cells following a signaling cascade, thus inducing sepsis progression (19, 20). Recently, several studies have reported that LPS is the main cofactor in sepsis development (21). LPS promotes endotoxemia, which is thought to be exclusively mediated via activating its cell surface receptor, Toll-like receptor 4 (TLR4), leading to the secretion of proinflammatory cytokines (22). LPS induces NF- κ B signaling pathway activation in podocytes and enhances the levels of proinflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor- α (TNF- α), and IL-1 β (23, 24). Thus, LPS can be used to induce inflammatory cell response for constructing sepsis cell models.

H9C2 cells have the characteristics of cardiomyocytes (25, 26). Therefore, in the present study, an LPS-induced H9C2 cell was used to build a sepsis cell model to provide new evidence for the relationship between sepsis and AATF. AATF-suppressed expression downregulated proliferation and facilitated apoptosis in H9C2 cells induced by LPS by enhancing *PUMA* and *NOXA* protein expression. Additionally, knockdown of AATF expression decreased LPS-induced IL-6 and TNF- α secretion in H9C2 cells. Collectively, our data revealed a critical mechanism for AATF regulation of sepsis.

MATERIALS AND METHODS

Cell culture and AATF-shRNA plasmids

Cardiomyocytes (H9C2 cells) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. The cells were maintained at 37°C in a 5% CO₂ incubator. Three shRNA plasmids were synthesized by Shanghai Jierui Biotechnology Co., Ltd. shAATF sequences are listed in Table I. We reinforced two restriction enzyme sites (AgeI and EcoRI) to the ends of the shRNA sequences, which were then ligated to the pLKO.1 plasmid vector.

Cell transfection and grouping

Cells were divided into blank, shRNA-162/558/975, and empty plasmid groups. For 24 h before transfection, 2×10⁵ cells were seeded into a 6-well plate and cultured in complete DMEM. The medium was changed with opti-MEM (2 mL/well) before transfection. H9C2 cells were then transfected with the above constructs and packaging lentivirus plasmids. Puromycin (1 µg/mL; Sigma, USA) was used to select stably infected cells. The cells were divided into mock (without transfection), shRNA (transfected with pLKO.1-AATF 162/558/975 plasmids), and shNC (transfected with empty plasmid) groups.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using RNAiso Plus reagent (TaKaRa, Kyoto-fu, Japan). RNA (of 1 µg) was reverse-transcribed using the PrimeScript™ RT Master Mix (TaKaRa). RT-qPCR was conducted using Power SYBR Green PCR Master (Thermo, NY, USA). A standard

Table I. Sequences of shAATF

shAATF	sequences
AATF-shRNA (162)-F	5'-CCGGGCTGGAACAGTTGTTGAATCCCTCGAGGGATTCAACAACGTGTTCCAGCTTTTTG-3'
AATF-shRNA (162)-R	5'-AATTCAAAAAGCTGGAACAGTTGTTGAATCCCTCGAGGGATTCAACAACGTGTTCCAGC-3'
AATF-shRNA (558)-F	5'-CCGGGGAGGAGGAGATCTCAGAAGACTCGAGTCTTCTGAGATCTCCTCCTCTTTTTG-3'
AATF-shRNA (558)-R	5'-AATTCAAAAAGGAGGAGGAGATCTCAGAAGACTCGAGTCTTCTGAGATCTCCTCCTCC-3'
AATF-shRNA (975)-F	5'-CCGGGGAGATTTCCAGTGAAGATGACTCGAGTCATCTTCACTGGAAATCTCCTTTTTG-3'
AATF-shRNA (975)-R	5'-AATTCAAAAAGGAGATTTCCAGTGAAGATGACTCGAGTCATCTTCACTGGAAATCTCC-3'

amplification protocol was used according to the manufacturer's instructions. *GAPDH* was used as the reference gene. Primers are listed in Table II.

Cell counting kit-8 (CCK-8) analysis of cell activity

H9C2 cells were divided into mock, shRNA-162, and shNC groups with or without LPS treatment. Cells (1×10^4) were seeded in a 96-well plate in triplicate, and the colorimetric CCK-8 assay kit (Keygenbio, Nanjing, China) was used to monitor the cell proliferation rate and inhibition rate of gene sensitivity continuously for 24 h.

Cell apoptosis

Cells were divided into mock, shRNA-162, and shNC groups with or without LPS treatment. Cell apoptosis was determined using the EBioscience™ Annexin V Apoptosis Detection Kit PE (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction and analyzed with flow cytometry using a BD FACSCalibur system (BD Biosciences, NY, USA). All analyses were performed in triplicate.

Western blotting analysis

Western blotting was performed as previously described. (27) Membranes were immunoblotted with the following antibodies: anti-rabbit PUMA (Abcam, ab9643), anti-mouse NOXA (Santa, sc-56169), and anti-mouse **β -actin** (Santa, sc-8432). Immunoblotting was performed as described previously using secondary (Jackson, 715-035-151) or rabbit antibodies (Jackson, 111-035-045) conjugated to horseradish peroxidase and visualized with chemiluminescence. β -actin was used

as the loading control. Protein grayscale was quantified using ImageJ software.

Enzyme-linked immunosorbent assay

A total of 4,000 cells were seeded in 96-well plates containing a complete medium. The supernatant was then tested using the rat IL-6 and TNF- α enzyme-linked immunosorbent assay (ELISA) kit (Huyu, Inc., Shanghai, China) according to the manufacturer's instructions. The absorbance value was measured using a microplate reader (BioTek, VT, USA). IL-6 and TNF- α concentrations were obtained based on standard curves.

Statistical analysis

Statistical analyses were performed with one-way analysis of variance and Newman-Keuls Multiple Comparison Test using GraphPad prism 5 (GraphPad Software, San Diego, CA, USA). Data are presented as means $\pm$ standard deviation (SD) of at least three independent experiments. A p-value of 0.05 or less was considered significant.

RESULTS

Comparisons of mRNA expressions of AATF among the mock, shRNA-162/558/975, and shNC groups

To investigate the role of AATF in sepsis, we constructed three shRNAs of the AATF plasmid (shAATF-162, shAATF-558, and shAATF-975). The knockdown efficiency in shAATF-162 was the highest among the three shRNAs. Therefore, we chose shAATF-162 as the candidate for further experiments (Fig. 1).

Table II. Primers for Reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Gene	Sequences
AATF-F	5'- GGCCAGGACCGAGTTGTATC-3'
AATF-R	5'- GGTCTGTGAGAGGTTAGCG-3'
IL-6-F	5'- TCTGGTCTTCTGGAGTTCCG-3'
IL-6-R	5'- AGCATTGGAAGTTGGGGTAGG-3'
TNF- α -F	5'- GCCTCTTCTCATTCTGCTCG-3'
TNF- α -R	5'- TCCGCTTGGTGGTTTGCTAC-3'
GAPDH-F	5'- AGACAGCCGCATCTTCTTGT-3'
GAPDH-R	5'- CTTGCCGTGGGTAGAGTCAT-3'

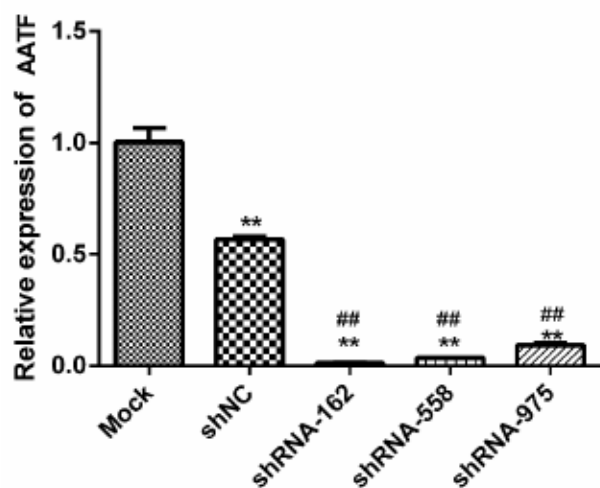


Fig. 1. Real-time quantitative PCR measures the interference efficiency of shAATF. AATF-shRNAs (162, 558, and 975) cloned the sequences into a pLKO.1 vector. H9C2 cells were transduced with a pseudovirus-containing culture medium. After 48 h, H9C2 cells were collected for real-time qPCR assay. Statistical analyses were performed using one-way analysis of variance and Newman-Keuls Multiple Comparison Test with GraphPad prism 5. Error bars represent data from three independent experiments (mean $\pm$ SD). **: $p < 0.01$ compared to the mock group; ##: $p < 0.01$ compared to the shNC group.

Knockdown of AATF inhibits H9C2 cell viability induced by LPS

We analyzed H9C2 cell viability using the CCK-8 assay. As shown in Fig. 2, AATF knockdown significantly decreased the viability of shAATF-162 H9C2 cells compared to that of the mock and shNC group cells ($p < 0.01$). After LPS treatment, cell viability was further suppressed. Therefore, knockdown of AATF expression attenuated cell proliferation, which was further retarded after LPS treatment.

Knockdown of AATF promotes H9C2 cell apoptosis induced by LPS

We also measured apoptosis in H9C2 cells using flow cytometry and western blotting. As shown in Fig. 3A and 3B, AATF knockdown H9C2 cells showed a higher apoptosis percentage than the mock and shNC groups. After LPS treatment, the number of apoptotic cells increased more than

that in the untreated group ($p < 0.01$). Previous studies demonstrated that during a cellular immune response, p53 as a tumor suppressor arrests cell cycle progression or increasingly upregulates cell apoptosis via overexpression of the BCL-2 family BH3-only protein PUMA. (28-31) Similarly, NOXA is a member of the pro-apoptotic BH3-only proteins of the BCL-2 family and plays a critical role in cell apoptosis by interacting with the prosurvival BCL-2-like proteins MCL1, BCL_{XL}, and BCL2A1. (32-34) As shown in Fig. 3C-3E, AATF knockdown significantly increased PUMA and NOXA protein expression and further upregulated PUMA and NOXA protein expression in H9C2 cell lines after LPS treatment ($p < 0.01$), taken together, AATF deficiency promoted H9C2 cell apoptosis induced by LPS. Mechanistically, AATF expression knockdown caused an increase in cell apoptosis following upregulation of PUMA and NOXA expression.

Knockdown of AATF suppresses LPS-induced inflammatory cytokine production in H9C2 cells

Both IL-6 and TNF- α are key factors in the inflammatory response to sepsis, and to elucidate how AATF regulates sepsis, we measured IL-6 and TNF- α secretion using ELISA and RT-qPCR.

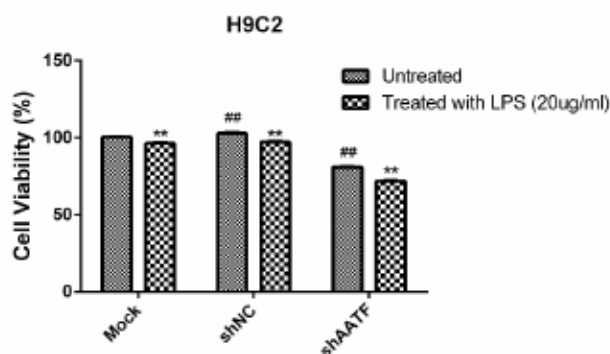


Fig. 2. CCK-8 assay for cell viability determination. H9C2 cell proliferation induced by LPS (20 μ g/mL) expressing the indicated plasmids was tested using the Cell Counting Kit-8 (CCK-8) assay. Statistical analyses were performed using two-way RM analysis of variance with GraphPad prism 5. Results represent the means of triplicate experiments (mean $\pm$ SD). ##: $p < 0.01$ compared to the mock group; **: $p < 0.01$ when compared between the LPS and no-LPS groups.

As shown in Figure 4A–D, AATF knockdown significantly decreased IL-6 and TNF- α expression in H9C2 cells after LPS treatment compared to that in the shNC group ($p < 0.05$). Therefore, AATF knockdown attenuated LPS-induced toxicity in H9C2 cells by reducing the levels of inflammatory factors (IL-6 and TNF- α).

DISCUSSION

Sepsis remains a critical health issue worldwide; therefore, it is essential to identify sepsis biomarkers that have sufficient discriminatory power to increase early detection rates. To the best of our knowledge, this is the first study to evaluate AATF effects in LPS-induced sepsis. The present study demonstrated

that *AATF* acts as a marker gene in regulating H9C2 cell proliferation and apoptosis induced by LPS. AATF knockdown significantly decreased H9C2 cell viability induced by LPS and elevated cell apoptosis by increasing the expression of the marker proteins PUMA and NOXA. Additionally, AATF suppression decreased the secretion of LPS-induced IL-6 and TNF- α in H9C2 cells.

AATF (Che-1) is a transcriptional cofactor that regulates gene expression by linking specific transcription factors with general transcription mechanisms. (35) More than two decades ago, Thomas et al. reported that AATF was essential for cell proliferation and survival. (36) AATF is involved in a wide range of cellular pathways, regulating proliferation and survival in physiological

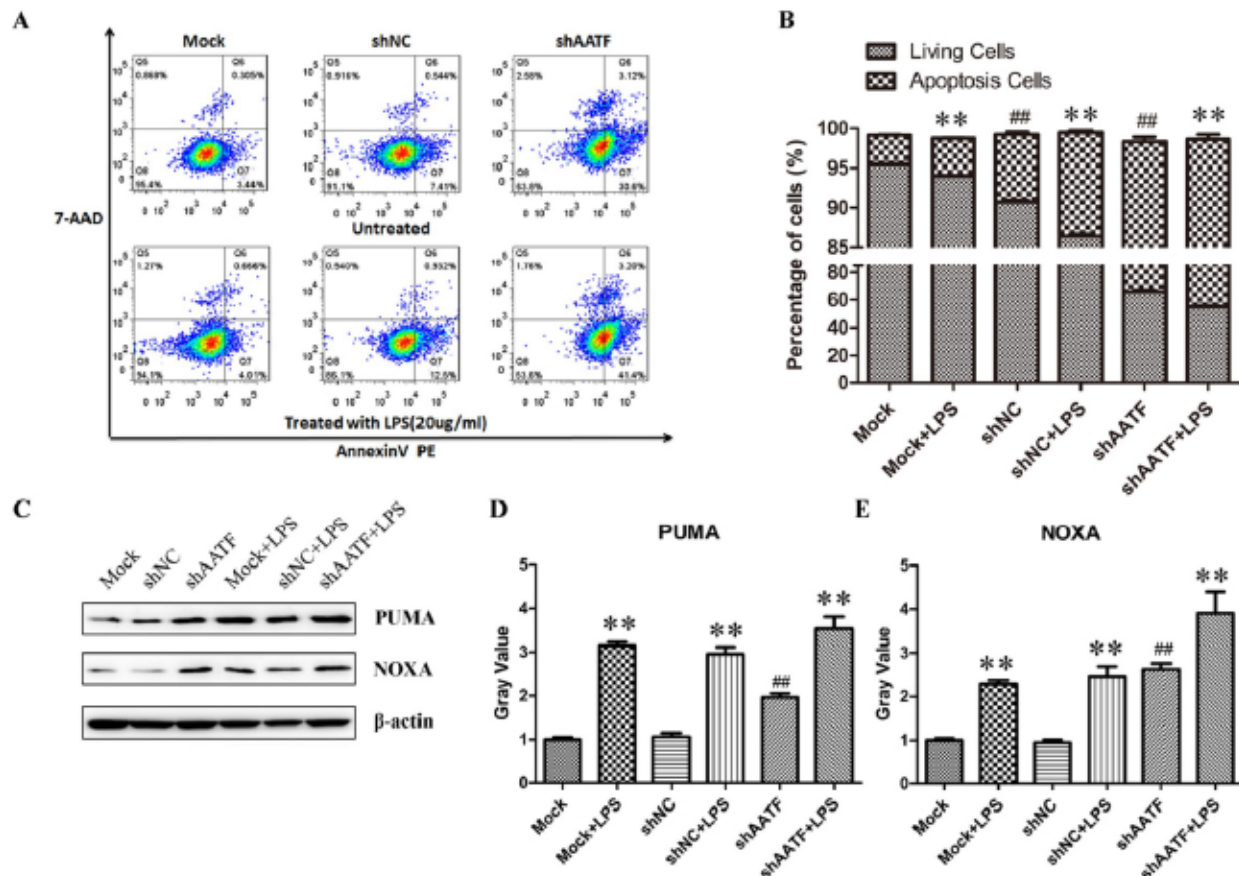


Fig. 3. Flow cytometry and western blotting for determining cell apoptosis. (A&B) Apoptosis in H9C2 cells expressing the indicated plasmids induced by LPS (20 $\mu\text{g/mL}$) was measured using flow cytometry analysis (A) and quantified (B). (C–E) PUMA and NOXA expression in H9C2 cells expressing the indicated plasmids induced by LPS (20 $\mu\text{g/mL}$) were tested with western blotting (C) and quantified (D, E). Statistical analyses were performed using two-way RM analysis of variance with GraphPad prism 5. Results represent the means of triplicate experiments (mean $\pm$ SD). **: $p < 0.01$ compared to the mock group; #: $p < 0.01$ compared to the shNC group.

and pathological conditions. (37) Similarly, AATF inhibited the transcription of pro-apoptotic genes induced by TP53, thus leading to cell cycle arrest and apoptosis decrease and contributing to the proliferation and tumor progression of lung adenocarcinoma. (10) In the present study, we found that AATF knockdown decreased cell viability and induced apoptosis in H9C2 cells induced by LPS, indicating that AATF plays an important role in sepsis progression. We also found that AATF knockdown significantly increased PUMA and NOXA protein expression in H9C2 cells induced by LPS compared to that in the shNC group. PUMA and NOXA proteins, which bind to B-cell lymphoma-2 (Bcl-2) family proteins, are major regulators of the apoptosis process. (38, 39) Thus, according to our observations, AATF knockdown reduced proliferation and promoted apoptosis of H9C2 cells by increasing PUMA and NOXA levels.

IL-6 and TNF- α are critical immune cofactors in sepsis development, which induce sepsis progression and mediate downstream regulatory signaling pathways. (40, 41) Previous studies have reported

that the upregulation of inflammatory cytokines IL-6 and TNF- α was induced by LPS, and we demonstrated similar results in the present study. (20, 42) To date, most studies of sepsis have focused on proinflammatory cofactor secretion, with excessive secretion of proinflammatory cytokines causing multiple organ failure. (4, 43) Therefore, decreasing the secretion of proinflammatory cytokines could be a therapeutic strategy for treating inflammatory diseases. Determining how to diminish the secretion of proinflammatory cytokines and attenuate the related inflammatory response might help in sepsis treatment. A previous study found that crocin might have beneficial effects in preventing sepsis cardiomyopathy, which is related to the inhibition of inflammatory factors (IL-6, TNF- α , and IL-1 β). (44) Consistent with the above results, AATF knockdown significantly decreased LPS-induced IL-6 and TNF- α secretion in H9C2 cells in the present study; therefore, AATF silencing attenuated LPS-induced toxicity in H9C2 cells via reducing the levels of inflammatory factors (IL-6 and TNF- α).

The present study has some limitations. First,

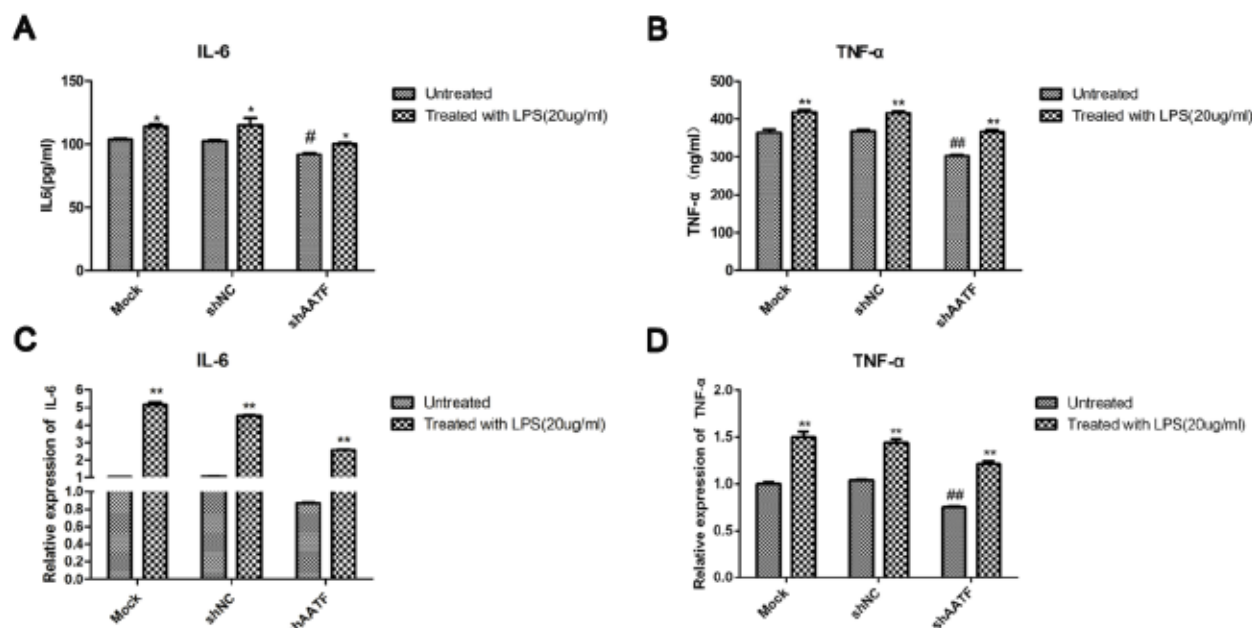


Fig. 4. Secretion of IL-6 and TNF- α determined by enzyme-linked immunosorbent assay (ELISA) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). ELISA and RT-qPCR were used to measure IL-6 (A, C) and TNF- α (B, D) secreted from H9C2 cells induced by LPS (20 μ g/mL) with or without AATF knockdown. Statistical analyses were performed using two-way RM analysis of variance with GraphPad prism 5. Results represent the means of triplicate experiments (mean $\pm$ SD). *compared to the mock group, # compared to the shNC group. */# $p < 0.05$, **/## $p < 0.01$.

we only used H9C2 cells to analyze the function of AATF in sepsis. We will further enrich the cell size to expand our conclusions. Second, compared to healthy individuals, there are no clinical data of patients with sepsis regarding AATF expression, the relationship between AATF expression and PUMA and NOXA, and IL-6 and TNF- α secretion. Third, the research only focused on the function of AATF treatment at the cellular level; thus, it is necessary to demonstrate its critical role using *in vivo* experiments. A previous study demonstrated that AATF mediates cell apoptosis and proliferation via the p53 signaling pathway, and AATF also interacts with STAT3 to upregulate the expression of monocyte chemoattractant protein-1, thus leading to cell proliferation, foci formation, migration, and invasion. (43) In future studies, we will focus on the cell cycle, cell migration, and the exact mechanism of the function of AATF during sepsis progression. A previous study demonstrated that AATF localizes at interphase centrosomes and regulates the duplication of centrosome and spindle formation, indicating a critical role of AATF in cell cycle progression. (45)

In conclusion, AATF deficiency negatively regulated cell viability, increased cell apoptosis by increasing PUMA and NOXA expression, and attenuated IL-6 and TNF- α secretion, thereby diminishing the stress response in sepsis. This might be a powerful therapeutic option for the treatment of patients with sepsis to specifically inhibit AATF.

Funding

None.

Conflicts of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Yin Li carried out the conception and design of the research, and Lei Feng participated in the acquisition of data. Yun Zhao performed the analysis and data interpretation. Yan Cong participated in the design of the study and performed the statistical analysis. Yin Li and Wenliang Tang conceived the study, participated in its design and coordination,

and helped draft and revise the manuscript for important intellectual content. All authors have read and approved the final manuscript.

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LMX1B positively regulates the expression of PHB1 in hypoxia-induced renal tubular epithelial cell injury

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Background and objective: Renal interstitial fibrosis (RIF) is a common pathological feature of various chronic kidney diseases that progress to end-stage renal failure. The LMX1B gene plays an important role in embryonic development, an important factor in regulating kidney development and maintaining podocyte function. In the present study, we aimed to explore whether LMX1B regulates PHB levels in the progression of RIF.

Methods: A hypoxic/reoxygenation model in renal tubular epithelial cells (RTECs) was used to simulate chronic injury in obstructive renal disease. Subcellular localization of LMX1B and prohibitin (PHB) 1 was observed using confocal laser microscopy. ROS and MDA levels were measured according to the reagent test kit protocols. The expression of LMX1B, PHB1, PHB2, TGF- β 1, Col-IV, and FN was tested by western blotting, and the gray value of the band was analyzed using Image J.

Results: LMX1B and PHB1 were both located in the nucleus of RTECs. The levels of PHB1 and PHB2 were closely associated with LMX1B levels. Overexpression of LMX1B in RTECs significantly increased the expression of PHB1 after H/R treatment. Moreover, the expression of TGF- β 1, Col-IV, and FN was significantly decreased in RTECs infected with LMX1B lentiviral activation particles than in control lentiviral activation-particle-infected RTECs. PHB1 levels were significantly decreased in LMX1B shRNA-transfected RTECs compared to control shRNA-transfected cells. Additionally, LMX1B overexpression effectively inhibited hypoxia/reoxygenation (H/R)-induced ROS and MDA levels.

Conclusion: The results demonstrated that LMX1B and PHB1 colocalized in the nucleus of RTECs, and LMX1B positively regulated PHB1 in an RTEC model system under H/R conditions. It plays a protective role in hypoxic RTECs and inhibits RIF caused by RTEC injury.

RIF is a common pathological feature of various chronic kidney diseases that progress to end-stage renal failure (1). It is marked by excessive accumulation of extracellular matrix proteins, which

ultimately leads to end-stage renal failure (2). RIF affects more than 10% of the population. Clinically, RIF is more closely related to chronic kidney disease (CKD) progression than the glomerular disease (3).

Key words: LMX1B; PHB 1; PHB 2; H/R injury

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Some studies believe that RTEC injury is the key factor and central link in RIF (4). RTEC injury refers to the activation, proliferation, tubule expansion, and cast formation of the renal tubules in response to hypoxia, poisoning, inflammatory factors, plasma proteins, or glucose, which eventually leads to apoptosis and cell death due to tubular atrophy and interstitial fibrosis (5). RIF caused by RTEC injury is the main cause and common pathological process of end-stage renal disease (ESRD). After injury, the RTEC movement, migration, and secretion functions are significantly enhanced, and various inflammatory factors, chemotactic factors, fibrogenic factors, vasoactive factors, and matrix proteins are produced (6). These pathological changes influence each other and mutually promote the entire process of RIF. After RTEC injury, the synthesis of transforming growth factor $\beta 1$ (TGF- $\beta 1$) and the expression of TGF- $\beta 1$ receptor on RTECs are rapidly upregulated, suggesting that under pathological conditions, RTECs are not only the cells that produce TGF- $\beta 1$, but also the target cells of TGF- $\beta 1$, which directly affect tubulointerstitial diseases (6). At the same time, TGF- $\beta 1$ induces the accumulation of ECM proteins, further aggravating the progression of RIF (5). However, the underlying mechanism of the occurrence and development of RIF is not fully understood, and effective prevention and treatment methods are lacking. Therefore, an in-depth study of the mechanisms that lead to RIF and promote disease progression is essential for effective prevention and treatment measures.

The LMX1B gene encodes a transcription factor with a LIM-homology region, belonging to the transcription factor family, rich in cysteine and zinc-binding regions. This characteristic helps the protein and other transcription factors to bind to each other (7, 8). Gene expression is an important factor in regulating kidney development and maintaining podocyte function (9). The LMX1B gene plays a variety of roles and is closely related to the production of bones, nails, eyeball retina, and kidneys and is also related to the function of the central nervous system. Mutations in the LMX1B gene can cause nail-patella syndrome and kidney disease (10). Current research suggests that LMX1B is closely

related to the maintenance of normal kidney function and is closely related to the production of podocyte processes and slit membranes (11, 12). He et al. found through podocyte research that LMX1B can regulate the expression of podocin to protect podocytes (13). Burghardt et al. found that LMX1B is an essential factor for maintaining podocyte differentiation (12). Haldin et al. found that LMX1B is closely related to the formation of glomeruli in *Xenopus laevis* (14). These studies suggest that LMX1B is closely related to glomerular diseases. However, no other reports are suggesting a role for LMX1B in the pathogenesis of RIF.

PHB is a tumor suppressor protein that is widely distributed in the cells of organisms such as bacteria, fungi, plants, protozoa, and mammals. PHBs are evolutionarily conserved proteins, including PHB1 and PHB2, which possess various cellular functions, such as cell proliferation, tumor suppression, apoptosis, transcription, and mitochondrial protein folding (15). At present, the subcellular location of PHB is still controversial, but most of it is believed to exist in the mitochondria and nucleus (16). PHB1 is mainly involved in maintaining the stability of mtRNA and performing normal physiological functions; PHB2 is mainly related to the permeability of mitochondrial membranes, which can maintain the normal permeability of mitochondrial membranes and make water-soluble CytC unable to penetrate the cytoplasm, thereby playing a role in protecting cells; PHB located in the nucleus mainly plays an anti-proliferative effect in the form of PHB1 (17). PHB expression was downregulated in RTECs injured by hypoxia. In the RIF rat model induced by a ureteral obstruction, PHB expression was decreased in renal interstitium and kidney tissues. The PHB expression was negatively correlated with the levels of TGF- $\beta 1$, Col-IV, FN, oxygen-free radicals (ROS) and activated caspase-3 (18, 19). It was reported that after knockdown of PHB expression in rat kidney tissues and in vitro cultured RTEC, the levels of ROS, malondialdehyde (MDA), TGF- $\beta 1$, and type IV collagen (Col-IV) in ECM components and fibronectin (FN) were all increased. Transfection of exogenous PHB upregulates PHB levels in rat kidney tissues and in vitro cultured RTECs. The

levels of ROS, MDA, TGF- β 1, Col-IV and FN were all decreased (20). Therefore, PHB may have an anti-fibrotic effect in the kidney.

Interestingly, we also found a positive correlation between LMX1B expression and PHB expression. As a nuclear transcription factor, LMX1B expression is positively correlated with PHB; thus, we explored whether or how LMX1B regulated PHB expression, which provided new ideas for the clinical prevention and treatment of tubular interstitial fibrosis and would be helpful to provide a reference in the clinical therapy of RIF.

MATERIALS AND METHODS

Cell lines and agents

The rat RTEC cell line NRK-52E cells (Cat. No. GNR8) were purchased from the Cell Bank and Type Culture Collection of the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shanghai, China) and maintained in DMEM (2 mM glutamine, 1% non-essential amino acids) supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere (37°C, 5% CO₂). The rPRTECs (Cat. CP-R062) were purchased from Procell Corporation (Wuhan, China) and cultured in Procell complete medium (Cat. CM-R062). LMX1B lentiviral activation particles (m): sc-421451-LAC were purchased from Santa Cruz Biotechnology, Inc. Control Lentiviral Activation Particles: sc-437282 (200 μ L) were used as negative controls.

Hypoxia-induced RTEC damage model

H/R (12/24 h) induced oxidative stress and cell injury, as described previously (20). The cells were divided as follows: untreated cells, RTEC injury induced by H/R treatment, RTEC transfected with LMX1BshRNA or control shRNA (interference with LMX1B), or RTEC infected with LMX1B lentiviral activation particles and control lentiviral activation particles (overexpression of LMX1B). The treated cells were harvested for further analysis 48 h after treatment.

Transfection

The transfection was according to the kit protocol from Santa Cruz Biotechnology, Inc. The lentiviral particles were transfection ready-to-use viruses, and 200

μ L virus stock solution contains 1x 10⁶ virus infection units (IFU). Lentiviral particles generally contain three to five expression vectors, and each vector can encode a 19-25 nt (with hairpin structure) shRNA targeting a specific site to knock out gene expression. After transfection, puromycin can be used to select cells stably expressing shRNA briefly, 293T cell line to package the virus. Lipo2000 transfection reagent is used for virus packaging plasmids and cultured at 37°C for 48 h. The virus supernatant was collected twice at 48 and 72 h after transfection (replacement of fresh culture solution after 24 h of collection). After collection, it was filtered with a 0.45 μ m filter and centrifuged in a 40 mL ultracentrifuge tube at 4°C, 72000g/min for 120 minutes. NRK52E and rPRTEC cells were transfected with Polybrene® (sc-134220) at a final concentration of 5 μ g/ml. The cell plate was mixed gently and incubated at 37°C overnight. The next day, remove the culture medium and replace it with 2 ml of complete medium without Polybrene and incubate the cells at 37°C for 48 h in a complete medium with 5% fetal bovine serum (FBS).

Western blotting analysis

For western blotting analysis, the primary antibodies for LMX1B, PHB1, PHB2, TGF- β 1, Col-IV, and FN, β -actin are recombinant Anti-LMX1b antibody (ab259926) was a rabbit monoclonal to LMX1b and purchased from Abcam. PHB1 antibody #2426S was a rabbit antibody to PHB1 and purchased from Cell signaling Technology. Anti-rabbit IgG, HRP-linked Antibody. PHB2 rabbit mAb #14085S was purchased from Cell signaling Technology. Recombinant anti-TGF beta 1 antibody (ab215715, Abcam) was a rabbit monoclonal to TGF beta 1. Recombinant Anti-collagen IV antibody (ab236640, Abcam) was a rabbit monoclonal to collagen IV. Recombinant anti-fibronectin antibody (ab268020, Abcam) was a rabbit monoclonal antibody to fibronectin. Anti- β actin antibody (ab6276) was a mouse monoclonal to β -actin. To detect the expression of LMX1B, PHB1, PHB2, TGF- β 1, Col-IV, and FN in RTECs, the total protein of RTECs was first extracted, and the total protein concentration was determined using the BCA method. Each lane was loaded with the sample at 30 μ g/well. SDS-PAGE (10%) was performed and used to separate the proteins. Then, the total protein was transferred to a PVDF membrane. The primary antibody was added at

1:500 to detect various proteins at 4°C overnight, and the secondary antibody was goat anti-rabbit or anti-mouse IgG labeled with horseradish peroxidase. The secondary antibodies were diluted at 1:10000 and incubated for 1 h at room temperature. The film was photographed, and the bands were analyzed using ImageJ software.

ROS and MDA measurements

ROS and MDA levels were measured as previously described (20). Briefly, cell supernatants were obtained by centrifugation (15000×g; 5 min) at 4°C, and 500 µL of the supernatant was incubated for 3 h at 37°C with 10 µL of a 10 µM DCF-DA (2,7-dichlorodihydrofluorescein diacetate) solution (Life Technologies/Molecular probes, Eugene, OR, USA). The fluorescence signal was measured at 485/525 nm using an S-3100 spectrofluorometer (Scinco Co. Ltd., Seoul, Korea). In addition, the supernatant was used to detect MDA levels. Absorbance was measured spectrophotometrically at 562 nm.

Immunofluorescence

RTECs were infected with LMX1B lentiviral activation

particles and control lentiviral activation particles for 48 h, followed by H/R treatment as described above. The cells were fixed with 100% methanol for 5 min and permeabilized with 0.1% Triton X-100 for 5 min. Next, the non-specific proteins were blocked by incubation in 2% BSA and 0.1% PBS-Tween for 1 h. The cells were then incubated with Anti-LMX1b antibody (Abcam ab272683, rabbit polyclonal to LMX1b) overnight at +4°C. The secondary antibody used was goat polyclonal secondary antibody to rabbit IgG - H&L (Alexa Fluor® 647) (ab150079) at 2 µg/ml for 1 h. All the antibodies were obtained from Abcam Corporation. DAPI was used to stain the cell nuclei at a final concentration of 1.43 µM. For PHB1 staining, the antibody information was as follows: the primary antibody was anti-PHB antibody (ab224653) and was a mouse monoclonal to PHB, and the secondary antibody was goat polyclonal secondary antibody to mouse IgG (ab150113).

Statistical analysis

All data were analyzed using the SPSS software (SPSS, Inc. version 20.0). The independent samples were analyzed

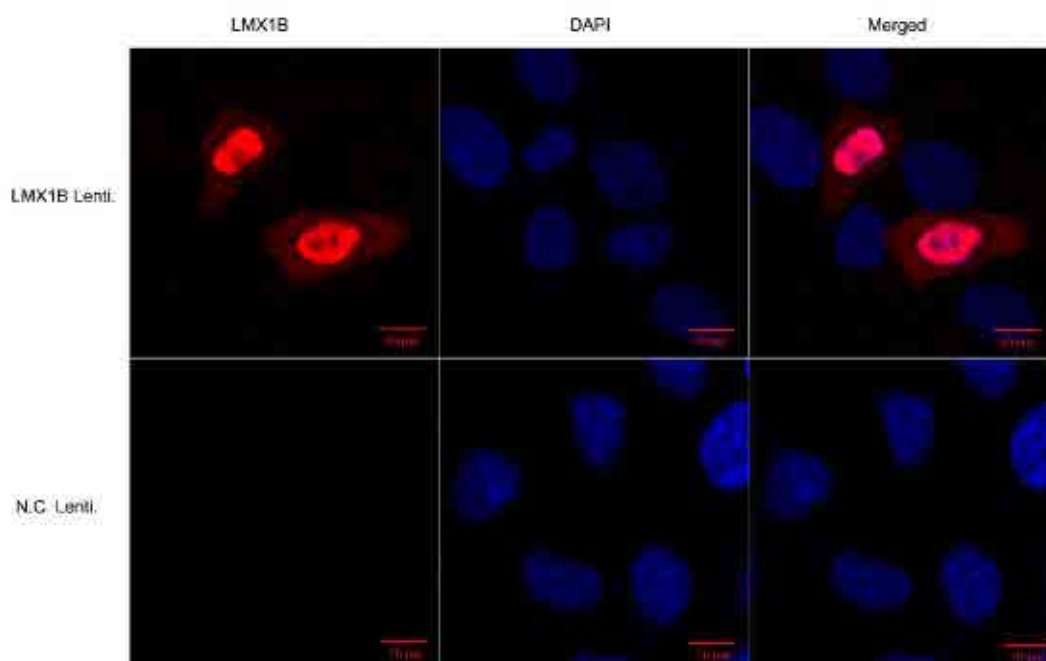


Fig. 1. Subcellular localization of LMX1B. RTECs were infected with LMX1B lentiviral activation particles and control lentiviral activation particles for 48 h. A confocal laser microscope was used to observe the subcellular localization of LMX1B. The magnification was 200×. DAPI was used to stain the nucleus of RTECs.

using an unpaired t-test, and multiple comparisons were analyzed using ANOVA, followed by Tukey's test. The results are presented as the mean $\pm$ standard deviation. $p < 0.05$ was considered statistically significant.

RESULTS

Subcellular localization of LMX1B and PHB1 under hypoxia

First, we detected the subcellular localization of LMX1B and PHB1 under H/R conditions, as described in the Materials and Methods. As shown in Fig. 1, LMX1B was mainly expressed in the nucleus, and a small amount of LMX1B was expressed in the cytoplasm of RTECs under hypoxic conditions. Moreover, we detected the subcellular localization of PHB1 using a confocal laser microscope. As shown in Fig. 2, we found that PHB1 was mainly located in the nucleus of RTECs under hypoxic conditions. All the results show that LMX1B and PHB1 were both located in the nucleus of RTECs under hypoxic conditions.

PHB1 and PHB2 levels are closely associated with LMX1B levels

Next, we detected the expression of LMX1B, PHB1, and PHB2 after H/R treatment. First, the RTECs were treated with the H/R treatment as described in the Materials and Methods section. The levels of LMX1B, PHB1, and PHB2 were detected by western blot analysis. As described in Fig. 3, the level of LMX1B was significantly decreased in

H/R-treated cells compared to that in the untreated RTECs (** $p < 0.01$). Moreover, the expression of PHB1 and PHB2 was significantly decreased in H/R-treated RTECs compared to that in untreated cells (** $p < 0.01$). This result was confirmed using rPRTECs (Fig. 3). All the data demonstrated that

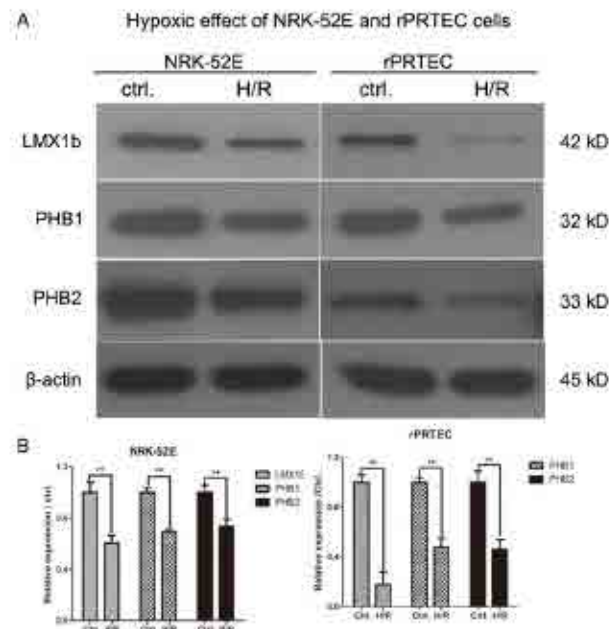


Fig. 3. The expression of LMX1B, PHB1 and PHB2 after H/R treatment. A): The expression of LMX1B, PHB1, and PHB2 were determined by western blotting in NRK-52E cells and rPRTECs after H/R treatment; B): The data are for proteins as quantitated by western blotting in NRK-52E cells and rPRTECs after H/R treatment. The relative expression of LMX1B, PHB1, and PHB2 was shown in the histogram. **: $p < 0.01$ compared with untreated group.

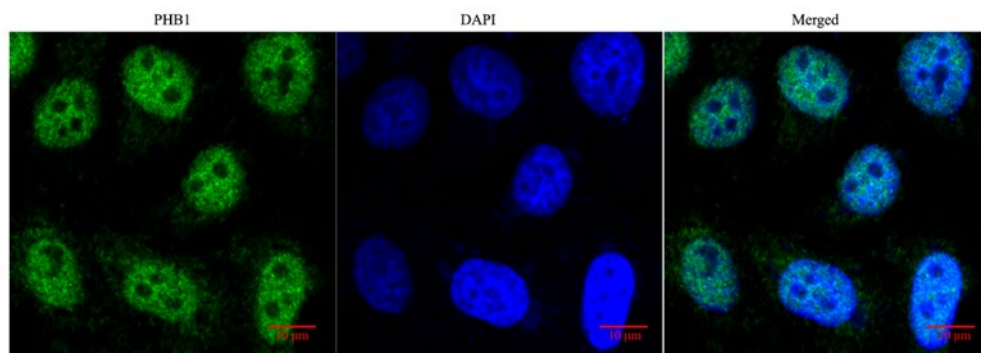


Fig. 2. Subcellular localization of PHB1. RTECs were plated into 24-well plates. Eight hours later, the subcellular distribution of PHB1 was observed using a confocal laser microscope. The magnification was 200 $\times$.

LMX1B expression was positively correlated with the levels of PHB1 and PHB2. However, further studies are needed to determine whether a regulatory relationship exists between LMX1B and PHB.

The expression of PHB1 is regulated by LMX1B in RTECs

To explore whether LMX1B regulated the expression of PHB1, RTECs were infected with LMX1B lentivirus for 48 h. The expression of LMX1B and PHB1, as well as that of TGF- β 1, Col-IV, and FN, was detected by western blotting. As shown in Fig. 4, overexpression of LMX1B in RTECs significantly increased the expression of PHB1 after H/R treatment (** $p < 0.01$, compared with control lentivirus-infected RTECs).

Importantly, the expression of TGF- β 1, Col-IV, and FN was significantly decreased in RTECs infected with LMX1B lentiviral activation particles than in control lentiviral activation-particle-infected RTECs. Furthermore, RTECs were transfected with LMX1B shRNA and control shRNA for 48 h, and the western blotting results showed that PHB1 levels were significantly decreased in LMX1B shRNA-transfected RTECs compared to control shRNA-transfected cells (** $p < 0.01$).

To determine whether the expression of TGF- β 1, Col-IV and FN regulated by LMX1B was mediated by PHB1, we knocked down PHB1 in NRK-52E cells and rPRTECs. NRK-52E cells and rPRTECs were infected with PHB shRNA (r) lentiviral

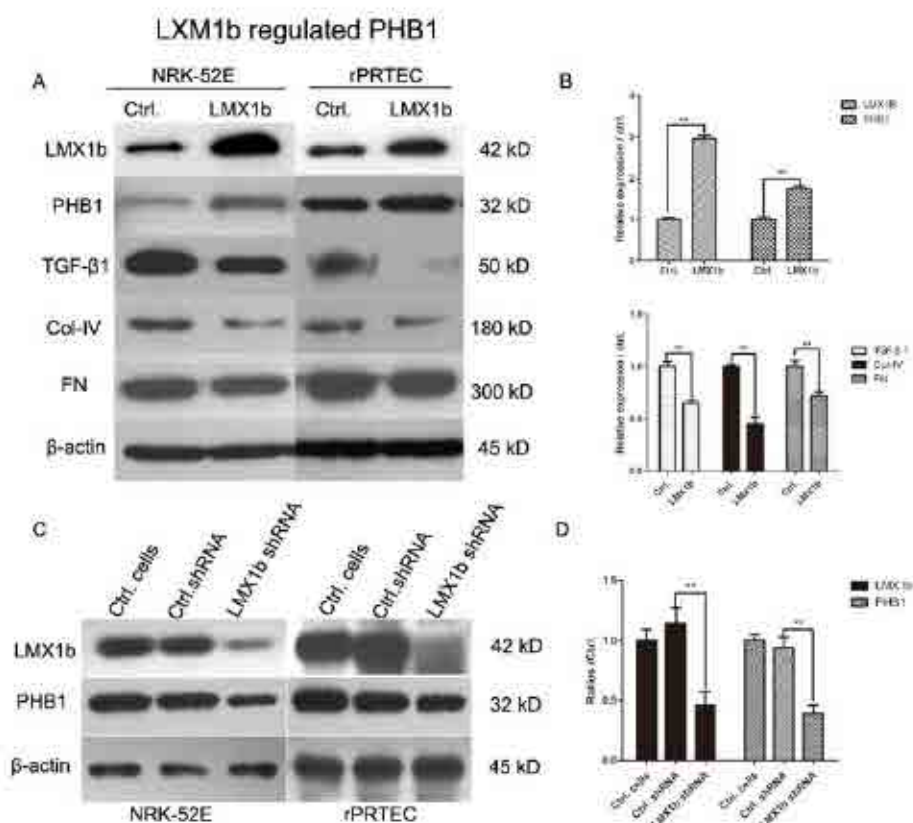


Fig. 4. The expression of PHB1 is regulated by LMX1B in RTECs. A): The levels of LMX1B, PHB1, TGF- β 1, Col-IV, and FN were determined by western blotting in RTECs; B): The gray value of the bands in western blotting analyzed with image J. The relative expression of LMX1B, PHB1, TGF- β 1, Col-IV, and FN are shown in the histogram. **: $p < 0.01$ compared with untreated group; C): The RTECs were transfected with LMX1B, shRNA, and control shRNA for 48 h. The expression of LMX1B and PHB1 were determined by western blotting; D): The data are for proteins as quantitated by western blotting. The relative expression of LMX1B and PHB1 is shown in the histogram. **: $p < 0.01$, compared with control shRNA transfected RTECs.

particles for 48 h, and the expression of PHB1 decreased. Moreover, overexpression of LMX1B in PHB1-knockdown RTECs (NRK-52E cells and rPRTECs) significantly increased the expression of PHB1 (** $p < 0.01$, compared with control lentivirus-infected RTECs). As expected, the expression of TGF- β 1, Col-IV, and FN was significantly decreased in NRK-52E cells and rPRTECs infected with LMX1B lentiviral activation particles compared to control lentiviral activation-particle-infected RTECs (Fig. 5). All the data revealed that LMX1B plays a protective role in hypoxic RTECs and that the effect of inhibiting RIF might be mediated by LMX1B-PHB1 signaling pathways.

Redox status measurements

Next, RTECs were infected with LMX1B lentiviral activation particles and control lentiviral activation particles for 48 h, and redox status was measured as shown in Fig. 6. H/R injury induces ROS and MDA production. LMX1B overexpression significantly decreased the formation of ROS and MDA in H/R-treated RTECs (** $p < 0.01$, compared with H/R-

treated RTECs). These results demonstrated that LMX1B overexpression effectively inhibited H/R-induced ROS and MDA levels.

DISCUSSION

The current common acute and chronic kidney diseases are mostly accompanied by fibrotic lesions in the late stages (21). Ischemia-reperfusion injury causes significant long-term damage to the kidney, mainly because of its impact on kidney function. During the entire process of kidney ischemia-reperfusion injury, the most serious pathological part is the tubule interstitium. After the kidney is injured, the persistent inflammatory response and the repeated existence of renal damage repair (or incomplete repair) form a vicious circle, which leads to renal tubular atrophy, progressive fibrosis, and decreased renal function, eventually leading to organ failure (22). RIF is a prominent pathological feature of CKD and a major factor in the progression of CKD to end-stage renal disease. Once RIF occurs, it progresses continuously (23). To date, its mechanism

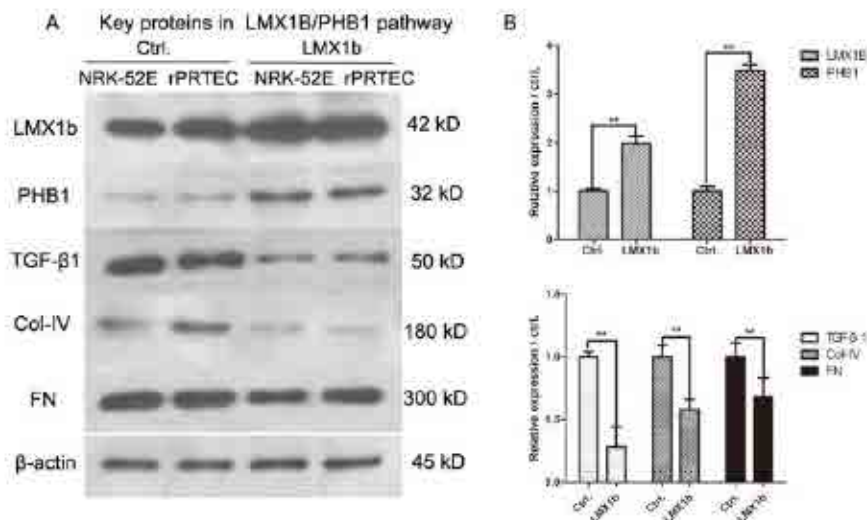


Fig. 5. The expression of key proteins in the LMX1B/PHB1 pathway in NRK-52E cells and rPRTECs. A): NRK-52E cells and rPRTECs were infected with PHB1 shRNA (r) lentiviral particles for 48 h. The expression of PHB1, LMX1b, TGF- β 1, Col-IV, and FN were determined by western blotting; B): The data are for proteins as quantitated by western blot in PHB1-knockdown NRK-52E cells and rPRTECs. The gray value of the bands in western blotting analyzed with image J. The relative expression of LMX1B, PHB1, TGF- β 1, Col-IV, and FN is shown in the histogram. **: $p < 0.01$ compared with Ctrl. group.

has not been fully elucidated. It has been found that upregulating the expression of LMX1B, PHB1, and PHB2 in RTECs resulted in a reduction in renal tubular damage and cell apoptosis. We found that the expression of LMX1B, PHB1, and PHB2 decreased in the hypoxia-injured RTECs; after overexpression of LMX1B, the expression of PHB1 and PHB2 increased (20). Interestingly, we found that LMX1B expression positively correlated with PHB1 and PHB2. This shows that LMX1B, PHB1, and PHB2 play a protective role in hypoxic RTECs.

In CKD, excessive deposition of ECM is the main reason for the gradual loss of renal function and the formation of RIF. Some scholars believe

that RTEC injury is the key factor and central link in RIF (24). RIF caused by RTEC injury is the main cause and common pathological process of ESRD. TGF- β is a small secretory signal protein involved in cell survival, inflammatory infiltration, cell differentiation, proliferation, and apoptosis (25). TGF- β 1 is considered an important factor in driving the development of RIF (26). At the same time, TGF- β 1 induces the accumulation of ECM, which further aggravates the progression of RIF. TGF- β production contributes to the accumulation of fibrous connective tissue, which is characterized by increased levels of FN and collagen. After RTEC hypoxia injury, the expression of pro-fibrosis genes FN1 and Col-IV was significantly upregulated, suggesting the formation of renal tubular fibrosis (27). It has been found the expression of LMX1B was increased in the kidney tissue of RIF rats, the RIF index decreased, and the apoptosis of RTECs was reduced. When inhibiting the expression of LMX1B in the kidney tissue of RIF rats, the RIF index increased, and the apoptosis of RTECs increased. It has been reported that upregulation of LMX1B expression in cultured RTECs reduced ECM secretion and RTEC apoptosis, whereas knockdown of LMX1B expression by siRNA in RTECs increased ECM secretion and RTEC apoptosis (28), and the expression of TGF- β 1, FN1, Col-IV decreased significantly after overexpression of LMX1B, indicating that LMX1B can inhibit RIF caused by RTEC injury; therefore, LMX1B may have anti-fibrotic effects in the kidney. Our previous experimental results showed that through gene interference, the expression of PHB, ROS, MDA, TGF- β 1, and Col-IV in ECM components in rat kidney tissue and RTECs cultured *in vitro* was downregulated, and FN expression increased; transfection of exogenous PHB upregulated PHB expression in rat kidney tissues and *in vitro* cultured RTECs, and the expression of ROS, MDA, TGF- β 1, Col-IV, and FN were decreased. Therefore, PHB may have an anti-fibrotic effect in the kidney. As we know, LMX1B is a nuclear transcription factor, and we found that the expression of LMX1B was positively correlated with PHB, and in addition, that LMX1B could regulate the expression of PHB in RTECs.

In the present study, by establishing a hypoxic

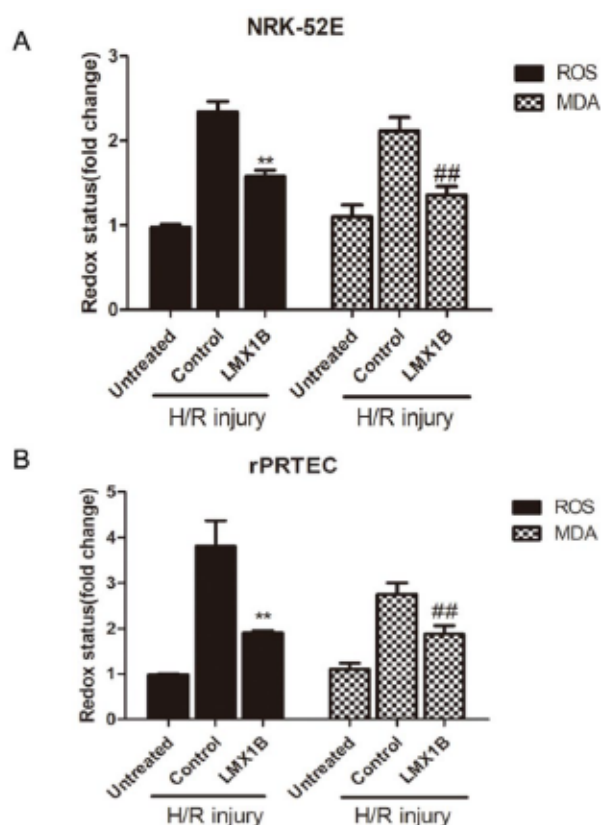


Fig. 6. Redox status measurements. RTECs were infected with LMX1B lentiviral activation particles and control lentiviral activation particles for 48 h. The LMX1B-transfected cells were treated with H/R treatment as described in the Materials and Methods section. The ROS and MDA level is shown in the histogram. ** $p < 0.01$, compared with H/R treated with negative control lentivirus and the H/R treated group. ### $p < 0.01$, compared with H/R treated with negative control lentivirus and the H/R treated group.

RTEC injury model, using a laser confocal microscope to observe the subcellular localization of LMX1B and PHB1 in RTECs; using siRNA to silence endogenous LMX1B and transfecting exogenous LMX1B to study different levels of LMX1B and the influence of PHB1 and PHB2 expression in RTECs; using western blot method to detect the changes in the expression of PHB1, PHB2, TGF- β 1, Col-IV, and FN after overexpression of LMX1B; and finally by using an ELISA kit to detect the levels of oxidation-related indicators, ROS and MDA, we were able to obtain results showing that LMX1B plays a protective role in hypoxic RTECs by regulating the expression of PHB and its related genes. In addition, we found that it inhibits RIF caused by RTEC injury and could be used as clinical prevention and treatment for renal tubular interstitial fibers, providing novel ideas for the treatment of RIF.

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Relationship between sTREM-1, MR-proADM, and IL-6 levels in the cord blood and infection of preterm premature rupture of membranes

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Background: There is an increase in the number of premature births due to the premature rupture of the fetal membrane (PROM). Premature infants are more susceptible to infection, but early signs of infection can be difficult to detect in neonates. Soluble trigger receptor -1 (sTREM-1) was expressed by myeloid cell, mid-regional pro-adrenomedullin (MR-proADM), and IL-6 may be useful markers for detecting early neonatal infection. **Methods:** A retrospective analysis was conducted in 689 premature infants with and without PROM to determine the risk factors for infection in PROM. The correlations between neonatal infection after PROM and sTREM-1, MR-proADM, and IL-6 levels of the umbilical cord blood were assessed in a cohort of 50 singleton infants. **Results:** Premature infants with PROM were divided into infected (n=151) and non-infected (n=234) groups and compared to 304 premature infants without PROM. No correlation was observed between PROM and ethnicity, gestational age, gender, or twin gestation. A significant correlation was observed between infection and PROM. In the PROM infants, there was a noteworthy correlation between gestational age and infection. Umbilical cord blood levels of sTREM-1, MR-proADM, and IL-6 were remarkably higher in the infected group than in the uninfected group. **Conclusion:** Infection during pregnancy is the main risk factor for PROM. In PROM neonates, gestational age could be a major risk factor for infection. Umbilical cord blood sTREM-1 and MR-proADM levels, and to a lesser degree IL-6 levels, may be useful to predict early PROM and neonatal infection.

Currently, social and environmental factors have led to an increase in the incidence of premature births, which can lead to a significant global healthcare burden.

About 85% of the world's preterm infants are middle or late preterm infants, while 10% are early preterm infants, and the remaining 5% are very early preterm

Key words: neonatal infection, MR-proADM, PROM, sTREM-1

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infants (1). The immune system in premature infants develops poorly, which increases their susceptibility to infection and in severe cases leads to septicemia. However, the early clinical manifestation of neonatal septicemia is atypical. Clinicians primarily rely on traditional biomarkers to identify infection, which can delay diagnosis and treatment and affect the prognosis.

PROM refers to the spontaneous rupture of amniotic sac before delivery, and PROM occurring before 37 weeks of pregnancy is called preterm premature rupture of membranes (PPROM). With the increase in the number of premature infants, PROM has attracted more attention, with PPRM and infection considered to have reciprocal cause and effect. Hence, Early neonatal infection index is required to be evaluated.

The hypersensitive C-reactive protein (CRP) and procalcitonin (PCT) are important for the diagnosis of infection; however, their low concentrations in neonates can confound the diagnosis of infection (2). Patients with the results of positive clinical blood culture clearly have septicemia, but in cases with PROM lasting more than 12 h, the prophylactic administration of antibiotics to pregnant women can result in false-negative results. Therefore, it is important to identify specific markers of inflammation for the early diagnosis of intrauterine infection, in order to facilitate prevention or treatment (3-6). The adrenomedullin in central MR-proADM and the Soluble trigger receptor -1 (sTREM-1) (sTREM-1) have attracted much attention in the diagnosis of infection. The sensitivity and specificity of MR-proADM are reported to be high, which allows prediction of the degree of infection. However, the relationship between MR-proADM and PPRM has rarely been studied in China.

This study was divided into two parts. In the first part, 689 premature infants with or without PROM were analyzed for infection and its risk factors. In the second part, we analyzed the relationship between infection and the levels of MR-proADM, sTREM-1 and IL-6 in umbilical cord blood in PROM cases.

MATERIALS AND METHODS

Clinical data

From January 2015 to December 2017, there were 689 premature infants treated in the Pediatrics department

of the Affiliated Hospital of Yanbian University, China. These included 385 cases of PPRM, of which 151 cases had combined infection and 234 cases had unincorporated infection. A retrospective analysis was conducted on this sample set.

A cohort of 50 singleton premature births with PROM treated in our department from September 2016 to November 2017 was selected as the observation group. They were divided into infection (n=16) and non-infection (n=34) groups. An additional 50 cases of single birth, normal full-term delivery with no complications in the same period were chosen carefully as the control group. The study received ethics approval from the Yanbian University and followed the principles outlined in the Declaration of Helsinki. Ethical agreements were obtained from the participants or their relatives by written informed consent.

Standard for neonates with PPRM

Infants were classified as PPRM with infection based on previously proposed diagnostic criteria (7, 8). The first set of criteria involved the clinical and laboratory diagnosis of navel infection, pneumonia, or septicemia after birth of the child. The clinical manifestations included fever or hypothermia, low appetite, decreased crying, decreased movement, poor facial appearance, cold limbs, no increase in weight or slow growth, jaundice, shock, pustules, abdominal distention, vomiting, diarrhea, cyanosis, irregular breathing or apnea, somnolence, irritation, convulsion, fontanelle tension, and increased muscle tension in the extremities. The laboratory criteria included white blood cell (WBC) count reduction ($<5 \times 10^9$ cells/L) or WBC increase (≤ 3 d, WBC $>2510^9$ cells/L; >3 d, WBC $>20 \times 10^9$ cells/L), WBC classification as immature/total neutrophils (I/T) ≥ 0.16 , CRP ≥ 8 mg/mL peripheral blood, and platelet count $\leq 10 \times 10^{10}$ cells/L.

Alternatively, pregnant mothers could be diagnosed with intrauterine infection if they showed three or more of the following: body temperature $\geq 38^\circ\text{C}$, pulse ≥ 100 beats/min, fetal heart rate >160 or <120 beats/min, WBC $>15 \times 10^9$ cells/L or nucleus left shift, CRP level increase, odor in amniotic fluid, and tenderness of uterus.

Collection and treatment of blood specimens

After the newborns were delivered, 5 mL of the umbilical vein blood was extracted, centrifuged at 3000 rpm for 10 minutes, and then the serum was stored at -80°C until

further use. Umbilical cord blood concentrations of MR-proADM and sTREM-1 were determined using ELISA, and IL-6 concentration was determined by flow cytometry.

Statistical analysis

The SPSS17 software package (SPSS, Inc., Chicago, IL) was used for all the statistical analyses.

RESULTS

Analysis of risk factors for PPRM

Of the 689 preterm infants, there were 385 cases of PPRM (55.89%), of which 87.81% (605/689) were middle and late preterm infants (32-36 weeks), and 12.19% (84/689) were early preterm infants (28-31 weeks). PPRM was observed in the observation group, but there was no early rupture in the control group. The rate of pregnancy and primipara, the

observation group were significantly higher than those of the control group, and the difference was statistically significant ($P < 0.05$). In contrast to the control group, the birth weight was higher and the gestational age was lower in the observation group. Statistically significant differences are indicated (see Table I).

Logistic regression analysis was presented on the risk factors of the observation group and infection was identified as the main risk factor for PROM, with OR = 1.679 and positive correlation. However, hypertension and placental abnormality were identified as the main risk factors for PPRM (Table II).

Comparison of sTREM-1, MR-proADM and IL-6 levels in umbilical cord blood between PPRM and normal group

There was no obvious difference in the levels of sTREM-1 and MR-proADM between the PPRM and full-term group ($P > 0.05$). The difference of IL-6

Table I. Comparison of risk factors for PPRM

Factor	Observation group n (%)	Control group n (%)	χ^2 (t/Z)	P
Gender (male/female)	215/170	165/139	0.169	0.681
Pregnant mothers (Chao/Han)	287/85	215/73	0.556	0.456
Primary maternal (primary/suffered)	272/113	186/118	6.829	0.009
Hypertension in pregnancy	23 (5.97)	75 (24.67)	48.673	0.001
Gestational diabetes	42 (10.91)	47 (15.46)	3.128	0.077
Pregnancy infection	61 (15.84)	27 (8.88)	7.329	0.007
Umbilical abnormality	11 (2.86)	4 (1.32)		0.169
Placental abnormalities	8 (2.08)	29 (9.54)	18.611	0.001
Others (anemia, hyperthyroidism, hypothyroidism)	25 (6.49)	22 (7.24)	0.148	0.701
Twins	72 (18.70)	65 (21.38)	0.766	0.381
Unknown reason	186 (48.31)	88 (28.95)	26.593	0.001
Gestational age $\bar{X} \pm S$	34.44 $\pm$ 3.74	34.27 $\pm$ 4.70	1.027	0.305
Weight $\bar{X} \pm S$	2.38 $\pm$ 0.39	2.26 $\pm$ 0.45	2.416	0.016
Mother's age (25–75 years)	29 (27–34)	31 (28–35)	–3.248	0.001
Pregnancy times $\bar{X} \pm S$	2.07 $\pm$ 1.573	2.35 $\pm$ 1.732	2.757	0.006

levels was statistically significant ($P < 0.05$), as shown in Table III.

Comparison between PPRM co-infected and non-infected group

In the PPRM group, 16 infection cases of premature infants and 34 cases were not infected. The concentrations of sTREM-1, MR-proADM, and IL-6 in the PPRM infection group were statistically higher than in the uninfected group (Table IV, Figure 1 and Figure 2).

DISCUSSION

In recent years, with environmental changes and increases in maternal age and multiple gestations,

there is a steadily increasing incidence of preterm birth. The occurrence of preterm birth directly affects general health in China, and the rate of preterm delivery is closely related to the economy, health, and civilization of a country and region (9). The immune system is less developed in preterm infants than in full-term infants, and they are susceptible to infection, and severe cases can develop septicemia. Early diagnosis of neonatal infection is challenging, and the prognosis may be poor with delayed treatment. This study demonstrated that sTREM-1 and MR-proADM levels may be important tools to identify early infection in these fragile patients.

PPROM, preterm premature rupture of membranes. When this rupture occurs before 37 weeks gestation it is named PPRM (10). PROM is the major factor of

Table II. Binary logistic regression analysis of PPRM risk factors

Factor	B	Wals	OR (95% ci)	P
Pregnancy times	-0.069	0.863	0.934 (0.808~1.079)	0.353
Weight	0.184	1.873	1.202 (0.923~1.566)	0.171
Age	-0.018	0.935	0.982 (0.946~1.019)	0.333
Hypertension	-1.555	35.084	0.211 (0.126~0.353)	0.000
Pregnancy infection	0.529	4.213	1.697 (1.024~2.810)	0.040
Placental abnormalities	-1.633	15.368	0.195 (0.086~0.442)	0.000
Primary maternal (primary/suffered)	-0.233	2.678	0.792 (0.543~1.156)	0.227

OR: odds ratio; PPRM: premature rupture of fetal membrane

Table III. Comparison of sTREM-1, MR-proADM, IL-6 content (pg/mL) in cord blood M (p25-p75)

Group	N	sTREM-1	proADM	IL-6
PPROM	50	28.36 (16.52-48.50)	42.39 (28.06-89.06)	5.61 (1.48-24.82)
Full term	50	20.00 (13.66-33.89)	36.17 (18.50-68.03)	1.48 (0.00-8.90)
Z		1.455	1.231	-2.314
P			0.218	0.021

IL-6: interleukin 6; MR-proADM: proadrenomedullin; sTREM-1: soluble triggering receptor expressed on myeloid cells-1

preterm labor, comprising 30–40% of all cases, and the incidence is rising every year (11). In this study, 55.89% (385/689) of preterm infants in the hospital were preterm due to PROM, which was higher than the rate reported in global literature.

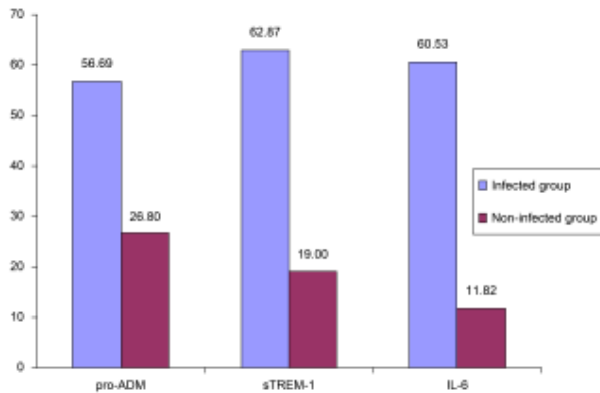


Fig. 1. Comparison of pro-ADM, sTREM-1 and IL-6 levels (pg/ml) in umbilical blood between infected group and non-infected group Comparison of IL-6 content in umbilical blood.

The pathogenesis of PROM is complicated. However, infection has been found to be the main reason for the occurrence of PROM, and there is a mutual cause and effect relationship between infection and PROM. Reproductive tract infection is the main route of infection and testing of cervical and vaginal secretions of pregnant women has shown that the incidence of premature delivery is increased by genital tract infection (12,13). Similar results were found in this study. Regression analysis of PROM susceptibility factors confirmed that infection was the most contributing risk factor for the occurrence of PROM, suggesting that the greater the infection, the greater the risk of PROM. A plausible mechanism could be that upon infection by pathogens, the body produces various enzymes, which degrade the collagen in fetal membranes, leading to PROM. Simultaneously, the pathogen stimulates the human body to secrete more probiotic bioactive substances,

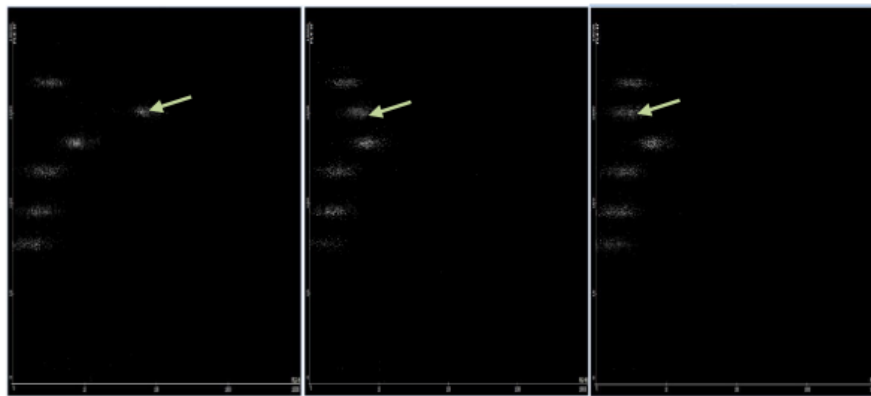


Fig. 2. Note: ← indicates the concentration of IL-6.

Table IV. Comparison of sTREM-1, MR-proADM, IL-6 content (pg/mL) in cord blood M (p25-p75).

Group	N	sTREM-1	proADM	IL-6
Infection	16	53.09 (32.59-88.30)	48.78 (16.53-82.56)	6.14 (2.79-88.78)
Non-infection	34	18.36 (11.14-23.16)	24.89 (9.50-37.94)	3.33 (0-11.08)
Z		-4.347	-2.452	-2.087
P		0.000	0.014	0.037

IL-6: interleukin 6; MR-proADM: proadrenomedullin; sTREM-1: soluble triggering receptor expressed on myeloid cells-1

causing uterine contractions leading to PROM. In addition, the invasion of pathogenic organisms can cause an imbalance of immune regulation in the uterus of the puerpera and decrease immune functions (14-16). One survey showed that neonatal infection increased twofold when the mother had chorioamnionitis (17).

In this study, the gestational age of the PPRM with infection group was distinctly lower than that of the uninfected group and was directly proportional to the infection degree. However, there was no discrepancy between the two groups in the duration of PROM, which is contrary to many reports. These discrepancies could be because of two reasons. First, when PROM exceeds 12 h, prophylactic antibiotics are administered to inhibit bacterial infection in the parturient and the premature infant. Secondly, the small sample size could have affected our observations and therefore larger studies need to be conducted. Our results also showed that hypertension in pregnancy may be a high-risk factor for PPRM and infection. However, owing to the small sample size, it is unclear whether this relationship is significant. This further emphasizes the need for larger cohort studies.

C-reactive protein (CRP) is an acute-phase reactant synthesized primarily by hepatocytes that is widely used in clinical practice as an indicator of infection. It is present in an extremely low level in normal human body fluids. The concentration of CRP begins to rise within 4-6 h of infection and reaches its peak at 36-50 h post-infection. When inflammation is effectively controlled, CRP levels are quickly restored to normal. However, CRP does not rise or rises insignificantly in early infection in preterm infants due to their incomplete liver function development. Therefore, CRP levels are of limited diagnostic use in early infection in these patients. CRP levels do show an upward trend in neonatal infections, suggesting a certain accuracy, but low sensitivity of diagnosis (18). Lai et al. reported that the sera of most infected newborns have less than 3 mg/L CRP, which is also the typical level in low birth weight or premature infants; therefore, the low level of CRP cannot exclude infection (19). CRP levels also have no statistical significance in predicting neonatal adverse outcomes (20, 21).

In recent years, the value of sTREM-1 about diagnosis in adults and children has been reported, but its significance in neonatal infection needs further investigation. TREM-1 is a member of the immunoglobulin superfamily receptors expressed on the surface of neutrophils and other cells (22). sTREM-1 is the product of hydrolysis and abscission of TREM-1, after ripening on the membrane surface (23). It increases rapidly within 48 h after infection. First, sTREM-1 selectively binds with extracellular ligands and promotes the increase of inflammatory factors under the stimulation of various pathogens (24). Second, it induces neutrophil degranulation, respiratory burst, and phagocytosis, and prolongs the lifetime of neutrophils (22). Finally, sTREM-1 promotes the production of monocyte chemoattractant protein-1 to enhance adhesion on the surface of the cell and accelerate chemotaxis of neutrophils and mononuclear cells to the inflammatory site (25).

Additionally, a study of septic and non-septic patients showed that the serum levels of both PCT and sTREM-1 could predict the degree and prognosis of sepsis, and the sensitivity and specificity were higher than those of other inflammatory factors (26). Thus, sTREM-1 was identified as a promising indicator of sepsis. Analysis of the serum of 85 children with sepsis showed that early detection of sTREM-1 was beneficial to the diagnosis and provided a reference value for clinical treatment (27). Dolin et al. also confirmed that sTREM-1 is an effective early indicator for the diagnosis of sepsis (28), and Cordeiro et al. reported that sTREM-1 could also predict the occurrence of premature birth and the outcome of the newborn by testing the umbilical cord blood of 926 newborns (29). In our experimental group of 50 premature infants, the level of sTREM-1 in the umbilical cord blood of the 16 infected children was significantly higher than in the 34 non-infected children. This result suggests that sTREM-1 levels could increase in early infection and could be used to evaluate neonatal infection and improve the prognosis.

Adrenomedullin is mainly derived from the adrenal medulla and pheochromocytoma tissue (30). Since ADM is cleared rapidly from circulation, the more stable MR-proADM molecules, which can directly reflect ADM levels, are measured. MR-proADM in

serum has extensive biological effects and is primarily concentrated in the vascular endothelial cells, smooth muscle cells, central nervous system, and blood of peripheral tissues. In the presence of pathogens, MR-proADM can have a bactericidal effect by increasing the complement activity. Simultaneously, the inflammatory factor also stimulates MR-proADM secretion, which makes the level of the biomarker significantly higher (31, 32).

Several studies have shown the diagnostic value of MR-proADM in children with pneumonia, but little has been done in the study of neonatal infection. Viasus et al. extensively studied literature analyzing several biomarkers in a total of 10,319 patients and found that mortality prediction as the area under the receiver operating characteristic curve was highest for adrenomedullin (33). MR-proADM can be elevated early in the sera of infected patients and is an independent predictor of infection (34). Li et al. studied 82 patients with systemic inflammatory response syndrome and showed that the elevation of serum MR-proADM could be an effective indicator for the early diagnosis and evaluation of the severity of the disease, as well as a warning sign for the prognosis of infection (35). In our study, similar results were obtained in the umbilical cord blood serum of infected newborns. The MR-proADM levels were increased significantly, suggesting that MR-proADM could be used for the early prediction of neonatal infection.

The diagnostic value of interleukin-6 (IL-6) has been well recognized in infection. It is produced rapidly in the acute inflammatory response and has a strong inflammatory effect (36). The level of serum IL-6 has been reported to predict infection and prognosis (37). This study showed that the level of IL-6 in the umbilical cord blood of the PPRM with infection group increased significantly, which suggests that IL-6 has diagnostic value for neonatal infection. However, there were also differences in the IL-6 levels in the umbilical cord blood of the PPRM group and the full-term group, suggesting a relationship between the IL-6 level and the stability of the membranes, and the specificity of the evaluation of infection with IL-6 levels was not as high as that of MR-proADM and sTREM-1 (38). In addition, most of the subjects in our study were middle and late

preterm infants. Therefore, the main cause of PROM may have been factors other than infection, and the mechanism is to be further explored.

Premature labor can be divided into spontaneous premature labor and therapeutic premature delivery. About 70% of premature birth is spontaneous preterm birth (sPTB) and 50% of sPTB corresponds to PROM (39). In addition, sPTB accounts for 23% of global PTB (40). Chorioamnionitis and intrauterine infection can lead to PROM, which occurs in more than 80% of Central Asian women with such infections (41). Subclinical chorioamnionitis usually has no typical clinical manifestations and can only be identified by placental pathology. Intrauterine subclinical infection can cause inflammatory cell exudation, edema of fetal membranes, increased brittleness, and PROM. At the same time, the uterus and vaginal environment of the pregnant woman can provide favorable conditions for bacterial growth after PROM (42). In this study, close to half of the infection group (47.02%, 71/151) of premature infants were infected with unknown causes. Although the pregnant mothers did not have clinical manifestations of infection, placental examination revealed amniotic changes in the preterm infants with subclinical infection. In this case, the fetus is infected in the uterine environment by consistent exposure to contaminated amniotic fluid, and when the fetus passes the birth canal, the pathogen can invade the blood circulation of the newborn, causing infection in the newborn. These results suggest that subclinical chorioamnionitis can increase the incidence of infection (43, 44) and mortality in newborns, and newborn mortality increases by four times if clinical chorioamnionitis develops (36). Therefore, it is suggested that routine placental pathological examination of PPRM infants be carried out in obstetrics. Based on the findings for parturients with subclinical chorioamnionitis and strong evidence for early premature infant infection, a rational guide for the clinical use of drugs to reduce mortality and improve infant prognosis should be provided.

Although infection is the greatest risk factor for PROM, several other factors require consideration. Risk factors such as environmental pollution and smoking are widespread. Smoking or passive smoking can lead to increased oxidative stress, decreased

antioxidant capacity, and DNA damage, leading to PPRM (45,46). Furthermore, a retrospective analysis showed that the onset of sPTB is seasonal, occurring especially in the summer (47). Therefore, the causes of PPRM may be related to behavioral (smoking, drug abuse), environmental (toxin exposure), and genetic factors, and it is necessary to further unravel the underlying mechanisms to reduce the occurrence of preterm labor.

This study confirms that maternal infection is the greatest risk factor for PROM. In PROM neonates, gestational age may also be a risk factor for infection. A key finding is that sTREM-1 and MR-proADM may be valuable predictors for the diagnosis of PPRM and combined infection. Future studies should expand the sample size and assess other indicators for the diagnosis of clinical infection. It is also necessary to explore the correlation between PPRM and environmental and genetic factors to take effective preventive measures to improve the outcomes of both mother and child.

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Hsa_circ_0005075 promotes the proliferation, migration, and invasion of colorectal cancer cells via miR-431/DDX5 axis

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Circular RNAs (circRNAs) serve as important regulators in cancer biology. The goal of the present research is to investigate the biological functions and mechanism of circ_0005075 in colorectal cancer (CRC). The expressions of circ_0005075, miR-431, and DDX5 mRNA were quantified in CRC tissues and cell lines by quantitative real-time polymerase chain reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay, Transwell assay, and flow cytometry were conducted for assessing CRC cell proliferation, migration, invasion, cell cycle distribution, and apoptosis. Bioinformatics analysis and luciferase reporter assay were used for identifying the binding site between circ_0005075 and miR-431. Western blotting was employed for the quantification of DDX5, E-cadherin, N-cadherin, and Vimentin protein expressions. The upregulation of circ_0005075 expression and the down-regulation of miR-431 expression were observed in CRC tissues and cell lines. Knockdown of circ_0005075 triggered the inhibition of proliferation, migration, and invasion of CRC cells, induced cell cycle arrest in G0/G1 phase, and promoted cell apoptosis. Circ_0005075 could function as a molecular sponge for miR-431. In addition, circ_0005075 could repress miR-431 expression to up-regulate DDX5 expression in CRC cells. Circ_0005075, regulating miR-431/DDX5 pathway, promotes CRC progression.

Colorectal cancer (CRC), one of the most common malignancies, currently is a leading cause of cancer-related deaths worldwide (1). According to the statistics, globally, there were approximately 1.8 million newly diagnosed CRC cases in 2018 (2). The average 5-year disease-free survival rate of CRC patients is only around 50%, and the prognosis of the patients with recurrence and distant metastases is more unfavorable (3, 4). There is a necessity for deciphering the molecular mechanism of CRC to

develop novel therapeutic strategies.

Circular RNAs (circRNAs), a subcategory of non-coding RNA (ncRNA) molecules, covalently formed by the 3' end polyadenylation and 5' end cap structures via back-splicing, is widely expressed in eukaryotic cells, with structural stability, sequence conservation, and cell/tissue specificity (5). CircRNA plays an important role in cell biology by acting as a sponge of microRNA (miRNA), regulator of RNA binding protein, transcription regulator, or

Key words: Colorectal cancer; circ_0005075; miR-431; DDX5

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protein translation template (6-8). Some circRNAs function as an oncogenic factor or tumor suppressor (9, 10). A previous study reported that circ_0005075 features prominently in the progression of cancers: circ_0005075 expedites the progression of hepatocellular carcinoma via the modulation of MAPK1 expression by sponging miR-335 (11). Another study reported that, circ_0005075, whose expression is up-regulated in CRC tissues and cells, is associated with the unfavorable prognosis of the patients; functionally, circ_0005075 facilitates the progression of CRC by the activation of the Wnt/ β -catenin pathway (12). Nonetheless, the explicit role and mechanism regarding circ_0005075 in CRC warrant in-depth exploration.

MicroRNA (miRNA, miR) binds to the 3'-untranslated region (3'UTR) of target mRNA and serves as a negative regulator of gene expression (13, 14). The reduction of miR-431 expression is reported in CRC; miR-431 suppresses the invasion of CRC cells by repressing CUL4B expression (15). In this work, we report that circ_0005075 adsorbs miR-431 and represses miR-431 expression in CRC cells, and in turn up-regulates DEAD-box helicase 5 (DDX5) expression, a previously reported oncogene in CRC (16). This study depicts a novel competitive endogenous RNA (ceRNA) network involved in CRC progression, which consists of circ_0005075, miR-431, and DDX5.

MATERIALS AND METHODS

Clinical samples

Forty-five CRC patients receiving surgical resection in Zhejiang Cancer Hospital were enrolled in this research. There were 29 males and 16 females aged 52.6 ± 5.3 years. None of the patients had received neoadjuvant therapy prior to surgery. All the tissues samples were obtained during surgery, after which the diagnosis was confirmed by pathological examination. CRC tissues and corresponding adjacent tissues were obtained and then stored at -80°C until RNA extraction. This study was supported by the Research Ethics Committee of Zhejiang Cancer Hospital. Written informed consent was obtained from all patients.

Cell cultivation and transfection

Human CRC cell lines (HCT116, HT-29, SW480,

and SW620) and the normal colonic epithelium cell line (FHC) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37°C in 5% CO_2 .

Circ_0005075 overexpression plasmid, pcDNA3.1 empty vector, small interfering RNA (siRNA) against circ_0005075 (si-circ_0005075) or negative control (si-NC), miR-431 mimics (miR-431), miR-431 inhibitors (anti-miR-431), and their negative controls (miR-NC and anti-miR-NC) were purchased from RiboBio (Guangzhou, China). All oligonucleotides or plasmid vectors were transiently transfected into HCT116 and SW480 cells employing Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

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

The extraction of total RNA from tissues and cells was conducted utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed employing the PrimeScriptTM RTKit with gDNA Eraser (TaKaRa, Dalian, China). Subsequently, qRT-PCR was carried out employing SYBR[®]Premix-Ex-TaqTM II kit (Takara, Tokyo, Japan) on an ABI7500 FAST RT-PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The primers for circ_0005075, miR-431, DDX5, GAPDH, and U6 were shown as follows: circ_0005075 (Forward, 5'-CAAATCTTGCGGCAACGC-3'; Reverse, 5'-GCGGGAGTGAAGATTCGA-3'); miR-431 (5'-CAGGCCGTCATGCAAA-3'); DDX5 (Forward, 5'-GCCGGGACCGAGGGTTTGGT-3'; Reverse, 5'-CTTGTGCTGTGCGCCTAGCCA-3'); GAPDH, (Forward, 5'-ATTCCATGGCACCCTCAAGGCTGA-3'; Reverse, 5'-TTCTCCATGGTGGTGAAGACGCCA-3'); U6, (Forward, 5'-CTCGCTTCGGCAGCACATATACT-3'; Reverse, 5'-CGCTTCACGAATTTGCGTGT-3'). The calculation of relative expressions of the genes was performed employing $2^{-\Delta\Delta\text{Ct}}$ method.

Subcellular fractionation and localization

A cytoplasmic and nuclear RNA Purification Kit (Amyjet, Wuhan, China) was used to isolate and extract cytoplasmic and nuclear RNA from HCT116 and SW480 cells. Then, the expression of circ_0005075 in the

cytoplasm and nucleus of HCT116 and SW480 cells was detected by qRT-PCR. GAPDH and U6 were used as cytoplasmic and nuclear controls, respectively.

Cell counting kit-8 (CCK-8) assay

CRC cells were transferred in 96-well plates (1×10^3 cells per well). The cells were cultured for 12, 24, 48, 72, and 96 h, respectively, before being incubated with 10 μ L of CCK-8 solution (Dojindo, Tokyo, Japan) at 37°C for 4 h. Then, the optical density of the cells was recorded at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

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

The DNA synthesis in proliferating cells was determined by BrdU assay. To be specific, the cells were transferred into a 96-well plate at a density of 2×10^3 cells/well, followed by 48 h of culture. Then, the cells were incubated with BrdU solution (10 μ M, BD Pharmingen, San Diego, CA, USA) for 4 h. Subsequently, the culture solution was discarded, and these cells were fixed and then incubated with anti-BrdU antibody (Beyotime, Shanghai, China) for 1 h at room temperature. After that, the cells were rinsed with PBS and then incubated with Hoechst staining solution (Beyotime, Shanghai, China). After rinsing with PBS again, the cells were observed under a fluorescence microscope.

Transwell assay

Transwell inserts (Costar, Cambridge, MA, USA) were used in Transwell assay. Matrigel (30 μ g/well, BD, San Jose, CA, USA) was used in the invasion assay but not in the migration assay. For each well, CRC cells (5×10^4) suspended in serum-free DMEM were seeded into the top compartment, and 500 μ L of DMEM containing 10% FBS was added to the lower compartment. After the cells were cultured for 24 h, the non-migrating or non-invading cells on the upper side of the membrane were scraped off using cotton swabs. The migrated/invaded cells were fixed in 95% methanol for 10 min and then stained with 0.1% crystal violet solution for 5 min. Finally, the number of cells was counted and then averaged from at least five random fields of view under a microscope.

Flow cytometry

Flow cytometry was used to analyze cell cycle and apoptosis. In cell cycle analysis, HCT116 and SW480

cells (1×10^6 cells per sample) were fixed with 70% ethanol overnight at 4°C. Then, the cells were rinsed twice with cold PBS, incubated with 100 μ L of RNase solution for 30 min, stained with 400 μ L of propidium iodide (PI) staining solution (BD Biosciences, San Jose, CA, USA) in the dark at 4°C for 30 min. Cell cycle analysis was performed on FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA), and BD Flowjo™ software (Becton Dickinson, Franklin Lakes, NJ, USA) was used for data analysis. For the detection of apoptosis, HCT116 and SW480 cells were harvested (5×10^5 cells per sample), centrifuged, and resuspended in 100 μ L of binding buffer. After that, the cells were stained with 5 μ L of AnnexinV-FITC kit (BD Bioscience, San Jose, CA, USA) and 5 μ L PI staining solution for 15 min in the dark. Ultimately, flow cytometry was performed to detect the apoptosis rate, and the data analysis was performed with BD Flowjo™ software.

Dual-luciferase reporter assay

The sequences of circ_0005075 and DDX5 3'-UTR containing putative miR-431 binding sites were amplified and inserted into psiCHECK-2 vector (Promega, Madison, WI, USA) for the generation of wild-type circ_0005075 reporter (circ_0005075-WT) and wild-type DDX5 reporter (DDX5-WT). GeneArt™ Site-Directed Mutagenesis System (Thermo Fisher Scientific, Wilmington, DE, USA) was used for constructing mutated circ_0005075 reporter (circ_0005075-MUT) and mutated DDX5 reporter (DDX5-MUT). Then, the constructed luciferase reporter vectors, miR-431 mimics, or miR-NC were transfected into CRC cells, respectively. Ultimately, the luciferase activity of each group was detected with the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot assay

After the cells were collected, the extraction of proteins was performed utilizing RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentrations were determined by a BCA Protein Assay Kit (Beyotime, Haimen, China). The equal amount of proteins in each group was separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Beyotime, Shanghai, China). After that, the membranes were blocked with

5% nonfat milk for 1 h at room temperature, followed by overnight incubation with the primary antibodies against DDX5 (Abcam, Shanghai, China, ab126730, 1:1000), E-cadherin (Abcam, Shanghai, China, ab40772, 1:1000), N-cadherin (Abcam, Shanghai, China, ab76011, 1:1000), Vimentin (Abcam, Shanghai, China, ab16700, 1:1000), and GAPDH (Abcam, Shanghai, China, ab9485, 1:2000) at 4°C. Next, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam, Shanghai, China, ab6721, 1:5000) at room temperature for 1 h. Lastly, the protein bands were

developed with enhanced chemiluminescent reagent (Biozym, Hessisch Oldendorf, Germany).

Statistical analysis

The data obtained from three independent experiments were processed by SPSS software 23.0 (SPSS Inc., Chicago, IL, USA) and presented as mean $\pm$ standard deviation. The comparison between two or more groups was conducted by Student's *t*-test or one-way ANOVA with Tukey or Dunnett post hoc test. The difference was deemed to be statistically significant when $P < 0.05$.

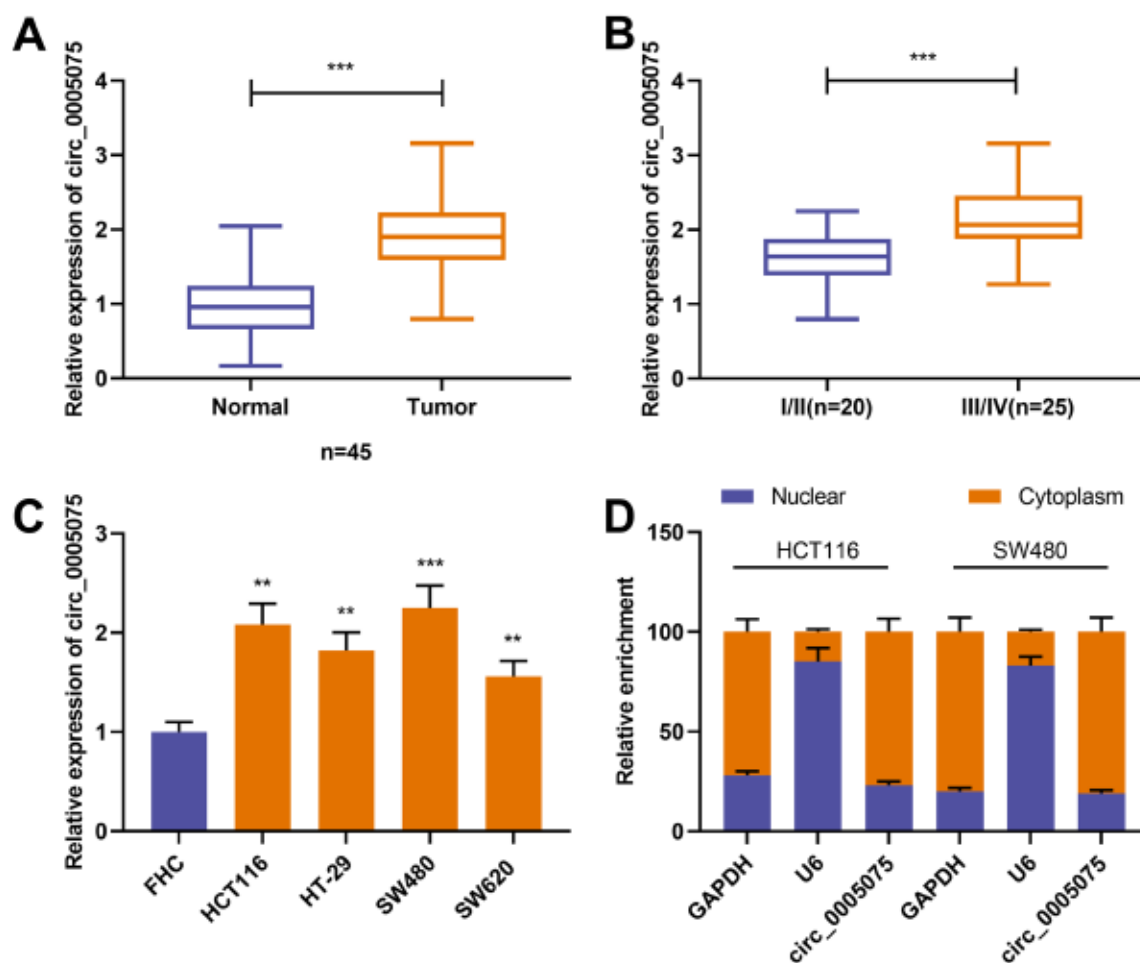


Fig. 1. Circ_0005075 was highly expressed in CRC. **A)** The relative expression of circ_0005075 in CRC tissues (Tumor) and adjacent tissues (Normal) was quantified with qRT-PCR. **B)** qRT-PCR was implemented for assaying the relative expression of circ_0005075 in CRC tissues at different clinical stages (I/II or III/IV). **C)** qRT-PCR was employed for assessing the relative expression of circ_0005075 in CRC cells (HCT116, HT-29, SW480 and SW620) and normal colonic cell line (FHC). **D)** Subcellular fractionation assay was used to measure the expression levels of circ_0005075, U6 and GAPDH in the nucleus and cytoplasm of HCT116 and SW480 cells. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ standard deviation (SD). ** $P < 0.01$ and *** $P < 0.001$ vs The normal group, clinical stage I/II group or FHC cells.

RESULTS

Circ_0005075 was highly expressed in CRC tissues and cells

To pinpoint the expression of *circ_0005075* in CRC, qRT-PCR was performed for the quantification of *circ_0005075* expression in 45 cases of CRC

tissues and corresponding adjacent tissues, which showed a significant augmentation of *circ_0005075* expression in CRC tissues as opposed to that in adjacent tissues (Fig. 1A). Moreover, a higher expression of *circ_0005075* was observed in tumor tissues in higher clinical stage (Fig. 1B). Additionally, compared with FHC cells, a marked up-regulation

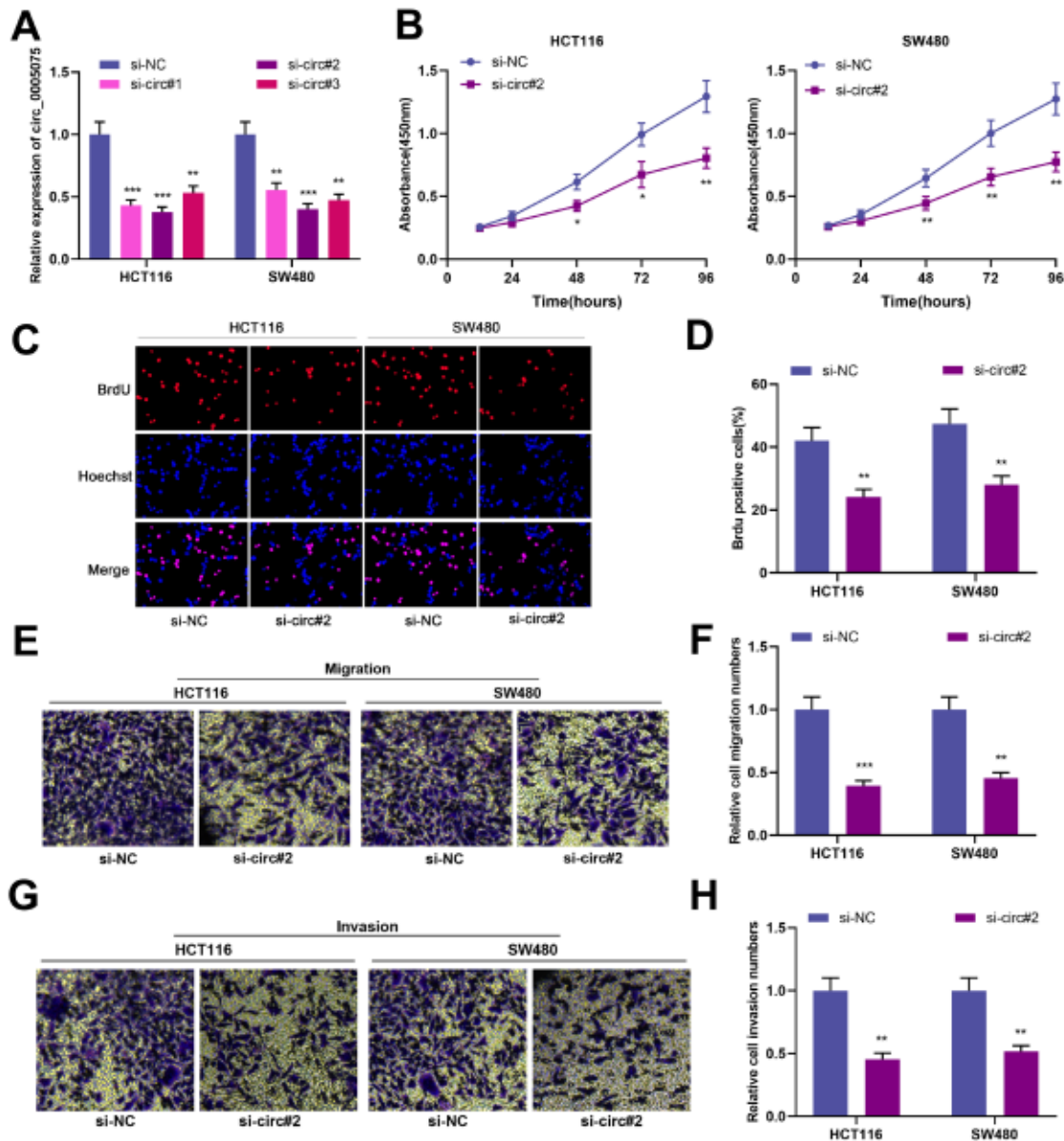


Fig. 2. Knockdown of *circ_0005075* repressed the proliferation, migration, and invasion of CRC cells. **A)** The relative expression of *circ_0005075* in HCT116 and SW480 cells was quantified with qRT-PCR after knockdown of *circ_0005075*. **B-D)** CCK-8 and BrdU assays were employed for monitoring the proliferation of HCT116 and SW480 cells after *circ_0005075* knockdown. **E-H)** Transwell assay was used for examining the migration and invasion of HCT116 and SW480 cells after *circ_0005075* knockdown. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. *P<0.05, **P<0.01, and ***P<0.001 vs The si-NC group.

of circ_0005075 expression was observed in CRC cell lines (Fig. 1C). In addition, the subcellular distribution of circ_0005075 was analyzed and found that circ_0005075 was mainly localized in the cytoplasm of HCT116 and SW480 cells, suggesting it may function as a ceRNA (Fig. 1D).

Knockdown of circ_0005075 repressed the proliferation, migration, and invasion of CRC cells

To investigate the biological function of circ_0005075, cell models of circ_0005075 knockdown were constructed. Circ_0005075 siRNAs were transfected into HCT116 and SW480 cells, respectively, and si-circ#2, with the highest knockdown efficiency, was selected for further experiments (Fig. 2A). CCK-8 and BrdU assays

showed that after circ_0005075 was silenced, the proliferation of CRC cells was significantly suppressed (Fig. 2 B-D). Furthermore, Transwell assay manifested that the migration and invasion of CRC cells in the si-circ#2 group were markedly inhibited (Fig. 2 E-H). Cell cycle analysis demonstrated that the percentage of HCT116 and SW480 cells in the G0/G1 phase was obviously increased in the si-circ#2 group compared with the si-NC group, whereas the percentage of cells in S phase was decreased significantly (Fig. 3 A-C). Additionally, knockdown of circ_0005075 promoted the apoptosis of HCT116 and SW480 cells (Fig. 3 D, E). Furthermore, Western blot showed that knockdown of circ_0005075 promoted the expression level of E-cadherin protein in HCT116

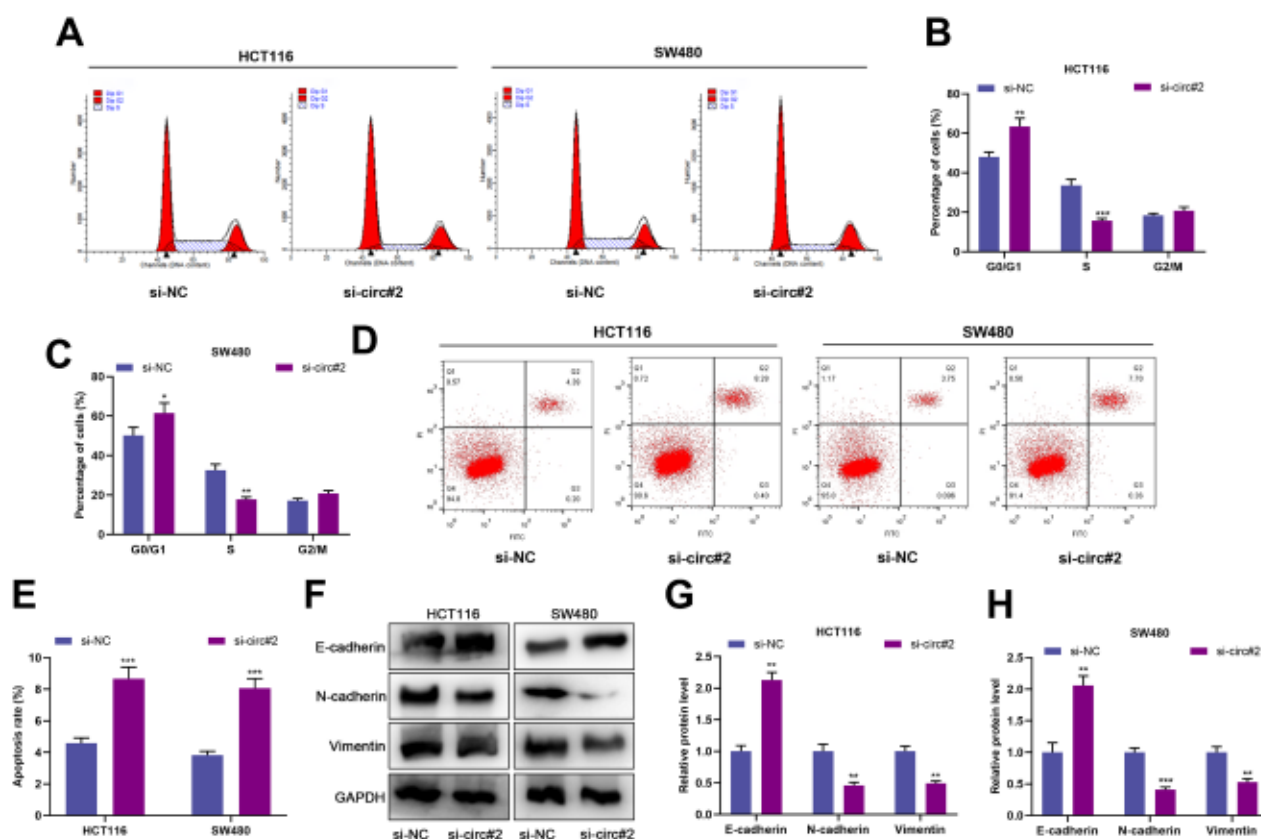


Fig. 3. The effect of knocking down circ_0005075 on cell cycle, apoptosis, and EMT of CRC cells. **A-C)** Flow cytometry was used to detect the effects of circ_0005075 knockdown on cell cycle distribution of HCT116 and SW480 cells. **D-E)** Flow cytometry was used to detect the effects of circ_0005075 knockdown on the apoptosis of HCT116 and SW480 cells. **F-H)** Western blot analysis was used to detect the expression levels of E-cadherin, N-cadherin, and Vimentin in HCT116 and SW480 cells after circ_0005075 was knocked down. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs the si-NC group.

and SW480 cells but reduced the expression levels of N-cadherin and Vimentin protein (Fig. 3 F-H).

Circ_0005075 was capable of repressing miR-431 expression in CRC cells

Next, the binding site between circ_0005075 and miR-431 was predicted by bioinformatics analysis (Fig. 4A). For validating the targeting relationship between circ_0005075 and miR-431, a dual-

luciferase reporter gene experiment was conducted. The results showed that the luciferase activity of wild-type circ_0005075 reporter was repressed by miR-431 mimics, and miR-431 mimics had no significant effect on the luciferase activity of mutant circ_0005075 reporter (Fig. 4B). Additionally, we investigated the expression of miR-431 in CRC. TCGA data showed that miR-431 expression was down-regulated in CRC samples. Furthermore, the

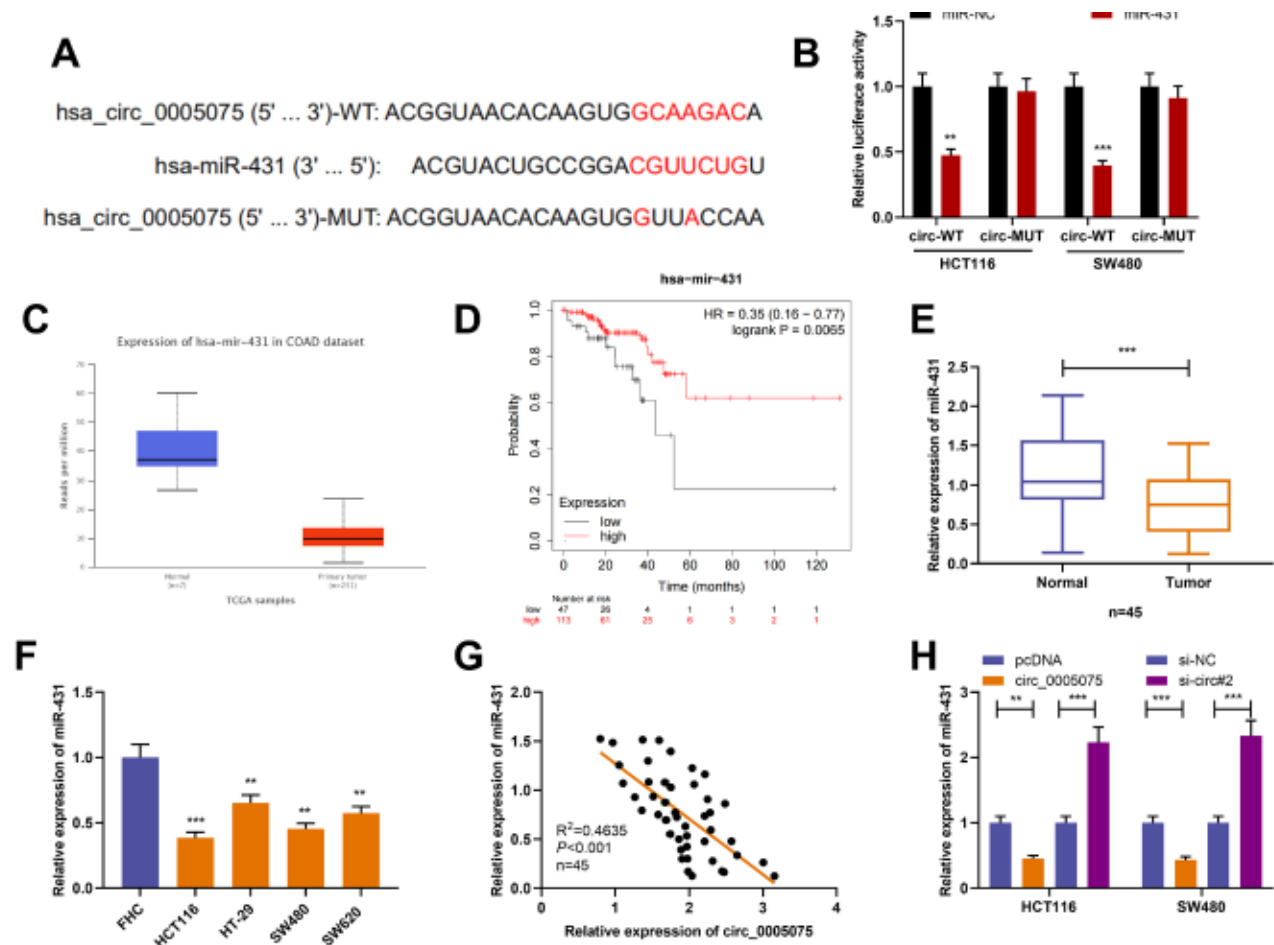


Fig. 4. MiR-431 was the target of circ_0005075. **A)** Circinteractome (<https://circinteractome.nia.nih.gov/>) was utilized for the prediction of the binding sequence between miR-431 and circ_0005075. **B)** Dual-luciferase reporter gene experiment was employed for validating the binding relationship between miR-431 and circ_0005075. **C)** The expression of miR-431 in CRC tissues was analyzed by TCGA database. **D)** Kaplan-Meier Plotter was used to analyze the relationship between miR-431 expression level and overall survival time of CRC patients. **E)** MiR-431 relative expression in CRC tissues (Tumor) and adjacent tissues (Normal) was detected by qRT-PCR. **F)** MiR-431 relative expression in CRC cells (HCT116, HT-29, SW480, and SW620) and the normal human colonic cell line (FHC) was assessed by qRT-PCR. **G)** The correlation between miR-431 and circ_0005075 expressions in CRC tissues was analyzed by Pearson's correlation analysis. **H)** qRT-PCR was utilized for the quantification of miR-431 relative expression in HCT116 and SW480 cells with circ_0005075 knockdown or overexpression. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. ** $P < 0.01$ and *** $P < 0.001$ vs The miR-NC group, normal group, FHC cells, pcDNA group or si-NC group.

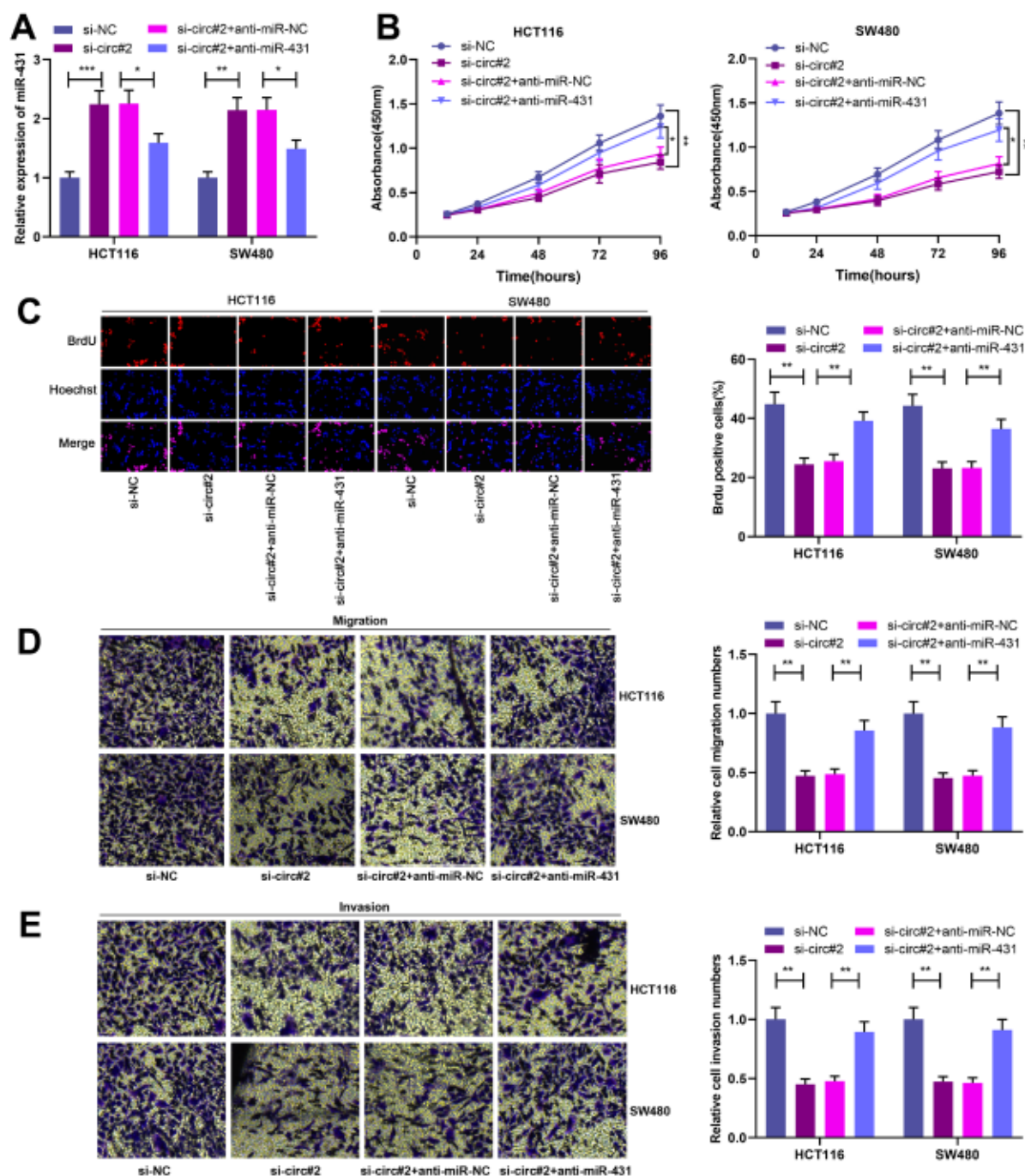


Fig. 5. Circ_0005075 targeted miR-431 to regulate proliferation, migration, and invasion of CRC cells. **A)** The relative expression of miR-431 in HCT116 and SW480 cells transfected with si-NC, si-circ#2, si-circ#2 + anti-miR-NC, si-circ#2 + anti-miR-431 was quantified with qRT-PCR, respectively. **B, C)** CCK-8 and BrdU assays were employed for detecting the proliferation of HCT116 and SW480 cells transfected with si-NC, si-circ#2, si-circ#2+anti-miR-NC and si-circ#2+anti-miR-431. **D, E)** Transwell assay was utilized for monitoring the migration and invasion of HCT116 and SW480 cells transfected with si-NC, si-circ#2, si-circ#2+anti-miR-NC and si-circ#2+anti-miR-431. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. * P <0.05, ** P <0.01, and *** P <0.001 vs the si-NC group or si-circ#2 + anti-miR-NC group.

results of Kaplan-Meier Plotter analysis showed that patients with low miR-431 expression had a shorter overall survival time than those with high miR-431 expression (Fig. 4 C, D). The data of

qRT-PCR indicated a down-regulation of miR-431 expression in CRC tissues and cell lines, compared with that in adjacent tissues and FHC cells (Figs. 4 E, F). Moreover, a negative correlation between

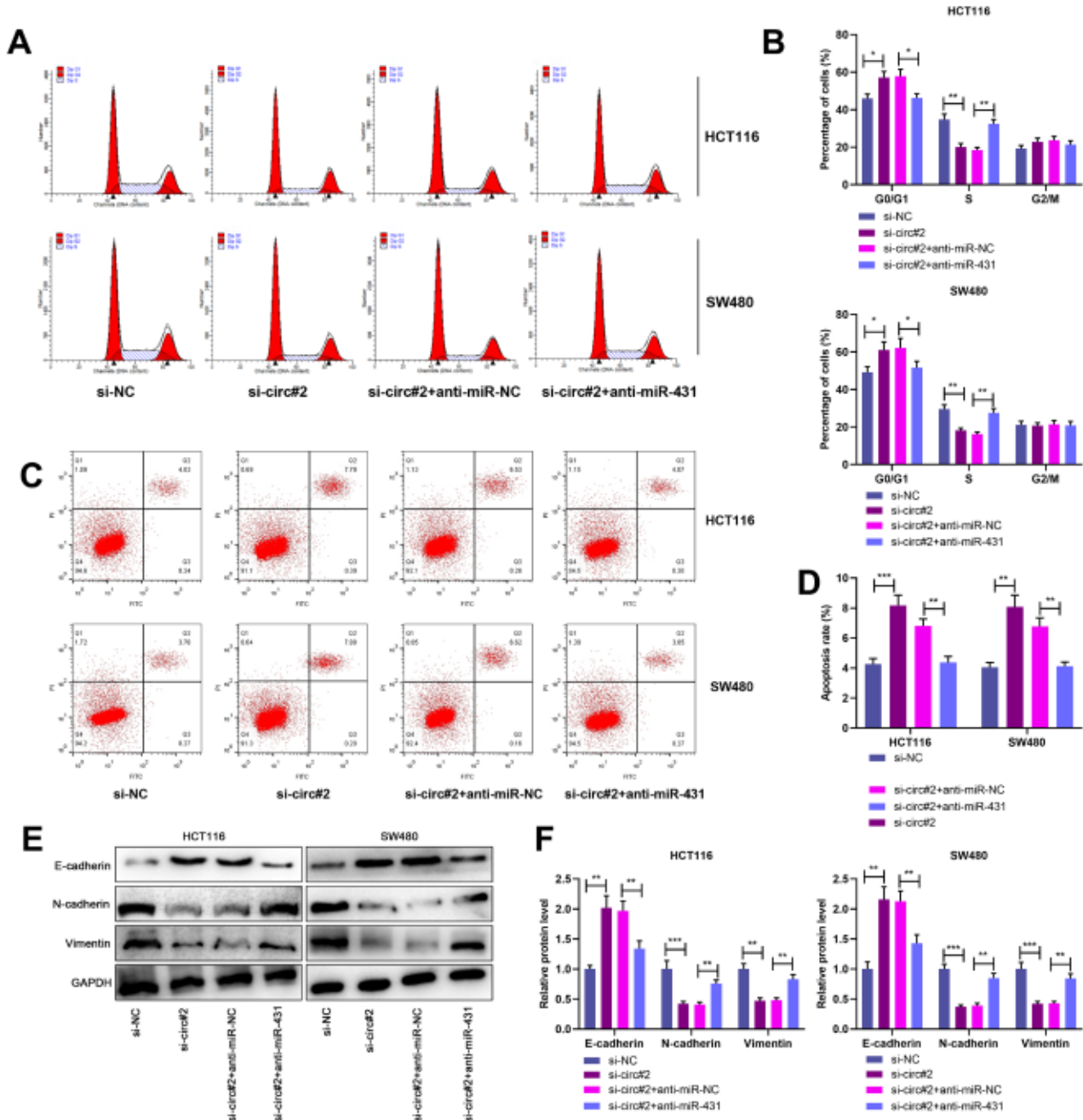


Fig. 6. Circ_0005075 targeted miR-431 to regulate cell cycle, apoptosis, and EMT of CRC cells. si-NC, si-circ#2, si-circ#2+anti-miR-NC, and si-circ#2+anti-miR-431 were transfected into HCT116 and SW480 cells, respectively. Then, the cell cycle distribution (A and B) and apoptosis rate (C and D) of HCT116 and SW480 cells were analyzed by flow cytometry. Western blot analysis was used to detect the expression levels of E-cadherin, N-cadherin, and Vimentin in HCT116 and SW480 cells (E and F). All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. * P <0.05, ** P <0.01, and *** P <0.001 vs the si-NC group or si-circ#2 + anti-miR-NC group.

circ_0005075 and miR-431 expression was demonstrated in CRC tissues (Fig. 4G). Additionally, miR-431 expression was down-regulated in HCT116

and SW480 cells overexpressing circ_0005075, whereas knockdown of circ_0005075 resulted in the up-regulation of miR-431 expression in CRC cells

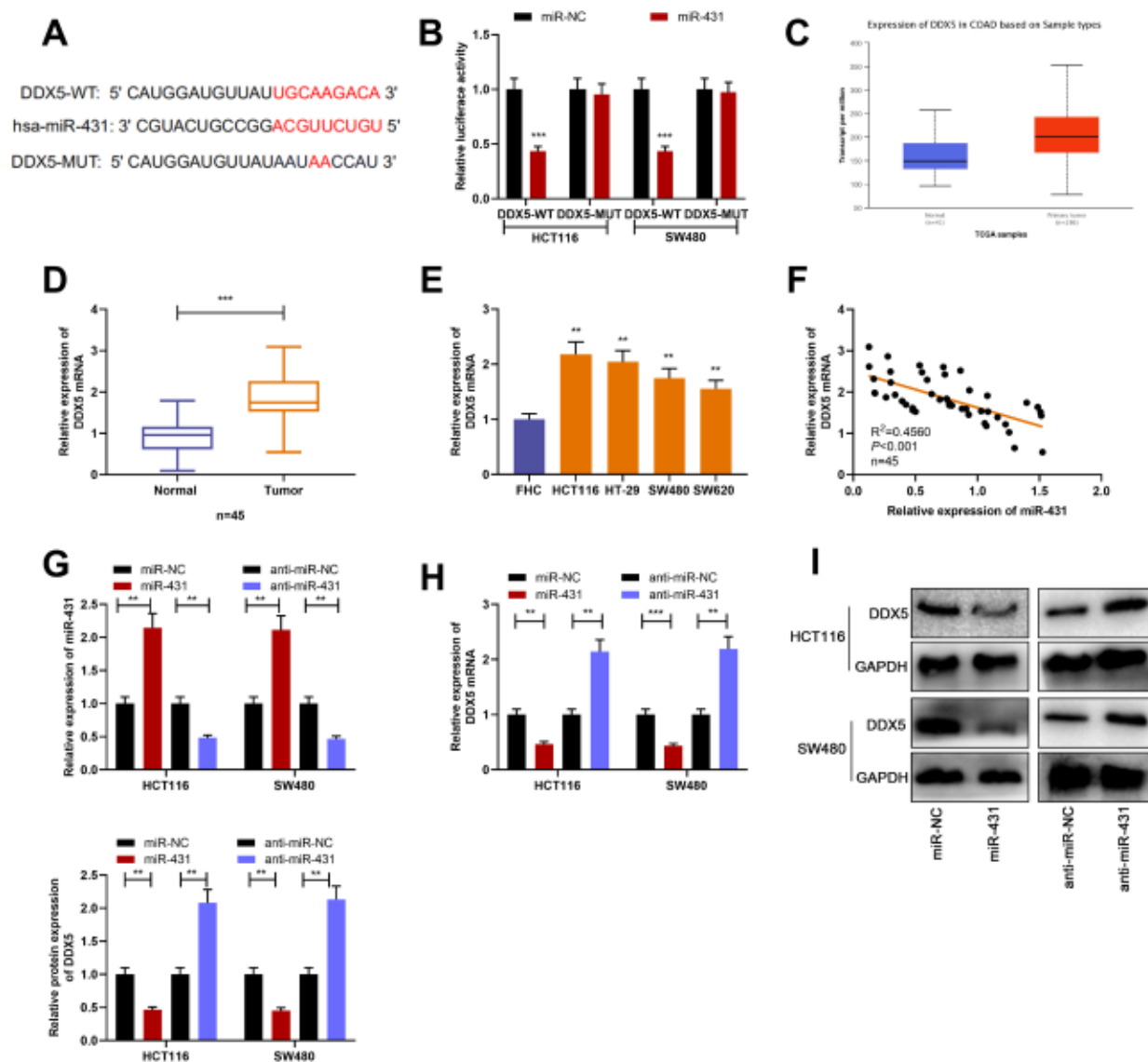


Fig. 7. DDX5 was the target of miR-431. **A)** Bioinformatics analysis was utilized for the prediction of the binding sequence between miR-431 and DDX5 3'UTR. **B)** The binding relationship between miR-431 and DDX5 was verified by dual-luciferase reporter gene experiment. **C)** The expression of DDX5 in CRC tissues was analyzed by TCGA database. **D)** The relative expression of DDX5 mRNA in CRC tissues (Tumor) and adjacent tissues (Normal) was quantified employing qRT-PCR. **E)** The relative expression of DDX5 mRNA in the normal human colonic cell line (FHC) and CRC cells (HCT116, HT-29, SW480, and SW620) was detected by qRT-PCR. **F)** The correlation between DDX5 mRNA and miR-431 expressions in CRC tissues was analyzed by Pearson's correlation analysis. **G)** The relative expression of miR-431 in HCT116 and SW480 cells transfected with miR-NC, miR-431 mimics, anti-miR-NC, or anti-miR-431 inhibitors was detected by qRT-PCR. **H, I)** The relative expressions of DDX5 mRNA and protein in HCT116 and SW480 cells transfected with miR-431 mimic (or miR-NC) and miR-431 inhibitor (or anti-miR-NC) were detected by qRT-PCR and Western blot. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. ** $P<0.01$ and *** $P<0.001$ vs the miR-NC group, normal group, FHC cells or anti-miR-NC group.

(Fig. 4H). These findings suggested that miR-431 was the target miRNA of circ_0005075 in CRC.

Inhibition of miR-431 could partly abrogate the effects of circ_0005075 depletion on CRC cells

Next, HCT116 and SW480 cells were transfected with si-NC, si-circ#2, si-circ#2+anti-miR-NC, and si-circ#2+anti-miR-431, respectively (Fig. 5A). Then, CCK-8 assay, BrdU assay, Transwell assay, and flow cytometry were performed. The results showed that the effects of knockdown of circ_0005075 on CRC cell proliferation, migration, invasion, cell cycle progression, and apoptosis were partly counteracted by miR-431 inhibition (Fig. 5 B-E, Fig. 6 A-D). In addition, Western blot showed that the inhibitory effect of circ_0005075 knockdown on epithelial-mesenchymal transition (EMT) was reversed by the inhibition of miR-431 expression (Fig. 6 E, F).

DDX5 was a target of miR-431

To further decipher the downstream mechanism of miR-431, the potential target genes of miR-431 were predicted via bioinformatics. As shown, a binding site for miR-431 was predicted in the sequence of

DDX5 3'UTR (Fig. 7A). Subsequently, the targeting relationship between miR-431 and DDX5 was verified by dual-luciferase reporter experiment (Fig. 7B). Additionally, the expression of DDX5 in CRC was investigated. TCGA data indicated that DDX5 was highly expressed in CRC tissues (Fig. 7C). The results of qRT-PCR implied the up-regulation of DDX5 mRNA expression in CRC tissues and cell lines (Figs. 7 D, E). Additionally, a negative correlation was observed between the expressions of DDX5 mRNA and miR-431 in CRC tissues (Fig. 7F). Next, miR-431 mimics and inhibitors were transfected into HCT116 and SW480 cells, respectively (Fig. 7G). qRT-PCR and Western blot indicated that overexpression of miR-431 led to the reduction of DDX5 expression whereas the inhibition of miR-431 expression triggered the up-regulation of DDX5 expression in CRC cells (Fig. 7 H, I).

Circ_0005075 promoted DDX5 expression via sponging miR-431 in CRC cells

For determining whether circ_0005075 regulated the expression of DDX5 by targeting miR-431, DDX5 expression was detected in CRC cells transfected with si-NC, si-circ#2, si-circ#2+anti-miR-NC, and

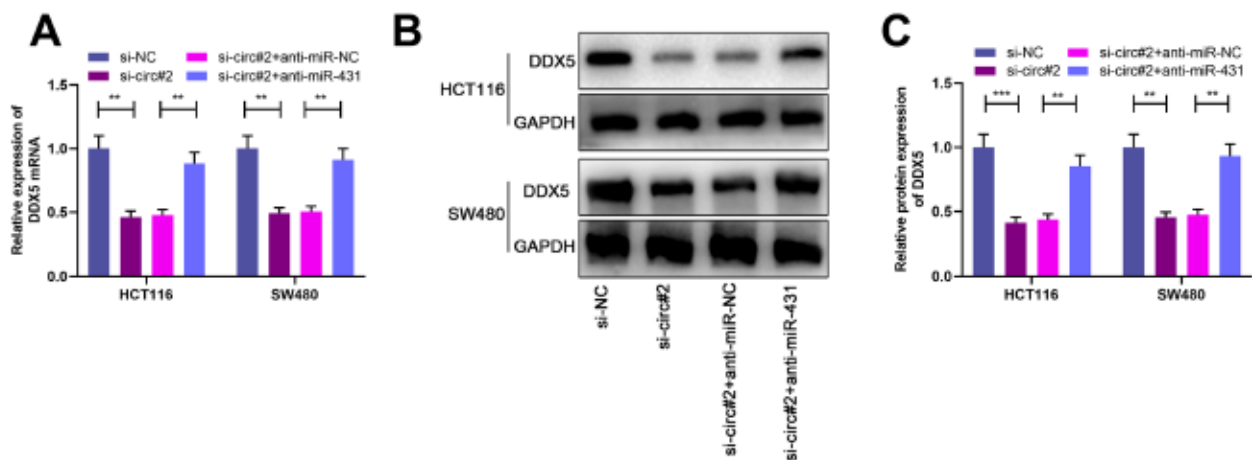


Fig. 8. Circ_0005075 regulated the expression level of DDX5 by regulation of miR-431 expression. **A-C**) The relative expression levels of DDX5 mRNA and protein in HCT116 and SW480 cells transfected with si-NC, si-circ#2, si-circ#2 + anti-miR-NC, si-circ#2 + anti-miR-NC were assessed using qRT-PCR and Western blot, respectively. All of the experiments were performed in triplicate, and the data are shown as mean $\pm$ SD. ** $P < 0.01$ and *** $P < 0.001$ vs the si-NC group or si-circ#2 + anti-miR-NC group.

si-circ#2+anti-miR-431, respectively. qRT-PCR and Western blot indicated that circ_0005075 knockdown repressed DDX5 expression in HCT116 and SW620 cells, while the co-transfection of miR-431 inhibitors could partly eliminate these effects (Fig. 8 A-C). Gene Set Enrichment Analysis (GSEA) revealed that, in the CRC samples with DDX5 high expression, some cancer-related gene sets were enriched, including the gene sets related to the positive regulation of signal transduction by p53 class mediator, regulation of RNA splicing, positive regulation of DNA damage response signal transduction by p53 class mediator, regulation of mRNA splicing via spliceosome, mitotic G2 M transition checkpoint, basal transcription factors, and ubiquitin mediated proteolysis (Fig. 9). These data suggest that circ_0005075 regulate these biological processes in CRC via regulating miR-413/DDX5 axis.

DISCUSSION

Unlike linear RNAs, such as long non-coding RNA (lncRNA) and miRNA, circRNAs are characterized with high stability and tissue specificity (17). CircRNA figures prominently in regulating multiple biological processes (18). The aberrant expression of circRNA is observed in CRC. For example, circZNF609 expression level is reduced in CRC tissues, and circZNF609 induces the apoptosis of CRC cells by

the up-regulation of p53 expression (19); circITGA7 suppresses the growth and metastasis of CRC cells by regulating Ras pathway and up-regulating ITGA7 transcription (20); serum exosomal circ_0004771, whose expression is significantly up-regulated in CRC patients, has the potential to be a novel diagnostic biomarker for CRC (21). It is noteworthy that, some studies have reported that, circ_0005075 is remarkably over-expressed in CRC tissues, potentiating the proliferation and invasion of CRC cells (12, 22). In our current research, significant up-regulation of circ_0005075 expression was observed in CRC tissues and cells. Additionally, functional experiments confirmed the carcinogenic effects of circ_0005075 on promoting CRC cell proliferation, migration, invasion, cell cycle progression, and EMT, and inhibiting cell apoptosis. These data were consistent with the previous reports (12, 22).

Reportedly, circRNAs regulate miRNA expression in the cytoplasm by acting as miRNA sponges (23, 24). To further elaborate on the mechanism of circ_0005075, we performed bioinformatics analysis, and it was predicted that miR-431 could probably be decoyed by circ_0005075. It is reported that miR-431, whose expression is down-regulated in pancreatic cancer and lung cancer, is a tumor-suppressive miRNA (25, 26). Importantly, the tumor-suppressive effect of miR-431 in CRC progression has also previously

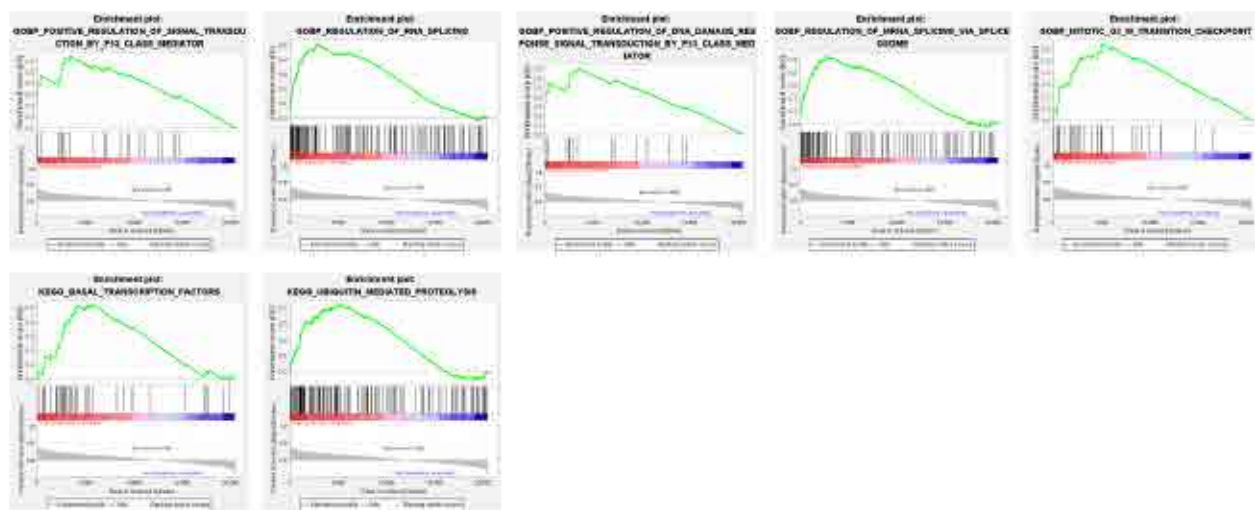


Fig. 9. GSEA was used to analyze the gene sets enriched in CRC samples with high DDX5 expression.

been reported (15). Herein, we provided the evidence that circ_0005075 functioned as a molecular sponge for miR-431 in CRC. In addition, inhibition of miR-431 reversed the inhibitory effects of circ_0005075 knockdown on CRC cell proliferation, migration, invasion, cell cycle progression, and EMT, as well as the promotion of apoptosis. These experimental data verified that circ_0005075 was capable of regulating the progression of CRC, at least partially, via sponging miR-431.

Previous reports have indicated that circRNAs regulate gene expression by directly binding with miRNAs to prevent them from interacting with target mRNA (24, 27, 28). In our study, DDX5 was predicted as the candidate target gene of miR-431 by bioinformatics analysis, which was further verified by a dual-luciferase reporter assay. DDX5, also called RNA helicase p68, belongs to the DEAD box family of RNA helicases subfamily (29). DDX5 is widely expressed in the nucleus and cytoplasm of human cells, regulates RNA metabolism, and partakes in multiple fundamental biological processes, such as cell proliferation and apoptosis, lipogenesis, embryo development (30, 31). In addition, reportedly, overexpression of DDX5 is associated with the detrimental prognosis of CRC patients (16). Functionally, DDX5 upregulates AKT and induces the nuclear exclusion and degradation of tumor suppressor FOXO3a in CRC cells (32). In the present research, a significant up-regulation of DDX5 expression was observed in CRC tissues and cells, supporting that DDX5 exhibited a role of a cancer-promoting factor in CRC. Additionally, we confirmed that DDX5 was the downstream target of miR-431 in CRC cells, and circ_0005075 could positively regulate the expression of DDX5 by competitively binding to miR-431. Based on these experimental data, it was concluded that circ_0005075 could indirectly up-regulate DDX5 expression by targeting miR-431, hence promoting the progression of CRC.

Collectively, this work defines the oncogenic characteristics of circ_0005075 in CRC. We demonstrate that circ_0005075 regulates DDX5 expression to promote CRC progression through adsorbing miR-431. This study is helpful to

expand our current understanding concerning the mechanisms of CRC progression.

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miR-449a inhibits cell viability of human prostate cancer cells by suppressing Notch1 expression and promoting cell autophagy

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Prostate cancer is a commonly seen male cancer in the world. In the present study, we explored the role of Notch1 and how it is regulated in the progression of prostate cancers. Firstly, Notch1 was highly expressed in prostate cancer cell lines PC3 and Du145, compared with normal human prostate cell control cell RWPE-1. Knockdown Notch1 by specific shRNA significantly suppressed cell proliferation of PC3 cells. Has-miR-449a is found to negatively regulate the expression of Notch1 in human prostate cancer cells. Moreover, MiR-449a mimics suppresses cell viability of PC3 cells, and miR-449a inhibitor promotes cell proliferation of PC3 cells. Furthermore, transfection with miR-449a decreased the level of Notch1 and increased the autophagy activity of human prostate cancer cells. In conclusion, miR-449a negatively regulates the expression of Notch1 and inhibits the cell proliferation of human prostate cancer cells partly due to increasing the cell autophagy of PC3 cells.

Prostate cancer is an epithelial malignancy that occurs in the prostate (1). The pathological types of prostate cancers mainly include adenocarcinoma, ductal adenocarcinoma, urothelial carcinoma, squamous cell carcinoma, and adenosquamous carcinoma. Among them, prostate adenocarcinoma accounts for more than 95% (2). The treatment of prostate cancer mainly includes radical surgery, radiotherapy, chemotherapy, and hormonal blockade palliative treatment (3). Many patients have advanced prostate cancer after developing symptoms such as progressive dysuria or hematuria, which has brought great obstacles to treatment (4). Many patients have developed advanced prostate cancer accompanied by the symptoms such as progressive dysuria or hematuria, which has a great

barrier in the clinical therapy of prostate cancers (5). Until now, the biological characteristics of prostate cancer are extremely complex, and the molecular biological basis of its pathogenesis, occurrence, and development is not fully understood. The clarification of molecular mechanisms is helpful for the diagnosis and treatment of human prostate cancer patients.

The Notch signaling pathway is widely present in vertebrates and invertebrates and is highly conservative in evolution. Notch1 is a kind of transmembrane protein, which is involved in the regulation of proliferation and apoptosis of tissues and organs and is closely related to the occurrence and development of tumors. It is expressed in various tumors of the whole body. More and more evidence shows that Notch1 can exert opposite functions in the progression of different

Key words: Notch1, miR-449a, cell autophagy, prostate cancer

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tumors, promoting cancer progression or inhibiting tumor growth. It has been reported that Notch1 is an effective therapeutic target in prostate cancer (6). Knockdown of NOTCH1 in aggressive prostate cancer cells, the cell proliferation, invasion, and tumorsphere formation ability is down-regulated (6). Moreover, the *in vivo* experiment in murine models revealed that Notch1 inhibition suppressed the xenograft growth. However, the molecular mechanisms in the androgen-dependent prostate cancer cell line and the androgen-independent prostate cancer cell line were different (7). CC chemokine receptor 7 (CCR7) promoted the invasion and metastasis of prostate cancer due to the upregulation of Notch1, which accelerated the epithelial-mesenchymal transition (EMT) (8). Deng, G. et al. found that Notch1 inhibition decreased the level of metastasis-associated 1 (MTA1) and increased the expression of KiSS-1 metastasis-suppressor (KiSS-1), mitogen-activated protein kinase 4 (MKK4), and a cluster of differentiation 82 (KAI1), suggesting that Notch1 inhibited the cell migration of prostate cancer cell through the metastasis-associated 1-KiSS-1 metastasis-suppressor pathway (9). Recently, it has been found that microRNA-139-5p inhibited human prostate cancer cell proliferation (10), and micro RNA34a decreased the paclitaxel resistance of prostate cancer cells via direct suppression of Notch1 related pathway (11).

The biological characteristics of prostate cancer are extremely complex, and the molecular biological basis of its pathogenesis, occurrence and development are not fully understood. In the present study, we identified the role of Notch1 in human prostate cancer cells and further explored how it is regulated in the proliferation of human prostate cancer cells. By using Targetscan software, we found that notch1 might be the target of has-miR-449a. Thus, we checked how has-miR-449a regulated cell proliferation by targeting Notch1 in human prostate cancer cells. The research might provide new molecular evidence for clinical therapy in human prostate cancers.

MATERIALS AND METHODS

Cell lines and agents

The human prostate cancer cell lines PC3, Du145 and

normal human prostate cell control RWPE-1 (ATCC® CRL-11609™) were purchased from ATCC. The PC3 and Du145 cells were cultured in a DMEM medium containing 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. The base medium for PWPE-1 was provided by Invitrogen (Gibco) as part of a kit Keratinocyte Serum-Free Medium (K-SFM, Cat. No. 17005-042). The kit was also provided with two additives, including 0.05 mg/mL of bovine pituitary extract (BPE) and 5 ng/ml of human recombinant epidermal growth factor (EGF) to make the complete growth medium. Lipofectamine™ 3000 was obtained from Invitrogen Corporation (Carlsbad, CA). Notch1 shRNA (cat. no. sc-36095-SH) and control shRNA plasmid (cat. no. sc-108060) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). hsa-miR-449a miRNA Mimic (Cat.No.: MCH02384), miRNA Mimic Negative Control (Cat. No. MCH00000), hsa-miR-449a miRNA Inhibitor (Cat. No.: MIH02384), and miRNA Inhibitor Negative Control (Cat. No. MIH00000) were purchased from Applied Biological Materials Inc. (Richmond, Canada).

MTT assay

The prostate cancer cell line PC3 cells were plated into 48 well plates and cultured for 8 h for adherence. The cells were transfected with Notch1 shRNA and control shRNA for 24, 48 and 72 h, respectively. The cell viability was determined by MTT assay. Briefly, before a test of four h, 10 µL of MTT (5 mg/mL) was added into the cultured medium. Finally, the absorbance of each well was read at 490 nm.

Antibodies

The primary antibody Notch 1 (A-8): sc-376403 is used to monitor the expression of Notch 1 by Western Blotting at the dilution 1:500. Beta-actin antibody was purchased from TransGen Biotechnology (Beijing, China). Beclin-1 antibody (Cat. No. Q14457) is purchased from Abgent. Anti-LC3 (Cat. No. L8918) can recognize human and rat LC3A/B-I and LC3A/B-II by immunoblotting, which is purchased from Sigma-Aldrich Corporation. Anti-p62 antibody (ab56416) is a mouse monoclonal to p62 and was purchased from Abcam Corporation. The secondary antibody was as follows: Goat anti-mouse IgG H&L (HRP) (ab205719) and goat Anti-Rabbit IgG H&L (HRP) (ab6721) were purchased from Abcam Corporation.

Western blotting

PC3 cells (2×10^3 cells/well) were plated into a 48-well plate and cultured for 8 h to make the cell density 70%~80%. Then, PC3 cells were transfected with miR-449a mimic and mimic negative control for 48 h. The cell lysates were collected by using RIPA buffer (Cat. No. P0013K, Beyotime, Shanghai, China). The total protein (30 μ g) was separated by 10% SDS-PAGE at the constant voltage of 80 v for 15 min followed by 120v for 80min. Next, proteins were transferred onto the NC membrane at a constant current of 300 mA for 2 h. The membrane was then blocked with 5% non-fat milk powder for 40 min at room temperature. The primary antibodies were added and incubated at 4°C overnight. The membrane was incubated with a secondary antibody for 1 h at room temperature.

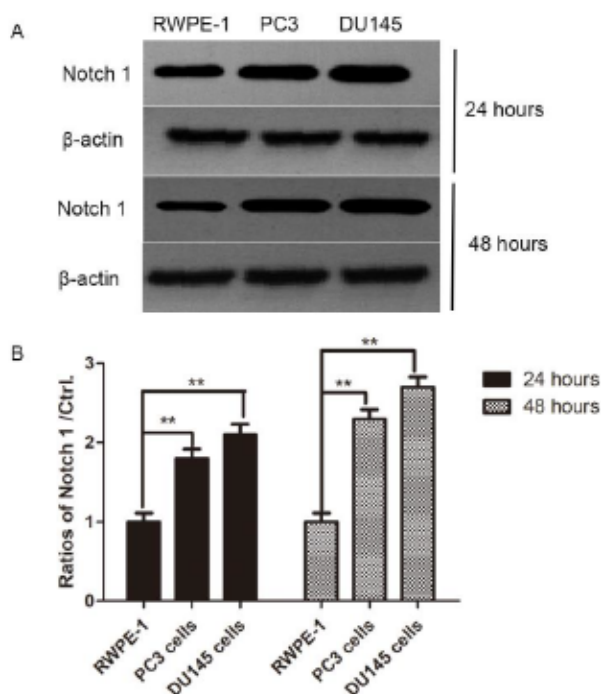


Fig. 1. Notch1 level increases in prostate cancer cell lines. A): The prostate cancer cell line PC3 and Du145 (2×10^5 cells/per well) were plated into 12-well plate. The cells were cultured for 24 h and 48 h, respectively. Western blotting was performed to detect the expression of Notch1 in PC3 cells and Du145 cells. Normal human prostate cell RWPE-1 was used as the control. β -actin was used as an internal reference gene. B): The relative expression of Notch 1 was shown in histogram in PC3 and Du145 cells. Ratios of Notch1 expression/Ctrl were shown as means $\pm$ S.D. of at least duplicates. $**p < 0.01$, compared with normal control cells.

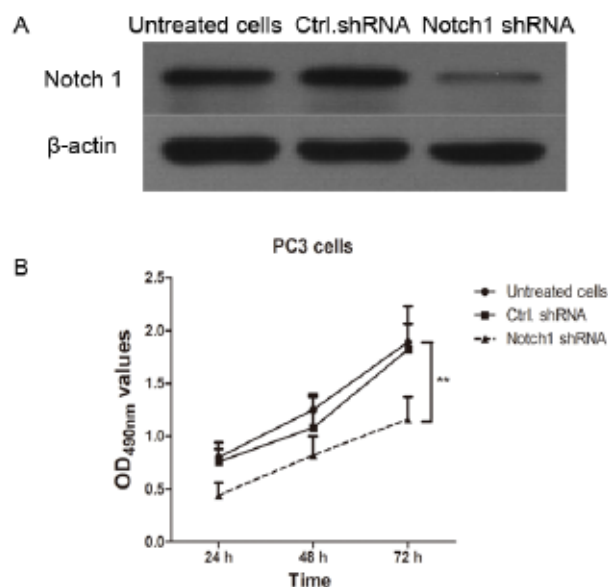


Fig. 2. Interference with endogenous Notch1 expression inhibits the expression of PC3 cells in a time-dependent manner. A): The PC3 cell at the cell density of 5×10^5 cells / per well was plated into 6-well plate. Eight hours later, the cells were transfected with Notch1 shRNA and Control shRNA for 48 h, and western blotting was used to test the expression of Notch1 in Notch1 shRNA transfected PC3 cells and control shRNA transfected PC3 cells. Here, lipofectamin 2000 treated cells were used as negative control. B): Cell viability of Notch1 shRNA and control shRNA transfected PC3 cells was tested by MTT assay. $**p < 0.01$, compared with that of untreated group.

Importantly, at each step for incubation of the antibodies, the membrane was washed three times with 1x TBST buffer for 10 min at each time. Finally, the bands were visualized using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis

The results of the present study were performed by using SPSS software. ANOVA and Tukey test is used to analyze the multiple comparisons. The results are expressed as mean values $\pm$ SD. The results were analyzed by using an independent samples t-test. Values of $p < 0.05$ were considered to be significant.

RESULTS

Notch1 level is high in human prostate cancer cells
Western blotting was performed to test the

expression of notch1 in human prostate cancer cells and to detect the expression of Notch1 in PC3 cells and Du145 cells at 24 h and 48 h. As shown in Fig. 1, the expression of Notch1 was significantly higher in human prostate cancer cell lines PC3 and Du145 than that in the normal human prostate cell control cell RWPE-1, which suggested that the Notch1 related pathway might be activated in human prostate cancers.

Interference with endogenous Notch1 inhibits the cell viability of human prostate cancer cells

The Notch1 gene is interfered with Notch1 shRNA for the indicated period of time to investigate whether the expression of Notch1 affects the cell

proliferation of prostate cancer cells. As shown in Fig. 2A, the endogenous Notch1 was interfered with Notch1 specific shRNA for 48 h, and the levels of Notch1 were detected by western blotting analysis. The results showed that the expression of Notch1 was obviously suppressed at 48 h by transfections with Notch1 specific shRNA. Moreover, the cell viability was detected by MTT assay when PC3 cells were interfered for 24 h, 48 h and 72 h, respectively. The results showed that the cell viability was significantly suppressed in Notch1 knockdown PC3 cells at 24 h, 48 h and 72 h, respectively. All the results showed that interference with Notch1 expression was helpful to inhibit the cell growth of human prostate cancer cells.

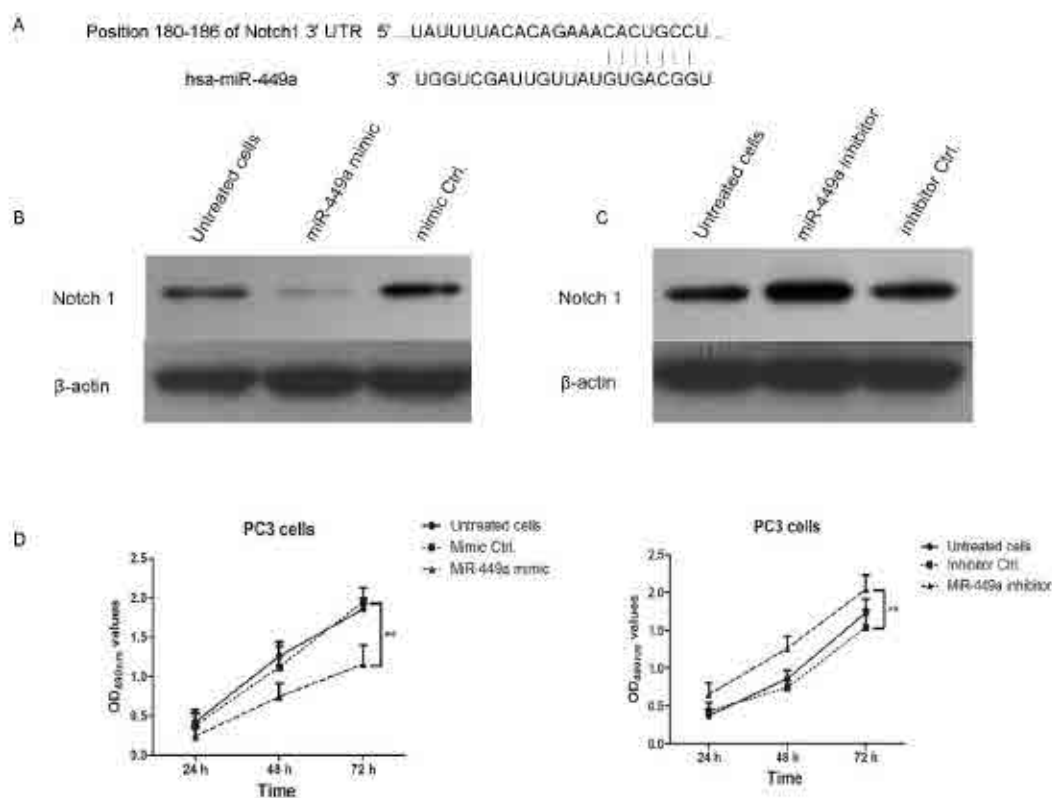


Fig. 3. miR449a inhibits the cell viability of PC3 cells by down-regulating the expression of Notch1. A): TargetsCan software predicts that hsa-miR-449a targeted at position 180-186 of Notch1 3'UTR. B): MiR-449a mimic and mimic control were transfected into PC3 cells. The expression of Notch1 was determined by Western blotting. Mimic control was used as the negative control. C): PC3 cells were plated into 48-well plate and cultured for 8 hours. The cells were transfected with miR-449 inhibitor and inhibitor control for 48 hours. The expression of Notch1 was determined by Western blotting. D): MTT assay. PC3 cells were transfected with miR-449a mimic and mimic control, miR-449a inhibitor and inhibitor control. The cells in each group were cultured for 24 hours, 48 hours and 72 hours, respectively. The OD490nm values were recorded. Each treatment was given three triplicates. Data was shown as mean value $\pm$ S.D. ** $p < 0.01$, compared with control group cells.

Notch1 expression is regulated by has-miR-449a in human prostate cancer cells

To identify how Notch1 is regulated in the progression of human prostate cancers, TargetScan software is used to predict the possible miRNAs. As shown in Fig. 3A, has-miR-449a is targeted on position 180-186 of Notch1 3'UTR. Next, human prostate cancer PC3 cells were transfected with miR-449a mimic and mimic control for 48 h, and the levels of Notch1 were detected by western blotting. As shown in Fig. 3B, Notch1 level was obviously decreased in miR-449a mimic transfected PC3 cells, compared with that in mimic control transfected PC3 cells. Conversely, Notch1 level was obviously increased in miR-449a inhibitor transfected PC3 cells, compared to that in inhibitor control transfected PC3 cells (Fig. 3C). All the data showed that has-miR-449a negatively regulated the expression of Notch1 in human prostate cancer cells.

MiR-449a mimics suppresses cell viability of PC3 cells, and miR-449a inhibitor promotes cell proliferation of PC3 cells

Next, we explored how mimic-449a regulated the cell proliferation of human prostate cancer cells. As shown in Fig. 3D, the OD490 values of miR-449a mimic transfected cells were significantly lower than those that mimic control transfected PC3 cells (** $p < 0.01$). Moreover, PC3 cells were miR-449a inhibitor and inhibitor control for 24 h, 48 h, and 72 h, and the results demonstrated that transfection with miR-449a inhibitor in human prostate cancer PC3 cells significantly inhibited cell viability than that of the inhibitor control group (** $p < 0.01$). All the results revealed that MiR-449a mimics suppresses cell viability of PC3 cells and miR-449a inhibitor promotes cell proliferation of PC3 cells

The levels of cell autophagy-related genes are detected by western blotting analysis

To further explore whether miR-449a expression affects cell autophagy of human prostate cancer PC3 cells and Du145 cells, western blotting is performed to test the levels of cell autophagy-related genes. PC3 cells and Du145 cells were transfected with miR-449a mimic and mimic negative control for 48 h and the

levels of beclin1, LC3-I, $\square$ and p62 were detected by western blotting. As shown in Fig. 4, the Notch1 level was obviously decreased and the expression levels of Beclin 1 and LC3- $\square$ were significantly higher in miR-449a mimic transfected PC3 cells and Du145 cells, compared with that of mimic negative control transfected PC3 cells and Du145 cells (** $p < 0.01$). P62 is an autophagy substrate protein, which is inversely proportional to autophagy activity and indirectly reflects autophagy activity. Here, we also detected the level of p62 in miR-449a mimic transfected PC3 cells and Du145 cells.

The data showed that transfection with miR-449a mimics significantly decreased p62 levels in human prostate cancer PC3 cells and Du145 cells, compared with that in mimic negative control transfected PC3 cells and Du145 cells (** $p < 0.01$). All the data demonstrated that miR-449a transfection in PC3 and Du145 cells decreased the level of Notch1 and increased the autophagy activity of human prostate cancer cells.

DISCUSSION

Prostate cancer is an epithelial malignancy that occurs in the prostate that seriously threatens the health of males (12). The early stage of prostate cancer is relatively hidden, and once found, it is often in the late stage of prostate cancer (13). The study of the pathogenesis of prostate cancer could provide an effective reference for the clinical treatment of prostate cancer patients (14). Notch1 signal pathway plays an important function in the embryonic development of animals and contributes to the progression of various human cancers. In the present study, we explored the function of Notch1 and the regulatory miRNAs in the progression of prostate cancers.

Firstly, we compared the expression of Notch1 in prostate cancer cell lines and normal human prostate cell control cells and the results revealed that Notch1 was highly expressed in prostate cancer cell lines. This was consistent with Deng's results that they found that the levels of Notch1 were obviously associated with the invasion of prostate cancer (9). Moreover, interference with Notch1 by specific shRNA also decreased the cell proliferation of PC3 cells. Some *in*

in vivo experiments showed that inhibiting the level of Notch1 with a Notch1 antibody, OMP-A2G1 could inhibit tumor growth in two murine models of prostate cancer (15). All the results demonstrated that Notch1 was an oncogene and involved in the progression of human prostate cancers.

MicroRNAs (miRNAs) are a class of endogenous regulatory non-coding RNAs found in eukaryotes that are about 20-25 nucleotides in length. It has been reported that miRNA 34a (11) and miR139-5p (10) inhibited the chemotherapeutic drug resistance or cell proliferation of human prostate cancer

cells. Our data found that hsa-miR-449a might target Notch1 and negatively regulated the level of Notch1 in human prostate cancer cells. Additionally, transfection with miR-449a mimics inhibited cell viability of PC3 cells, while transfection with miR-449a inhibitor increased cell viability of PC3 cells. Furthermore, the relationship between miR-449a and cell autophagy was tested, and the results showed that miR-449a increased the autophagy activity of human prostate cancer cells. It has been reported that miR-449a could inhibit tumor growth, migration, and invasion in breast cancer (16), non-

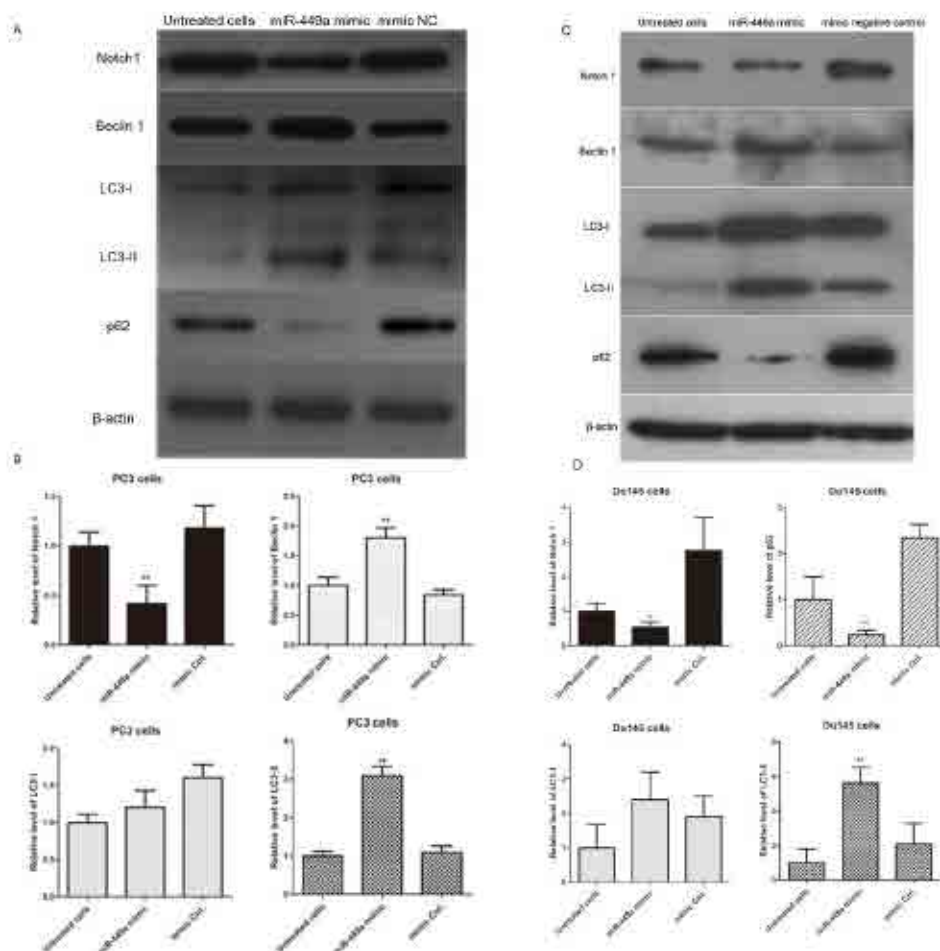


Fig. 4. The levels of cell autophagy related genes are detected by western blotting analysis. PC3 cells (A) and DU145 cells (C) (2×10^3 cells/well) were plated into 48-well plate and cultured for 8 hours for adherence. The cells were transfected with miR-449a mimic and mimic negative control for 48 hours. The expression of beclin1, LC3-I, II and p62 was detected by western blotting. Beta-actin was used as an internal reference gene. The relative expression of Notch1, beclin1, LC3-I, LC3-II and p62 was shown in histogram in PC3 cells (B) and (D). $**p < 0.01$, compared with mimic control transfected PC3 cells.

small cell lung cancer (17), colorectal cancer (18) and ovarian cancer (19), etc. Chen et al. has also found that loss of miR-449a promoted metastasis of prostate cancer cells (20), which was consistent with the results in our study. However, there was a study reported that miR-449a worked as a tumor suppressor gene in endometrial cancer by targeting CDC25A (21). The opposite role of miR-449a might be due to the different tumor samples; thus, it needs to be clearly explored in the progression of various human cancers.

In conclusion, Notch1 was found to be highly expressed in human prostate cancer cells and miR-449a negatively regulated the level of Notch1. Moreover, miR-449a inhibits the cell proliferation of human prostate cancer cells partly due to increasing the cell autophagy of PC3 cells.

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An analysis of the risk factors that predispose heatstroke patients to develop acute kidney injury

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This study aimed to analyze the factors related to acute kidney injury (AKI) in patients with heatstroke (HS). Patients with HS who visited the hospital from January 2013 to December 2019 were retrospectively analyzed. These patients were divided into an AKI and a non-AKI group based on the presence of AKI. Patients in the AKI group were further divided into the transient and persistent AKI groups. The differences in clinical characteristics between the AKI and the non-AKI group and the persistent AKI group and transient AKI group were analyzed to study the related factors of the occurrence of AKI. Male patients were more prone to AKI ($P = 0.01$). Compared with the non-AKI group, HS patients with AKI had a higher core body temperature (CBT) ($P < 0.001$) and lower mean arterial pressure (MAP) ($P = 0.004$). In addition, the incidence of mechanical ventilation, renal replacement therapy (RRT), and blood transfusion were higher in the AKI group ($P < 0.001$). However, the proportion of patients among whom cooling the CBT to 38.9°C within 30 min had taken place was lower ($P = 0.007$), and the risk of Disseminated Intravascular Coagulation (DIC) and death was higher ($P < 0.001$). There was no statistical difference in age, basic disease, heat index, and other indexes ($P > 0.05$). Compared with the transient AKI group, patients with persistent AKI had higher serum creatinine (Scr) levels, WBC counts, a higher rate of transfusion, DIC and death ($P < 0.05$), and a lower rate of cooling the CBT to 38.9°C within 30 min from the discovery of unconsciousness ($P = 0.006$). There were no significant differences between the two groups in terms of gender, age, highest CBT and MAP at admission, RRT, mechanical ventilation, or other factors ($P > 0.05$). Multivariate logistic regression analysis showed that the highest CBT (OR2.54; 1.007–6.42) and the Scr level on admission (OR 1.13; 1.07–1.18) were risk factors for AKI in HS patients ($P < 0.05$). The highest CBT and Scr levels on admission are independent risk factors for AKI, while the duration and progression of AKI may be associated with delayed CBT cooling.

Heatstroke (HS) affects the body through thermal damaging factors that can lead to serious and even fatal diseases. Due to the rapid onset, HS can cause

multiple organ function damage and has a high mortality rate (1). HS patients are prone to acute kidney injury (AKI) (2, 3). For some of these patients,

Keywords: heatstroke, acute kidney injury, transient AKI, persistent AKI

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heat-related kidney injury will be transient, and they will be able to recover quickly (4–6). However, other patients with kidney injuries may eventually progress to developing chronic kidney disease (7, 8). Presently, clinical research on HS patients with AKI risk factors is limited; sustained research is needed in this area to illuminate the role of related factors. We retrospectively analyzed the clinical data of HS patients from three centers to explore the risk factors for the occurrence and progression of AKI to help clinicians identify and actively treat such patients promptly.

MATERIALS AND METHODS

General information

This study represents retrospective research. We searched for patients with HS who were treated in three hospitals (General Hospital of the People's Liberation Army, Tongren Hospital, and the People's Liberation Army Hospital, China) from January 2013 to December 2019. Herein, HS is defined as a Core Body Temperature (CBT) that rises above 40°C, accompanied by hot, dry skin and central nervous system abnormalities (9). Patients younger than 14 years, patients with burns, and patients with previous severe liver and kidney dysfunction were excluded.

Data collection and processing

General information, such as age, gender, previous hypertension, coronary heart disease, and cerebrovascular disease, were collected. Other underlying issues, e.g., a history of the previous use of anesthetics, or a history of similar episodes, were also noted. Information regarding the presence of respiratory tract infections, a lack of sleep (less than 7 hours a day), and abdominal discomfort was obtained. Other triggers prior to the onset of HS, symptoms during the onset (including convulsions, diarrhea, abdominal pain, vomiting, and muscle pain) were collected. Temperature and humidity at the time of onset were collected, and the heat index was calculated.

The heat index calculation was based on the following formula: $\text{heat index} = -8.7847 + 1.6114 T_c - 0.012308 T_c^2 + H [2.3385 - 0.14612 T_c + (2.2117 \times 10^{-3}) T_c^2] + H^2 [-0.016425 + (7.2546 \times 10^{-4}) T_c + (-3.582 \times 10^{-6}) T_c^2]$ (T_c = ambient temperature, H = ambient humidity) (10).

Collect the type of HS. The classification of HS was

based on the following aspects. HS acquired as a result of engaging in physical activities, such as military training, long-distance running, moving, fighting, pushing/pulling, or outdoor work within 24 hours of the onset of HS was defined as “exertional heatstroke” (EHS). Conversely, “classical heatstroke” (CHS) was defined as the result of having engaged in sitting, lying, standing, or doing paperwork within 24 h before the onset of HS.

The highest CBT after heatstroke and blood pressure at admission were collected; their mean arterial pressure (MAP) values, vital signs, collecting its after admission WBC count, serum creatinine (Scr) level, and coagulation test. We evaluated the presence of Disseminated intravascular coagulation (DIC) using the DIC diagnostic scoring system (11).

Daily urine output was noted, and the need for a blood transfusion, mechanical ventilation, and renal replacement therapy (RRT) was calculated; additionally, whether the CBT had decreased to 38.9°C within 30 minutes after admission was recorded.

Grouping and diagnostic criteria

The patients were divided into an AKI and a non-AKI group based on the presence of AKI. Patients in the AKI group were further divided into a transient and a persistent AKI group.

AKI was defined using the Kidney Disease Improving Global Outcomes criteria. An increase in Scr ≥ 0.3 mg/dl (26.5 $\mu\text{mol/L}$) within 48 hours following admission to the hospital or an increase $\geq 50\%$ of the baseline level within 7 days was used as the diagnostic criteria for AKI. Persistent AKI was defined as the presence of a persistently elevated Scr level or oliguria (including cases of AKI where the patient died within 3 days following admission to the hospital) (12). Transient AKI was defined as cases in which the Scr level decreased by $>50\%$ or returned to the baseline level within 3 days, or when normal urine output returned without the use of diuretics (>0.5 ml/(kg·h) (4–6, 13).

The mean Scr level within 6 months of admission to the hospital was used as the baseline level. If it was not available, the baseline Scr level was estimated according to the Modification of Diet in Renal Disease formula (12).

The processing method of missing data

The missing ambient temperature and humidity data

were based on the local average temperature and humidity data of the day queried by the National Meteorological Administration. For districts and counties where there was no temperature monitoring station and no temperature data at the location of the disease, the average data measured by monitoring stations within 50 kilometers of the location was included in the study.

For the information that could not be obtained from the medical records, such as past medical history, causes, and the situation at the onset, the researchers contacted patients or their family members through telephone inquiries, mailing forms, and e-mail to collect and summarize the information.

When important biomarker parameter such as one day's blood creatinine levels was missing, missing data was imputed five times with the "PMM" (predictive mean matching) method containing all the predictor variables and the median ones of these five values were imputed as missing data.

Statistical analysis

According to the data distribution, normally distributed measurement data were expressed as the mean $\pm$ the standard deviation ($\bar{x} \pm SD$) and were compared using t-tests. Non-normally distributed measurement data were expressed as the median and interquartile data and were compared between groups using non-parametric tests. Count data were expressed as frequency and expectations

and were compared between two groups using Chi-square tests. We included variables with $P < 0.1$, as well as clinical significance in multivariate regression analysis to identify the risk factors for AKI; $P < 0.05$ was considered statistically significant.

RESULTS

Differences in general characteristics between AKI and non-AKI patients

A total of 175 patients with HS were enrolled (Fig.1), including 166 males and 9 females. There were 40 patients in the 14–19-year-old age group, 40 in the 20–22-year-old age group, 45 in the 23–33-year-old age group, and 42 patients in the ≥ 34 -year-old age group.

AKI occurred in 117 of the 175 patients with HS. Compared with the non-AKI group, the AKI group included more males ($P = 0.01$), a higher CBT ($P < 0.001$), and lower MAP ($P = 0.004$) levels. Patients with AKI had an increased proportion of blood transfusions, increased rates of mechanical ventilation and RRT ($P < 0.001$), and a greater risk of DIC and death ($P < 0.001$). In addition, the percentage of patients for whom the CBT decreased to 38.9°C within 30 minutes of admission to the hospital was low ($P = 0.007$).

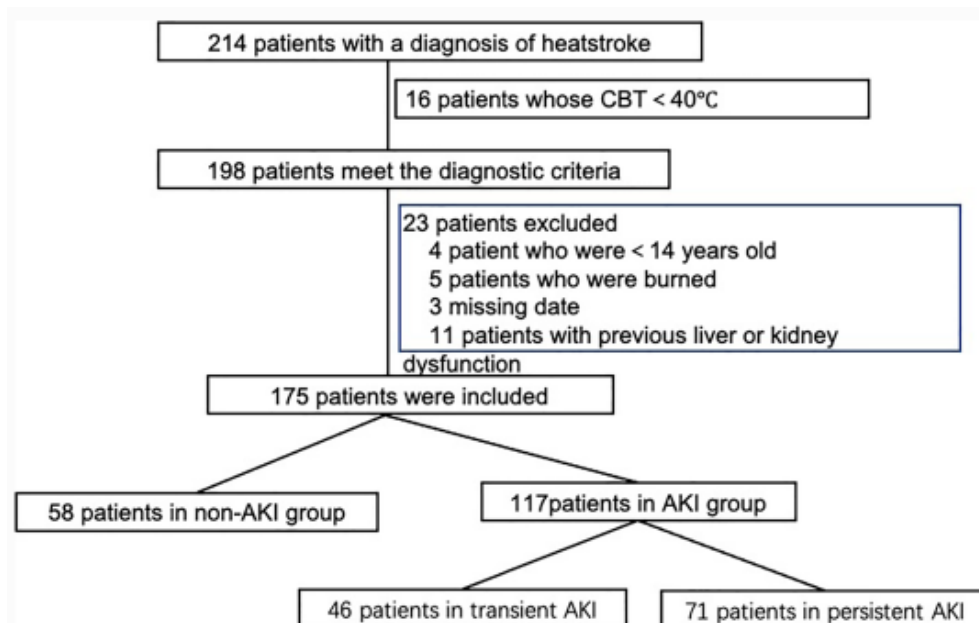


Fig. 1. Enrolment of study patients.

Based on age, as well as body mass index, the presence of a pre-existing underlying disease, as well as heat index, indicated no statistical differences between the two groups; based on the classification of HS, no statistical differences ($P > 0.05$) were observed. (Table I).

Comparison of the clinical characteristics between patients with persistent AKI and patients with transient AKI

Of the 117 patients with AKI, 71 had this condition

for more than 3 days. Compared with transient AKI, patients with persistent AKI had higher levels of WBC, Scr ($P < 0.05$). The persistent AKI group had a significantly increased risk of needing a blood transfusion and had a higher chance of DIC ($P < 0.001$). In addition, patients with persistent AKI had a lower rate of cooling to 38.9°C within 30 minutes after onset of HS ($P = 0.006$). There were no statistically significant differences between the

Table I. Comparison of clinical characteristics between AKI and non AKI patients with HS

	ALL, n=175	AKI group N=117	Non-AKI group N=58	t/chi-square value/Z	p-value
Age, y	22 (19,33)	22 (19,33.25)	20 (19,31.75)	-1.67	0.16
Gender (male)	166	115 (111.0)	51 (55.0)	6.54	0.01
Past medical history					
Cardiovascular disease	6	5 (4.0)	1 (2.0)	0.19	0.67
Diabetes	2	1 (1.3)	1 (0.7)		1.0
Cerebrovascular disease	3	3 (2.0)	0 (1.0)	0.37	0.54
Previous history of similar attacks	5	2 (3.3)	3 (1.7)	0.66	0.42
A history of anesthetic drug use	8	7 (5.3)	1 (2.7)	0.78	0.38
Discomfort 3 days before onset					
Respiratory tract infection	9	4 (6.0)	5 (3.0)	1.22	0.27
Abdominal pain or diarrhea	7	5 (4.7)	2 (2.3)	0	1.0
Sleep less than 7 hours a day	2	1 (1.3)	1 (0.7)		1.0
Others	6	4 (4.0)	2 (2.0)	0	1.0
Heat index	84.20±7.02	83.61 ±7.26	85.34±6.46	0.510	0.13
Symptoms at onset					
Sweating	44	31 (29.4)	13 (14.6)	0.16	0.59
Diarrhea	30	24 (20.1)	6 (9.9)	2.82	0.09
Myalgia	6	2 (4.0)	4 (2.0)	1.779	0.18
Chest tightness	26	15 (17.4)	11 (8.6)	1.158	0.28
Abdominal pain	9	6 (6.0)	3 (3.0)	0	1.0
Vomiting	68	47 (45.5)	21 (22.5)	0.256	0.61
Convulsions	90	64 (60.2)	26 (29.8)	1.51	0.22
Urinary and fecal incontinence	30	23 (20.1)	7 (9.9)	1.57	0.21
Classification (Exertional heatstroke)	156	106 (104.3)	50 (51.7)	0.773	0.38
Signs					
Highest CBT, $^{\circ}\text{C}$	40.5 (40,41.7)	41.2 (40.3,41.75)	40.2 (40.0,41.32)	-1.822	<0.001
BMI	24.26 (22.49,27.69)	24.49 (22.70,27.86)	23.94 (21.26,27.7)	-0.83	0.40
On admission MAP, mmHg	76.47±20.84	73.13±20.67	82.97±19.76	0.70	0.004
On admission Scr, $\mu\text{mol/l}$	123.1(88.8,183.3)	156.0(123.0,227.0)	80.0(72.0,92.1)	-9.74	<0.001
Whether the CBT drops to 38.9°C within 30 minutes	36	17(23.9)	19(12.1)	7.40	0.007
Invasive mechanical ventilation	66	55(42.8)	11(23.2)	17.03	<0.001
Blood transfusion	74	65(49.5)	9(24.5)	25.47	<0.001
RRT	76	63 (50)	13 (26)	18.29	<0.001
DIC presence	69	60(46.1)	9(22.9)	20.77	<0.001
Death	35 (35.0)	32 (23.4)	3 (11.6)	11.921	<0.001

HS: heatstroke; **CBT:** core body temperature; **BMI:** Body mass index; **MAP:** mean arterial pressure **Scr:** serum creatinine; **DIC:** Disseminated intravascular coagulation; **RRT:** renal replacement therapy

two groups in terms of age, gender, classification of HS, or highest CBT and MAP levels on admission. In addition, there was no significant difference in the ratio of mechanical ventilation and RRT between the two groups ($P > 0.05$) (Table II).

Differences in the highest CBT between the AKI and non-AKI groups and between the transient and persistent AKI groups

The peak CBT of patients with HS was significantly higher in the AKI compared with the

Table II. Comparison of clinical characteristics between persistent and transient AKI

	Persistent AKI N=71	Transient AKI N=46	t/chi-square value/Z	p-value
Age, y	23 (20.0, 39.05)	22 (19.0, 30.0)	-1.26	0.21
Gender (male)	70 (69.8)	45 (45.2)		1.0
Past medical history				
Cardiovascular disease	4 (3.0)	1 (2.0)	0.19	0.66
Diabetes	1 (0.6)	0 (0.4)		1.0
Cerebrovascular disease	3 (1.8)	0 (1.2)	0.662	0.42
Previous history of similar attacks	2 (1.2)	0 (0.8)		0.52
A history of anesthetic drug use	4 (4.2)	3 (2.8)	0.039	0.84
Discomfort 3 days before onset				
Respiratory tract infection	3 (2.4)	1 (1.6)	0.006	0.94
Abdominal pain or diarrhea	5 (3.0)	0 (2.0)	1.882	0.17
Sleep less than 7 hours a day	1 (0.6)	0 (0.4)		1.0
Others	46 (47.2)	2 (0.8)		0.166
Heat index	83.73 ± 7.40	83.39 ± 7.10	0.280	0.81
Symptoms at onset				
Sweating	19 (18.8)	12 (12.2)	0.007	0.93
Diarrhea	17 (14.6)	7 (9.4)	1.30	0.25
Myalgia	2 (1.2)	0 (0.8)		0.52
Chest tightness	10 (9.1)	5 (5.9)	0.26	0.61
Abdominal pain	6 (3.6)	0 (2.4)	2.55	0.11
Vomiting	17 (14.6)	7 (9.4)	1.30	0.25
Convulsions	39 (38.2)	24 (24.8)	0.085	0.77
Urinary and fecal incontinence	13 (14.0)	10 (9.0)	0.208	0.65
Classification (Exertional heatstroke)	66 (64.9)	41 (42.1)	0.148	0.7
Signs				
Highest CBT, °C	41.3 (40.38, 41.80)	41.2 (40.2, 41.40)	-1.22	0.22
BMI	24.59 (22.80, 30.27)	23.54 (22.35, 26.10)	-1.331	0.18
On admission MAP, mmHg	74.49 ± 21.0	70.67 ± 20.19	-0.92	0.36
On admission Scr, ummol/l	184.0 (136.68, 294.403)	144.0 (112.2, 162.90)	-3.51	<0.001
WBC (× 10 ⁹ /L)	13.46 (6.8, 15.02)	11.11 (8.10, 16.16)	-2.22	0.046
Whether the CBT drops to 38.9°C within 30 minutes	5 (10.1)	12 (6.9)	6.19	0.006
Invasive mechanical ventilation	38 (34.7)	17 (20.7)	1.82	0.18
Blood transfusion	52 (38.9)	13 (26.1)	24.76	<0.001
RRT	44 (39.7)	20 (24.3)	3.00	0.08
DIC presence	50 (36.4)	10 (23.6)	26.48	<0.001
Death	28 (19.4)	4 (12.6)	13.28	<0.001

HS: heatstroke; **CBT:** core body temperature; **BMI:** Body mass index; **MAP:** mean arterial pressure **Scr:** serum creatinine; **DIC:** Disseminated intravascular coagulation; **RRT:** renal replacement therapy

non-AKI group ($P < 0.001$) (Fig. 2). However, there was no significant difference in the peak of CBT between the persistent and the transient AKI groups ($P = 0.22$) (Fig. 3).

The difference in MAP on admission between the AKI and non-AKI groups and between the transient and persistent AKI groups

The MAP levels were significantly lower in the AKI than in the non-AKI group ($P = 0.004$; Fig. 4). However, there was no significant difference in the MAP levels between the persistent and the transient AKI group ($P = 0.36$) (Fig. 5).

Multiple logistic regression analysis of AKI in patients with HS

Rhabdomyolysis in EHS increases the clinical risk of AKI. Therefore, both the classification of HS and the potential risk factors (using $P < 0.1$ in the different analyses to indicate a statistical significance) were included in the multivariate logistic regression. The regression results showed that the highest CBT (OR2.54; 1.007–6.42) and Scr (OR1.13; 1.07–1.18) on admission were risk factors for AKI in HS patients. Gender, HS classification, diarrhea symptoms, MAP level on admission, and

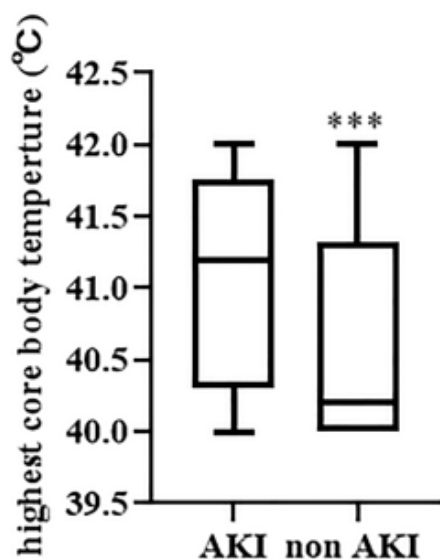


Fig. 2. Comparison of highest core body temperature between AKI and non-AKI group. *** $p < 0.001$.

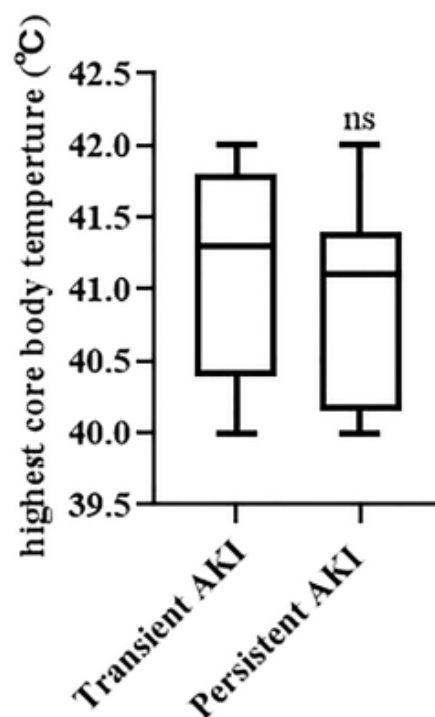


Fig. 3. Comparison of highest core body temperature between transient AKI group and persistent AKI group; $^{ns}p > 0.05$.

early CBT cooling were not independent risk factors for AKI ($P > 0.05$), as shown in Table III.

DISCUSSION

AKI is one of the most common complications of HS. The impact of AKI on HS is significant. It is associated with several complications, including metabolic imbalances, sepsis, and end-organ damage, and leads to higher mortality rates and hospitalization costs (14). We examined the records of 175 patients with HS and found that the peak CBT and Scr levels on admission were independent prognostic factors of AKI in cases of HS. However, the persistence and progression of AKI were associated with delayed cooling and Scr levels at admission.

AKI occurred in 117 of the 175 patients included in this study, an incidence rate of 67%. In an existing study, Thongprayoon et al. (14) analyzed the clinical data of HS patients using the National Hospital Sample database of the United States and showed

that the prevalence of AKI in 3,346 HS patients was 36%. Comparatively, the incidence of AKI in the present study was mainly due to differences in the severity of the disease. Additionally, in the present study, all HS patients showed symptoms of nervous system failure (100% vs 19.2%). The proportion of invasive mechanical ventilation was high (37.7% vs 20.4%), the proportion of blood transfusions required was high (42.3% vs 4.90%), and the mortality rate while in the hospital was high (20.0% vs 5.0%). Fan et al. (15) retrospectively analyzed the data of 176 HS patients and found that approximately 58% of patients developed AKI, which was similar to the incidence found in the current study. However, this study only analyzed the relationship between the blood-test index level and AKI after admission to the hospital; it did not consider the relationship between the occurrence of AKI and CBT, factors such as previous underlying diseases, or external environment. Therefore, the present study has independent added value.

The current study revealed that hyperthermia was a risk factor for the occurrence of AKI in cases of HS. Researchers first observed in the 1990s that the incidence of AKI or chronic kidney disease was

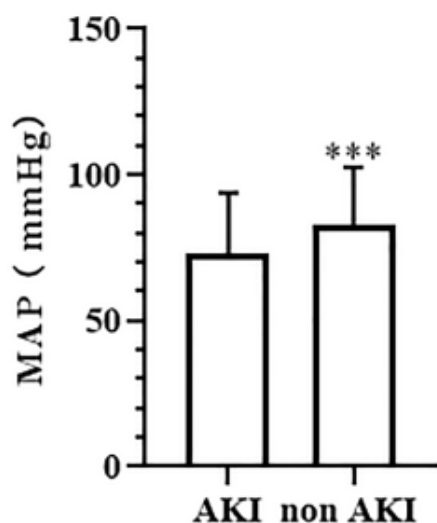


Fig. 4. Comparison of MAP levels between AKI and non-AKI group. MAP: mean arterial pressure; *** $p < 0.001$.

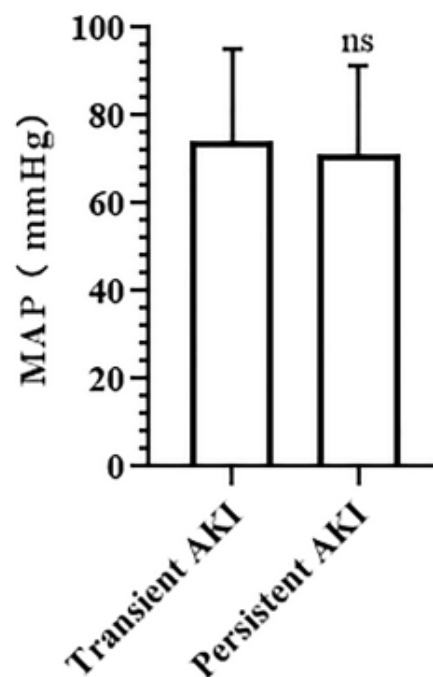


Fig. 5. Comparison of MAP levels between transient AKI group and persistent AKI group; $^{ns}p > 0.05$.

much higher among workers exposed to hot climates compared with those in cool climates who did the same work. Renal biopsies of workers exposed to heat for an extended time revealed glomerulosclerosis, glomerular hypertrophy, signs of chronic glomerular ischemia, and evidence of mild to moderate chronic renal tubulointerstitial damage. Accordingly, researchers put forward the theory that heat may be a risk factor for kidney damage (16). Subsequently, an animal experiment confirmed that intermittent, repetitive passive (i.e., resting) heat exposure could cause renal damage (17). The mechanism of renal injury caused by hyperthermia is unknown. It may be related to pathophysiological processes, such as tissue damage directly caused by heat stress, secondary inflammatory reactions, oxidative stress, and the increased activation of apoptotic factors (18). Contrary to expectations, regression analysis suggested that delayed cooling was not an independent risk factor for AKI. In previous animal experiments (19), mice were exposed to a

high thermal environment in a hot chamber and their CBT was raised to 41.0°C, after which they were immediately removed from the chamber. Once removed from the heated environment, the CBT of the mice dropped rapidly and HS did not occur. Nevertheless, these mice showed moderate levels of kidney damage. As such, the authors of the paper suggest that even a brief increase in CBT may lead to serious kidney damage. In the current study, regression analysis also showed that extreme body temperature (the peak CBT), rather than cooling time, was an important cause of AKI in patients with HS.

However, some heat-related AKI is transient with a duration of fewer than 3 days (4–6). Kidney function improved within 3 days in nearly 40% of AKI patients in this study. We compared the clinical characteristics of transient and persistent AKI and found that patients in both groups had similar peaks at their highest CBT level and different rates of early cooling. In most patients with persistent AKI, cooling is delayed. This suggested that although the peak CBT led to the occurrence of AKI in HS patients, the persistence or progression of AKI was related to the persistence and the buildup of hyperthermia. Kidney damage may be temporary and reversible if the CBT is rapidly reduced. When cooling is delayed, AKI will continue

and progress further. This reaffirms the importance of early rapid cooling in HS patients.

Traditional concepts posit that AKI occurs due to renal hypoperfusion, pre-renal injury caused by a decrease in renal blood flow. Contrary to expectations, MAP levels were not found to be an independent risk factor for the occurrence of AKI, nor were they significantly associated with the persistence or progression of AKI. The results of an existing study revealed that when heat stress occurred, the decrease in renal perfusion was only confined to the renal cortex blood flow, and there was no significant change in renal medullary blood flow (20). The results of an existing clinical trial revealed that when exercising in hot weather, Ibuprofen was given to reduce renal perfusion. Although it caused a decrease in the glomerular filtration rate, the risk of AKI did not increase (21). In addition, heat acclimatization was carried out on rats by repeated heat exposure, and the experimental results revealed that the decrease in renal blood flow could protect the body. This will help improve the body's heat resistance capacity (20, 22). Accordingly, the occurrence of AKI in patients with HS cannot be attributed solely to pre-renal AKI caused by decreased renal blood flow, and lower blood pressure does not necessarily cause internal kidney damage.

In contrast to CHS, patients with EHS often

Table III. Multivariate analysis of factors associated with AKI in HS patients.

Variables	OR	95% CI	p-value
Highest CBT, °C	2.54	1.007-6.42	0.04
Classification (Exertional heatstroke)	0.73	0.46-23.80	0.23
Gender (male)	0.61	0.04-9.71	0.73
Whether the CBT drops to 38.9°C within 30 minutes	0.36	0.08-1.47	0.15
On admission SCr, ummol/l	1.13	1.07-1.18	<0.001
On admission MAP, mmHg	0.99	0.96-1.03	0.83
Diarrhea	4.78	0.59-38.60	0.14

OR: odds ratio; **CI:** confidence interval; **CBT:** core body temperature; **Scr:** serum creatinine; **MAP:** mean arterial pressure. After collinearity test, VIF of included parameters <2, so it is considered that there is no collinearity between parameters. Regression equation fitting: Cox-Snell $R^2 = 0.58$, Nagelkerke $R^2 = 0.801$. The fitting degree is satisfactory.

develop fatigued rhabdomyolysis due to intense exercise. The destruction of rhabdomyocytes causes myoglobin and other cellular components to be released into the blood and deposited in renal tubules. These precipitates can obstruct the renal tubules, leading to tubular necrosis and kidney damage. However, EHS was not found to be an independent risk factor for the occurrence and persistence of AKI. This result was similar to a previous conclusion (23). Existing studies found that strenuous exercise could significantly increase the markers of muscle injury (e.g., creatine kinase and myoglobin); however, most patients did not develop AKI (14, 23). This is because exercise-induced fatigue rhabdomyolysis can often be spontaneously relieved without the need for special therapeutic intervention when other conditions are not involved (23). When combined with multiple factors such as hyperthermia, dehydration, and alcohol consumption, fatigued rhabdomyolysis can lead to AKI.

There were some limitations in this study. One limitation was that, due to the low incidence of HS (24), the sample size included in this study may be insufficient, which weakens the conclusion about the meaning of the results. Second, the majority of patients included in the study were young men, which may affect the generality of the conclusions. In addition, due to the COX regression equation for AKI persistence was not well-fitting and convincing after several attempts, the results of some parameters are not shown in this study, which may affect the comprehensiveness of the conclusion. Prospective studies that include larger samples are needed for additional verification of our results.

In summary, the occurrence of AKI in patients with HS is related to a high CBT. Accordingly, timely and effective cooling maybe could improve or even reverse AKI.

Competing interests

The authors declare that they have no competing interests.

Funding

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Ethics approval and consent to participate

The study was conducted following the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the Chinese PLA General Hospital. Written informed consent was obtained from all participants.

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Screening amino acid metabolism-related gene signature for recurrence prediction of colon adenocarcinoma

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Objective: This study aimed to develop an amino acid metabolism-related gene signature for recurrence prediction in colon adenocarcinoma (COAD).

Methods: We downloaded RNA sequencing profiles of COAD from The Cancer Genome Atlas (TCGA) as a training set and GSE39582 from the Gene Expression Omnibus database as a validation set. Differentially expressed RNAs (DERs) were screened from recurrence tumor samples by defined thresholds. The amino acid metabolism-related gene signature was identified by LASSO cox regression analysis. Independent prognostic clinical factors were also assessed by using survival analysis. A risk score (RS) signature was established and validated in two independent datasets.

Results: We obtained 498 differentially expressed mRNAs and 71 differentially expressed lncRNAs based on data mining. Compared with amino acid metabolism genes of Gene Set Enrichment Analysis database, we screened 197 overlapped DERs. A twelve genes-based RS signature was established. This model exhibited a high accuracy for recurrence prediction with an area under the ROC curve (AUC) of 0.924 and 0.843 in TCGA and GSE39582, respectively. In addition, pathological stage and RS model status were identified as independent clinical factors associated with recurrence. The combined model integrating these two factors reached a higher AUC value of 0.940 in the TCGA dataset and 0.876 in the validation set.

Conclusion: We established a high accuracy prognostic model for recurrence prediction. Our findings suggested that the combined model can identify high-risk recurrence COAD patients and might be a reliable tool for decision-making in a clinic.

Colon adenocarcinoma (COAD) is one of the most common malignancies in the gastrointestinal tract with an increasing occurrence worldwide. According to cancer statistics, in 2021, an estimated 104,270 new diagnoses cases and 52,980 deaths of colon cancers in the United States (1). Risk factors include lifestyle, obesity and dietary patterns, such as consumption of processed meat

and low uptake of dietary fibres. Currently, the primary treatments for COAD are chemo-radiotherapy, targeted and immunotherapy (vascular endothelial growth factor inhibitor bevacizumab and epidermal growth factor receptor inhibitor cetuximab) (2). However, despite excellent cancer diagnosis and therapy achievement, many patients still suffered from drug resistance and

Keywords: amino acid metabolism; COAD; prognosis; recurrence

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disease recurrence. Notably, if diagnosed at an advanced stage, patients would have less than 10% of 5 years survival rate (3). Thus, developing novel biomolecules and targets for cancer treatment is necessary.

Emerging evidence has demonstrated the association between long non-coding RNAs (lncRNAs) and various cancers (4). lncRNA is a kind of non-coding RNA with more than 200 nucleotides. In general, lncRNAs regulated gene expression in tumorigenesis through various mechanisms, such as epigenetic modifying, transcriptional and post-transcriptional regulation. The rapid development of multi-omics technologies has enabled breakthroughs to identify novel biomarkers in cancer diagnosis and prognosis. By comparing RNA sequencing profiles, Li et al. screened differentially expressed RNAs (DERs) between left- and right-side colon cancers and three lncRNAs were related to the overall survival of patients (5). lncRNA *LINC01578* was identified as a potential prognostic biomarker for colon cancer metastasis, and the mechanism might be involved in regulation of Nuclear Factor-kappa B/ YinYang1 pathway (6). A recent paper identified six prognosis-related genes and constructed a risk score system for recurrence prediction in colon cancer (7). Taken together, the development of candidate biomarkers, especially combined gene signatures, provides potential to identify high-risk patients and might help clinicians to design proper therapeutic interventions.

Since metabolic reprogram has become a hot topic in cancer biology, this study focused on altering amino acid metabolism-related genes in recurrence COAD. Initially, we downloaded mRNA expression profiles from a public database and screened DERs between recurrent and non-recurrent tumor samples, including lncRNAs and mRNAs. As a result, a twelve-gene signature was constructed and validated to identify high-risk patients for recurrence. Furthermore, the prognostic model integrated clinical factors and signature genes exhibited high accuracy for recurrence predicting of COAD patients.

MATERIALS AND METHODS

Data resource

The profiles of RNA sequencing data were downloaded from The Cancer Genome Atlas (TCGA) database

and tested on a platform of Illumina Hiseq 2000 RNA Sequencing. The data set included 465 COAD samples, and among which 363 samples with recurrence prognostic information. By researching the key words of “Homo sapiens”, “colon adenocarcinoma”, we also downloaded datasets from Gene Expression Omnibus (GEO) database. Inclusion criteria were as follows: tumor specimens were derived from COAD; sample number should be more than 200; containing prognosis information. Finally, we obtained one dataset, GSE39582 (8), tested on the GPL570 Affymetrix Human Genome U133 Plus 2.0 Array platform. There were 585 specimens in GSE39582, of which 519 COAD specimens contained prognostic information. The TCGA set was used as a training set, and the GSE39582 dataset was considered a validation set.

Screening differentially expressed mRNAs and lncRNAs

Firstly, the lncRNAs and mRNAs in the two datasets were annotated according to the HUGO Gene Nomenclature Committee (HGNC) database (9), containing 4904 lncRNA and 19203 coding genes.

Additionally, samples in the TCGA set were divided into recurrent and non-recurrent groups. Limma package (10) of version 3.34.7 was applied to identify DERs (including lncRNAs and mRNAs) from samples of the two groups by criteria of false discovery rate (FDR) value less than 0.05 and $|\log_2 \text{fold change (FC)}| > 0.263$. Finally, the screened DERs were obtained for further analysis.

Screening amino acid metabolism-related RNAs

The amino acid metabolism genes were downloaded from Gene Set Enrichment Analysis (11) (GSEA) database. By comparison with the above screened DERs, we obtained the overlapped genes associated with amino acids metabolism.

Furthermore, the correlations between the expression status of lncRNAs and amino acid metabolism-related genes were also analyzed using cor function in R 3.6.1. Pearson correlation coefficient (PCC) between lncRNAs and amino acid metabolism-related genes were calculated, and those with significant correlation were used to build a lncRNA-mRNA network by Cytoscape software (12). DAVID online tools (13) were applied to conduct functional analysis, including biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. FDR < 0.05 was considered as a threshold.

Construction of risk model for prediction of recurrence prognosis

According to corresponding prognosis information, the genes in the above lncRNA-mRNA network associated with disease recurrence were screened by univariate and multivariate analysis (survival pack version 2.41-1) (14). Log-rank $P < 0.05$ was regarded as a threshold.

The LASSO cox regression (15) model in penalized package version 0.9.50 (<https://cran.r-project.org/web/packages/penalized/index.html>) (16) was utilized to screen optimal genes that correlated with recurrence (parameter “lambda” was derived from 1000 cross-validation likelihood cycling calculation). According to the prognostic coefficient value and expression level of

genes, we established a risk score (RS) system:

$$\text{Risk score (RS)} = \sum \beta_{\text{RNA}} \times \text{Exp}_{\text{RNA}}$$

Here, the β_{RNA} means the Lasso prognostic coefficient of RNA, and Exp_{RNA} indicates RNA expression level in the training set.

Testing and validation of risk model

After calculating the RS values of each sample, the patients in the training set were divided into high-risk and low-risk groups by setting median RS values as threshold. The association between risk grouping and actual recurrence prognosis was assessed using Kaplan Meier method in survival package in R3.6.1.

Similarly, the expression value of corresponding

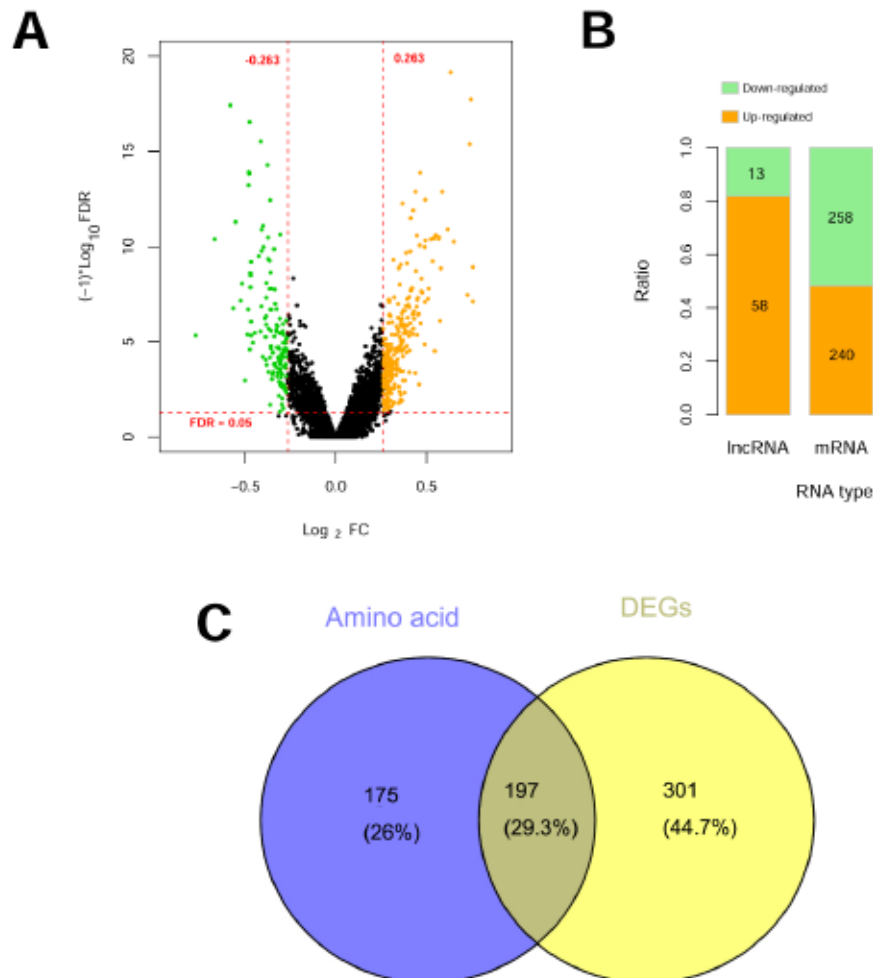


Fig. 1. Results of screening differentially expressed RNAs (DERs). The volcano plot (A) and column chart (B) of DERs. The orange and green dots in diagram A represent up- and down-regulated genes, respectively. Red horizontal line means $\text{FDR} < 0.05$, while two dashed lines in vertical direction means $|\log_2 \text{FC}| > 0.263$; C. Venn diagram shows the comparison of amino acid metabolic genes and differentially expressed genes.

RNAs was extracted from the validation set, and RS value of each sample was also obtained according to the calculation formula. Tumor samples in the validation set were divided into high and low-risk group. The relation between risk grouping and recurrence prognosis was evaluated using survival package in R3.6.1.

Independent clinical factors correlated with COAD recurrence

Univariate and multivariate analyses were conducted to screen prognostic factors associated with recurrence by the threshold of Log-rank P value < 0.05 . Moreover, the prognostic model of clinical factors was established based on tumor samples in the training and validation set. The accuracy was also compared with the above RS model. The pROC package (17) Version 1.14.0 (<https://cran.r-project.org/web/packages/pROC/index.html>) was used to quantify the performance of prognostic models by calculating the area under ROC curves (AUROC). AUROC is a quantitative indicator of ROC curve, and its value is distributed between 0.5 and 1. The larger the AUC value, the more accurate the test model.

RESULTS

Amino acid metabolism-related genes in COADs

A total of 12008 mRNAs and 827 lncRNAs were annotated from TCGA training set and GSE39582 validation set. In addition, combined with clinical prognosis information, multiple DERs were identified from recurrence group samples compared with the non-recurrence group (Fig. 1A, B), including 498 mRNAs and 71 lncRNAs.

Based on the profiles from the GSEA database, we obtained a total of 372 genes related to amino acid metabolism and derivatives. Furthermore, by comparing the screened DERs and amino acid metabolism-related genes, we finally obtained 197 overlapped genes for further analysis (Fig. 1C).

CeRNA network analysis and functional analysis for crucial genes in COADs

The correlation of expression levels between acid metabolism-related genes and the 71 lncRNAs was analyzed, and defined thresholds selected eligible

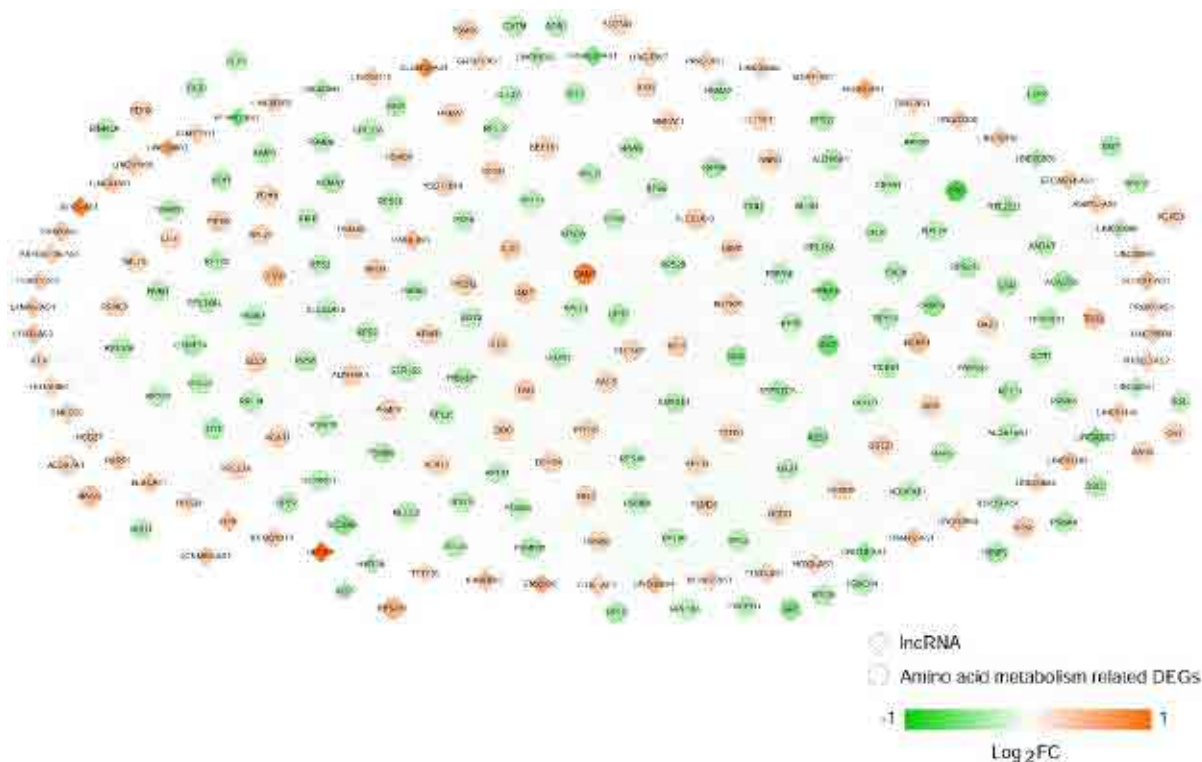


Fig 2. CeRNA regulatory network of lncRNAs and amino acid metabolic genes. The diamond and circle nodes represent lncRNAs and amino acid metabolic genes, respectively. Color changes from green to orange indicate the variate of logFC value from low to high. The green and gray lines indicate negative and positive correlations, respectively.

gene pairs. Finally, we obtained 927 genes pairs to construct a lncRNA-mRNA network. The network was consisting of 243 nodes, including 66 lncRNAs and 177 mRNAs (Fig. 2), indicating a complex regulatory mechanism in COAD progression.

Functional enrichment analysis revealed that these 243 RNAs were mainly associated with 63 processes and 24 pathways (Fig. 3). The top twenty biological processes or pathways included biological processes of viral transcription, nonsense-mediated decay, translation, and pathways of ribosome, metabolic pathways, alanine, aspartate and glutamate metabolism.

Risk prediction model construction and performance validation

Univariate and multivariate survival analyses were conducted for these 243 RNAs. We screened 75 RNAs associated with recurrence prognosis, including 40 lncRNAs and 35 mRNAs. Multivariate analysis revealed only 23 DERs (10 lncRNAs and 13 mRNAs) were independently correlated with disease recurrence (Fig. 4).

By using cox-proportional hazards model, we optimized the DERs combination and finally obtained 12 crucial genes, such as *CIRL-AS1*, *CECR7*, *LINC01301*, *PRKAG2-AS1*, *BCKDK* and *GSR* (Table I).

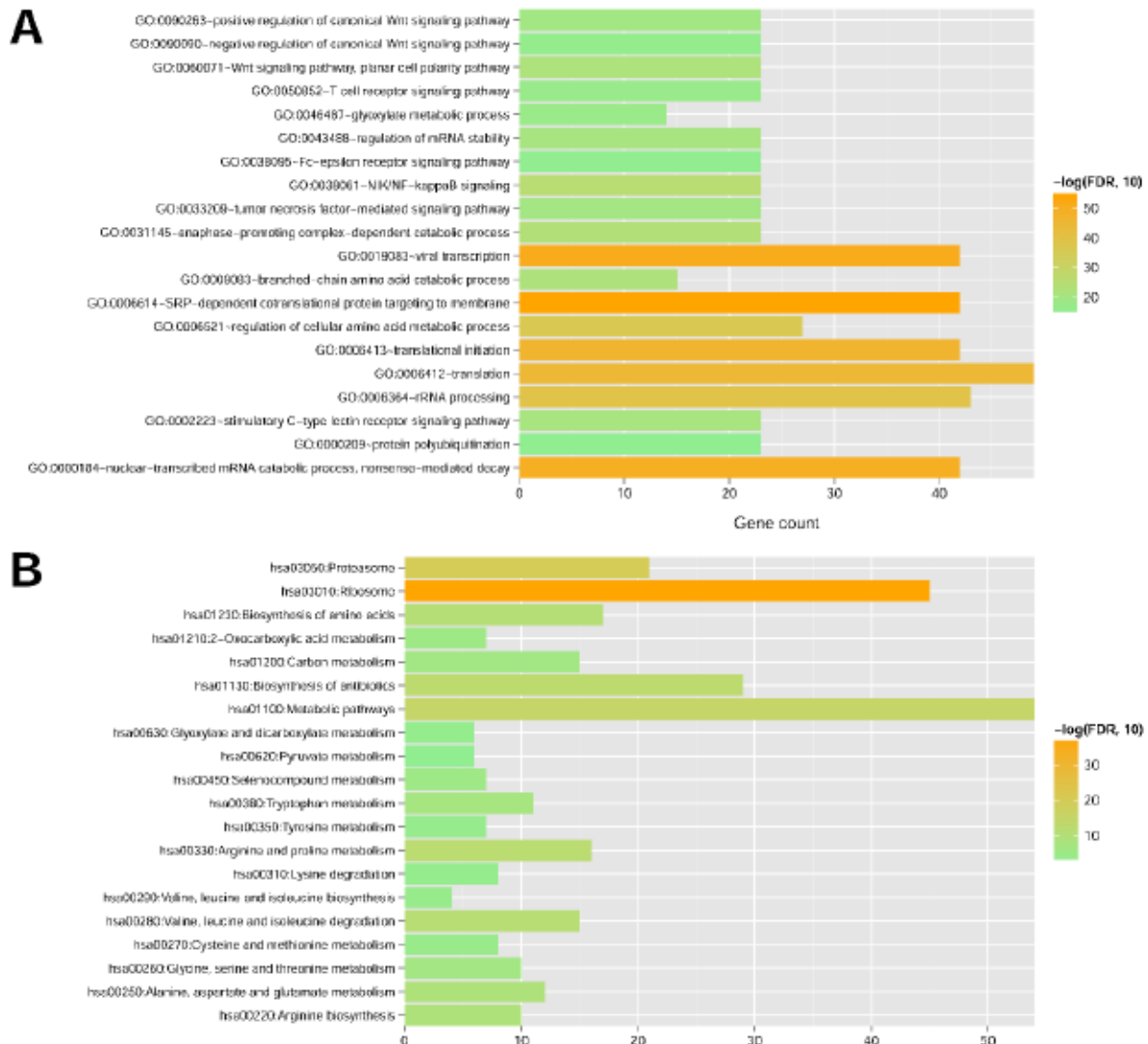


Fig. 3. Biological processes (A) and KEGG signaling pathways (B) associated with genes in ceRNA network. The column color closer to salmon pink represents a more significance.

Assessment and validation of prognostic risk models

Having screened twelve DERs, we established a 12 signature genes-based RS model by analysis of LASSO coefficients values and expression level in the training set. RS of each patient was calculated, and samples in the two datasets (TCGA training set and GEO validation set) were divided into two groups. Kaplan-Meier curve showed the grouping

was significantly correlated with actual disease recurrence prognosis. Patients in the high-risk group suffered from a lower survival ratio than those in the low-risk group, and the results were consistent in the training and the validation set. After the generation of ROC curves, the values of AUC were 0.924 and 0.843, indicating the risk model showed high accuracy for recurrence prediction (Fig. 5).

Table I. Optimization of differential expressed mRNAs associated with recurrence prognosis of COAD

ID	Type	Multi-variable cox regression			LASSO Coef
		Pvalue	Hazard Ratio	95%CI	
C1RL-AS1	lncRNA	1.498E-02	2.814	1.223-6.475	0.35121
CECR7	lncRNA	9.970E-03	1.849	1.159-2.951	0.24541
H19	lncRNA	3.846E-02	1.059	1.031-1.205	0.08482
LINC01301	lncRNA	3.500E-02	1.588	1.033-2.441	0.17807
LINC01503	lncRNA	1.200E-03	1.986	1.311-3.008	0.41218
PRKAG2-AS1	lncRNA	4.525E-02	0.718	0.514-0.935	-0.23703
BCKDK	mRNA	1.445E-02	2.492	1.199-5.180	0.12188
CSAD	mRNA	4.750E-02	1.280	1.167-1.734	0.07491
GSR	mRNA	4.536E-02	0.774	0.671-0.901	-0.00122
PSMB10	mRNA	2.160E-03	0.539	0.363-0.800	-0.22789
PSMC5	mRNA	1.590E-03	3.075	1.553-6.162	0.43150
RPL9	mRNA	4.878E-02	0.973	0.665-0.988	-0.13052

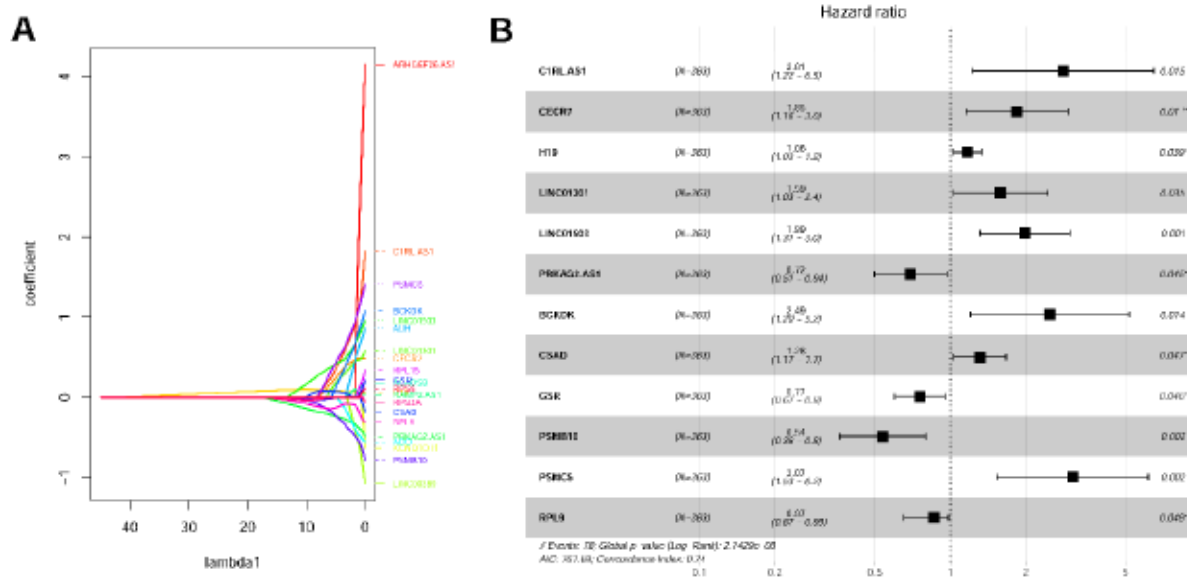


Fig. 4. Coefficient diagram of Lasso algorithm (A) and forest map of optimized DERs (B) related to recurrence prognosis.

Identifying independent prognostic clinical factors for COAD

Combining with clinical information of TCGA samples, we conducted cox regression analysis to identify clinical factors related to the patient's prognosis (Table II). Our results showed that pathologic stage (HR (95% CI): 1.423[1.055-1.920], $P < 0.05$) and RS status (HR (95% CI): 2.697 [1.574-4.623], $P < 0.001$) were two independent clinical factors correlated with recurrence prognosis of COAD patients.

In addition, we compared the predictive accuracy of the two independent clinical factors (pathologic stage and RS status) and the combined model (pathologic

stage combined with RS status) with ROC analysis in the training set and validation set, respectively. The results showed that the AUC value of the combined model (0.940) was higher than that of RS (0.924) or pathologic stage (0.605) in the TCGA training set (Fig. 6A). Besides, the AUC value of the combined model (0.876) was also higher than that of RS (0.842) or pathologic stage (0.574) in the GEO validation set (Fig. 6B). These results indicated a high accuracy of the combined model.

DISCUSSION

This present study, we analyzed the transcriptome

Table II. Univariable and multivariable cox regression analysis to identify independent clinical factor associated with COAD recurrence prognosis

Clinical characteristics	TCGA (N=363)	Univariable cox		Multivariable cox	
		HR (95% CI)	P value	HR (95% CI)	P value
Age (years, mean $\pm$ SD)	65.79 $\pm$ 12.83	0.989[0.971-1.006]	1.94E-01	-	-
Gender (Male/Female)	194/169	1.630[1.030-2.579]	3.52E-02	1.524[0.959-2.424]	7.49E-02
Pathologic M(M0/M1/-)	263/47/53	1.498[0.815-6.072]	2.16E-01	-	-
Pathologic N(N0/N1/N2)	210/90/63	0.909[0.537-1.540]	7.23E-01	-	-
Pathologic T(T1/T2/T3/T4)	10/61/249/42/1	2.310[1.484-3.597]	3.17E-04	1.575[0.916-2.707]	1.01E-01
Pathologic stage (I / II / III / IV /-)	59/140/107/47/10	1.849[1.423-2.402]	2.87E-06	1.423[1.055-1.920]	2.12E-02
Risk score status (High/Low)	181/182	3.523[2.099-5.912]	2.28E-07	2.697[1.574-4.623]	3.07E-04
Recurrence (Yes/No)	78/285	-	-	-	-
Recurrence free survival time (months, mean $\pm$ SD)	29.67 $\pm$ 25.91	-	-	-	-

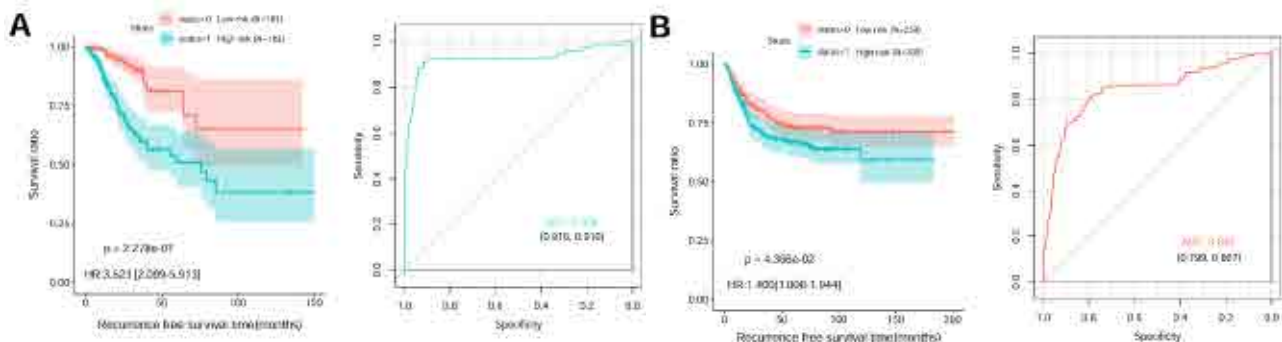


Fig 5. Kaplan-Meier curves (left) plot and receiver operating characteristic analysis (right) in TCGA training set and testing dataset. Green and red color represents risk grouping individuals.

sequencing data and screened numerous DERs from recurrence COAD patients, including a cohort of lncRNAs. A total of 197 amino acid metabolism-related genes were identified. ceRNAs regulatory network were constructed and these DERs might regulate COAD progression through several biological processes and pathways, viral transcription, translation, ribosome, metabolic pathways, alanine, aspartate and glutamate metabolism. A twelve signature genes-based RS model was established and exhibited a high accuracy for recurrence prognosis prediction.

Amino acids metabolism is indispensable for rapid proliferation of cancer cells and metabolic changes are considered as a crucial hallmark in cancer development (18). Metabolism reprogram enables tumor cells to adapt to a nutrition deficient environment through increasing metabolic rate of glucose, fatty acids and glutamine (19). Our results demonstrated that several DERs were enriched in pathway of alanine, aspartate and glutamate metabolism, including *ALDH4A1*, *GLUD1*, *GLUL*, and *GLS*. Here, we focused on the glutamate metabolism in cancer progression. Glutamine is

a conditionally amino acid in human body, and cancer cells display higher demand for glutamine than normal cells (20; 21). Tumor cells favor Gln addiction by upregulating the cell membrane transporter protein (22). Glutamine is imported to cells by transporters of ASCT2 (SLC1A5) or other transporters, then metabolized to produce energy through α -ketoglutarate generation in tricarboxylic acid cycle (23; 24). SLC1A5 is a main amino acid transporter and is required for colorectal cancer growth in KRAS-mutant types (25). The *SLC1A5* expression level was significantly correlated with tumor depth and vascular invasion. *SLC1A5*-knockdown resulted in suppression of malignant survival and cell migration (26). Many studies have indicated that *SLC1A5* could be an effective therapeutic target against KRAS-mutant colorectal cancer. Glutaminolysis begins with conversion of glutamate. Glutamate not only provides the amino groups for non-essential amino acids biosynthesis, but also is involved in synthesis of glutathione (GSH), which is the major cellular antioxidant (27). The catalyzed reaction of glutamate is limited

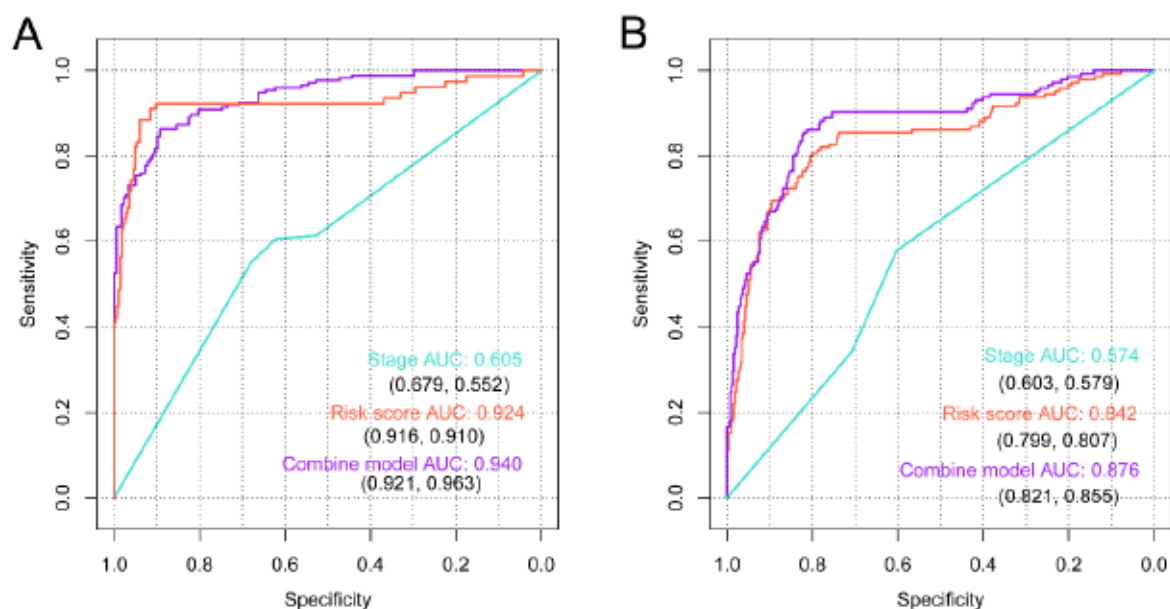


Fig. 6. ROC curve of independent prognostic clinical factors and the combined model. **A)** ROC curves for accuracy assessment based on independent clinical factors of stage and risk score or their combined model in the TCGA set. **B)** ROC curves for accuracy assessment based on independent clinical factors of stage and risk score or their combined model in the GSE39582 set. The number in the brackets represents the specificity and sensitivity of ROC curves.

by mitochondrial glutaminase (GLS), an enzyme encoded by two genes, kidney-type (GLS1) and liver-type (GLS2) glutaminases (28). GLS2 functions as a tumor suppressor, while GLS1 acts as an oncogene overexpressed in multiple cancer types. Inhibition of GLS-1 could suppress cancer cell proliferation through deprivation of glutamine metabolism, thus several GLS1 inhibitors have been developed into clinical trials (29).

A twelve genes signature was constructed for disease prediction, including six lncRNAs and six mRNAs. The lncRNA *LINC01503* has been well-characterized in several cancer types and high expression level predicts worse prognosis in glioma, squamous cell carcinoma, and colorectal cancer (30-32). It operates cellular functions through multiple different mechanisms. Lu et al. reported *LINC01503* promotes colorectal cancer growth by regulating miR-4492 and downstream pathway (30). In the non-small cell lung cancer, *LINC01503* could be regulated by transcription factor *c-MYC* and thus activates the ERK/MAPK signaling pathway to drive disease progression (33). Moreover, the gene of *CIRL* is correlated with immunological activity regulation, and act as an unfavorable prognostic biomarker in glioma, hepatocellular carcinoma (34). The antisense lncRNA *CIRL-AS1* was reported being upregulated in gastric cancer and probably exerted its biological function through AKT/ β -catenin pathway (35). Meanwhile, a recent study showed a novel lncRNA *PRKAG2-AS1* was upregulated in hepatocellular carcinoma and could promote cancer progression through binding with miR-502-3p (36). Taken together, lncRNAs of *CIRL-AS1*, *PRKAG2-AS1* and *LINC01503* might be potential prognostic markers for COADs.

As for the six genes in RS model, we found glutathione reductase (GSR) is involved in oxidation-reduction process, *BCKDK* is related to branched-chain amino acid catabolic process and CSAD was involved in metabolic pathways. It is well known that GSR and glutathione peroxidase (Gpx1) catalyze the redox metabolic cycle of GSH, and thus maintain intracellular redox homeostasis via thiol/disulfide bonds and NADPH/NADP⁺ exchange (37). GSR mediating oxidative stress was involved in

modulation of tumor metastasis and drug resistance in some cancer types (38; 39). In colorectal cancer, a recent study reported AMPK α 1, as an upstream regulator of GSR, confers survival advantage of cancer cells by regulating GSR phosphorylated level (40). These findings revealed GSR could be potential target for improving cancer therapy. Meanwhile, two genes (*PSMB10* and *PSMC5*) were related to proteasome pathway and several biological processes. PSMB10 (proteasome subunit beta type-10) is a major subunit for 20S proteasome complex. After exposure to interferon- γ , three β subunits of 20S proteasome are replaced by immunoproteasome subunits PSMB8, 9, 10 (41). Proteasome subunits differential assembly is enabled to modulate tumor cell viability and proteasome inhibitor sensitivity in cancer (42). For example, myeloma cell expressing special-KRAS mutant types showed increased expression of proteasome maturation protein and proteasome subunit β of PSMB 8,9,10 by an ETS -dependent manner, which finally induced proteasome inhibitor resistance (43). Overexpression of PSMB10 was linked to adverse prognosis in acute myelocytic leukemias (44). In addition, PSMC5 is also known as 26S protease regulatory subunit 8 and typically expressed in the cytoplasm. The 26S proteasome is responsible for protein degradation. It is enabled to unfold polyubiquitin-modified proteins by using energy from ATP hydrolysis, and translocate proteins to degradation chamber for proteolysis (41). Previous study has revealed that PSMC5 promoted radiational damage effect in lung cancer cells and may serve as a prognostic biomarker for radiotherapy and target therapy (45). Jhaveri et al. showed that increased tissue expression levels of *PSMC5*, *MYPT1* and *TFRC* were correlated with pancreatic cancer development (46). The potential role of PSMC5 in COAD was fewer reported. Here, our resulted identified that PSMC5 and PSMB8 were dysregulated in tumor samples and associated with COAD recurrence prognosis, indicating the two genes might play crucial roles in cancer development. However, further studies are needed to verify whether the two targets could be possible prognostic biomarkers in COAD.

There were still some limitations in our study.

Although the RS model exhibited a high accuracy performance for recurrence prediction, the amino acids metabolism-related genes in our model need further experimental verification, especially by clinical COAD specimens. Besides, several genes of risk model were fewer reported in COAD progression and the biological function should be explored. Last, the samples from TCGA dataset are limited and more data are required to fully verify reliability of the combined prognostic model.

In summary, we developed a twelve-gene signature for recurrence prognosis prediction of COAD patients. The combined prognostic model integrated clinical factors and risk score status displayed high predictive performance. Our study provides a novel insight for understanding COAD progression.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of interest

No conflict of interest.

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Long non-coding RNA SNHG7 silencing confers protection against epithelial-mesenchymal transition in ovarian cancer cells via microRNA-34a-targeted EDG4 and the PI3K/AKT pathway

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Long non-coding RNA small nucleolar RNA host gene 7 (lncRNA SNHG7) and microRNA-34a (miR-34a) have been implicated in ovarian cancer (OC) development. Furthermore, the binding of SNHG7 to miR-34a is widely recognized in numerous cancers. Therefore, our research intended to explore the specific mechanism of SNHG7 and miR-34a in OC. SNHG7, miR-34a, and endothelial differentiation gene (EDG) 4 expression in harvested OC tissues and cells were measured by RT-qPCR. Dual-luciferase reporter gene assay and RIP assay were implemented to assess the binding of SNHG7 to miR-34a, and of miR-34a to EDG4. Gain- and loss-of-function assays were implemented in OC cells, followed by scratch test and Transwell assay of cell migration and invasion, respectively. The epithelial-mesenchymal transition (EMT) and the PI3K/AKT pathway-related proteins were measured by Western blot analysis. SNHG7 and EDG4 were overexpressed, but miR-34a was poorly expressed in OC tissues and cells. miR-34a bound to SNHG7 and targeted directly EDG4, and SNHG7 activated the PI3K/AKT pathway in OC cells. Furthermore, SNHG7 or EDG4 silencing, miR-34a overexpression, or PI3K/AKT pathway bluntness diminished OC cell invasion, migration, and EMT. Collectively, SNHG7 downregulation repressed EMT of OC cells via miR-34a-targeted EDG4 and the PI3K/AKT pathway.

Ovarian cancer (OC) arises from serous intraepithelial carcinoma or ovarian surface epithelium (1). OC ranks the 7th most prevalent cancer among women and is the 8th most principal cause of cancer-induced mortality on a global scale (2). Early diagnosis improves survival of OC patients, but unfortunately, only 15% OCs are diagnosed early or locally (3). Defined as a process that epithelial cells transform into mesenchymal cells, epithelial-mesenchymal transition (EMT) is strongly linked to cancer cell migratory, invasive, stem-like properties, and drug resistance (4). EMT is regarded as a pivotal orchestrator of the post-ovulation repair process in ovarian surface epithelium, which can be triggered by a series of environmental stimuli (5). Moreover,

it is documented that EMT assumes a critical role in OC development and that alterations in EMT-related cellular pathways can be utilized as useful candidate target for OC treatment (6). Therefore, it is critical for OC therapy to figure out the molecular mechanism underlying EMT in OC.

Mounting evidence illustrates the involvement of long non-coding RNAs (lncRNAs) in various physiological and pathological tumor activities, including EMT and cancer metabolism (7). Furthermore, lncRNA small nucleolar RNA host gene 7 (SNHG7) was capable of stimulating tumorigenesis by orchestrating EMT in nasopharyngeal carcinoma (8). More importantly, the oncogenic role of SNHG7 has been detected in OC development (9).

Key words: Ovarian cancer; Long non-coding RNA SNHG7; microRNA-34a; EDG4; PI3K/AKT pathway; Epithelial-mesenchymal transition

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Interestingly, it was noted that microRNA (miR)-34a was a target miR of SNHG7 (10). miR-34a was able to repress EMT of lens epithelial cells (11). Moreover, a prior study illustrated the anti-oncogenic role of miR-34a in OC by hampering cell proliferation, colony formation, invasion and migration (12).

Intriguingly, the targeting relationship was predicted between miR-34a and endothelial differentiation gene (EDG) 4 by TargetScan in our study. EDG4, also named as lysophosphatidic acid receptor 2 (LPA2), has been detected to be upregulated in numerous cancers (13). For instance, EDG4 functioned as an orchestrator in the metastasis, invasion, and EMT of gastric cancer cells (14). EDG4 could assume a critical role in aggressiveness and carcinogenesis of OC (15). It was established that LPA modulated phosphatidylinositol 3-kinase (PI3K)/AKT pathway to increase intestinal DRA gene transcription (16). Blocked PI3K/AKT pathway by TRPM7 silencing was able to contribute to suppression of the EMT and metastasis of OC cells (17). In this context, it was hypothesized that the SNHG7/miR-34a/EDG4/PI3K/AKT regulatory network was involved in OC development. Thus, tissue and cell experiments were implemented in this study to validate this network in OC.

MATERIALS AND METHODS

Compliance with ethical standards

The experiments were implemented under ratification from the Ethics Committee of the Second People's Hospital of Lanzhou and following the principles outlined in the *Declaration of Helsinki*. Ethical agreements were obtained from the donors or their relatives by written informed consent.

Study subject

Thirty-six OC tissue specimens surgically resected in the Second People's Hospital of Lanzhou were harvested from September 2015 to September 2018. None of the patients received chemotherapy or radiotherapy before surgery, and the diagnosis was made by postoperative pathological examination. Paracancerous tissues were obtained from normal ovarian tissues more than 5 cm from the margin of cancer tissues. Each tissue specimen was frozen in liquid nitrogen.

Cell treatment

OC cell lines A2780 (BeNa Culture Collection, Beijing, China), OVCA433 (Bioshhy, Shanghai, China), and SKOV-3 (American Type Culture Collection, Manassas, VA, USA) and normal human ovarian cell IOSE-80 (BeNa Culture Collection) were included in this study. These cells were cultured with Roswell Park Memorial Institute 1640 medium (Gibco, Carlsbad, CA, USA) encompassing 10% fetal bovine serum (FBS, Gibco) in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) with saturated humidity and 5% CO₂ at 37°C after recovery. When the cells were adherent to the wells and the cell density reached 80%, the cells were subcultured at 1:3 ratio following 3-4 day of trypsinization, then the cells in the logarithmic growth period were collected for the subsequent assays.

Transfection was conducted on OVCA433 and SKOV-3 cells in logarithmic growth phase. One day before transfection, logarithmically growing OVCA433 and SKOV-3 cells were seeded in 12-well plates at a concentration of 1×10^5 cells/mL. When the cell confluence achieved 50%-70%, 800 µl serum-free medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to each well containing cells. The transfection experiment was carried out as per the methods provided by the manuals of lipo2000 (11668027, Thermo Fisher Scientific) reagent. After 6-h cell transfection, the medium was renewed for continuous cell incubation. All plasmids were attained from Miaolingbio (Wuhan, Hubei, China). LY294002 was attained from Cell Signaling Technology (#9901, CST, Beverly, MA, USA). The concentration of LY294002, which was dissolved in dimethyl sulfoxide (DMSO), was 20 µmol/L.

Scratch test

A marker pen was utilized to draw horizontal lines evenly behind a 6-well plate at intervals of 0.8 cm, with at least five lines through each well. The logarithmically growing cells were collected and cultured in the 6-well plate for 24 h. The 6-well plate was vertically and evenly lined with a 200-mL pipette. The scratched cells were washed off by 3 sterile PBS washes, and the plate was added with serum-free medium (Thermo Fisher Scientific) before incubation with 5% CO₂ at 37°C. Samples were photographed at 0 and 48 h after seeding under the microscope (Olympus, Tokyo, Japan), followed by measurement of cell migration distance.

Transwell assay

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was melted at 4°C on ice, mixed with serum-free medium at 1:1 and polymerized in a Transwell chamber (50 µL/well, Corning Glass Works, Corning, N.Y., USA) at 37°C. Cells in logarithmic phase were starved with culture medium containing 1% FBS for 24 h. The apical chamber was filled with culture medium plus 2% FBS medium, and the basolateral chamber with culture medium plus 10% FBS medium. After detachment and centrifugation, the starved cells were attained and resuspended with serum-free medium for counting. Following preparation of 5×10^6 cells/mL cell suspension, 100 µL cell suspension was supplemented into the corresponding apical chamber. Besides, 800 µL medium encompassing 10% FBS was supplemented into the basolateral chamber. The cells were cultured in a 37°C incubator with 5% CO₂ for 24 h. The Transwell chamber was fixed with 4% paraformaldehyde before crystal violet staining and returning to blue with water. The cells on the chamber were wiped with cotton balls, dehydrated, and cleared. Five fields of view were selected using the five-point sampling method under an inverted microscope (400 ×, Olympus) to count the invasive cells, followed by calculation of the mean value.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated as per the protocols of a Trizol Kit (Invitrogen, Carlsbad, CA, USA). SNHG7 and EDG4 primers were designed and synthesized by AuGCT Biotechnology Company (Beijing, China) (Table I). The RNA was dissolved with diethyl pyrocarbonate

ultrapure water. The optical density (OD) value at 260 nm and 280 nm was measured by a 752/752N ultraviolet/visible spectrophotometer (752N, Beijing UESIPHY Development Co., Ltd., Beijing, China). The total RNA was qualitatively identified, followed by estimation of the concentration. An eppendorf tube were added with 5 µL Mix (T025A, Guangzhou Yingzan Biotechnology Co., Ltd., Guangdong, China) reagent, 5 µL total RNA, and 10 µL RNase Free H₂O, centrifuged, and mixed. The mixture was plated into the PCR instrument (T100, Bio-Rad Laboratories, Shanghai, China) for reaction under the following conditions: at 37°C for 15 min; at 85°C for 5 s. The reaction was terminated at 4°C, and the generated cDNA was stored at 20°C. RT-qPCR was implemented on an ABI StepOnePlus Quantitative PCR Instrument (StepOnePlus, ABI Company, Oyster Bay, NY, USA) using SYBRGreen Fluorescent Dye (163795-75-3, Shanghai Jining Industrial Co., Ltd., Shanghai, China) Method. Relative level of target mRNAs and miRs was calculated using the $2^{-\Delta\Delta C_t}$ methods standardized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, for EDG4 and SNHG7) and U6 (for miR-34a) (18).

Western blot analysis

Cells were lysed with radio-immunoprecipitation assay (RIPA) lysis at 4°C and 8000 rpm for 30 min and shaken every 10 min, followed by estimation of protein concentration using bicinchoninic acid protein quantification kit (Solabio, Beijing, China). Subsequent to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, 50 µg proteins were electroblotted into a nitrocellulose membrane (LC2005, Thermo Fisher Scientific) which underwent 2-h sealing

Table I. *Primer sequences for RT-qPCR*

Targets	Primers
SNHG7	F: 5'-AGCGCTTACCACTGTCCATT-3' R: 5'-TGCCCAGGTGAACTGTTTCT-3'
miR-34a	F: 5'-ACACTCCAGCTGGGTGGCAGTGTCTTAGCT-3' R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAAT-3'
EDG4	F: 5'-TTGTCTTCCTGCTCATGGTG-3' R: 5'-TCAGCATCTCGGCAAGAGTA-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	F: 5'-ACCACACTTTCTACAATGAGCTG-3' R: 5'-CTTCTCTTTAATGTCACGCACG-3'

with 5% skimmed milk powder at room temperature. Overnight membrane incubation was implemented at 4°C with primary rabbit antibodies from Abcam (Cambridge, UK) to EDG4 (ab135980), matrix metalloproteinase (MMP)-2 (ab37150), MMP-9 (ab73734), E-cadherin (ab40772), Vimentin (ab92547), AKT (ab8805), phosphorylation (p)-AKT (ab38449), PI3K (ab139307), and GAPDH (ab181602) and primary rabbit antibody from CST to p-PI3K (#17366). Then, horseradish peroxidase-tagged goat anti-rabbit Immunoglobulin G (IgG) secondary antibody (ab97051, Abcam) was supplemented for 1-h membrane incubation at room temperature. After 1-min development in electrogenerated chemiluminescence working fluid (BM101, Biomiga, San Diego, CA, USA), the relative level of proteins was calculated using the ratio of gray value of target band to internal reference band (GAPDH).

Dual-luciferase reporter gene assay

To evaluate the relationship between SNHG7 and miR-34a as well as between miR-34a and EDG4, the artificially synthesized SNHG7 3'untranslated region (UTR) and EDG4 3'UTR gene fragments were inserted into pMIR-reporter plasmids (Beijing Huayueyang Biotechnology, Beijing, China) by double enzymatic digestion. Complementary sequence mutation sites of seed sequences were designed on SNHG7 and EDG4 wild-type (WT), respectively, and inserted into pMIR-reporter reporter plasmids using T4 DNA ligase after restriction endonuclease digestion. The correctly sequenced WT or mutant type (MUT) luciferase reporter plasmids were co-transfected with negative control (NC) mimic or miR-34a mimic into HEK-293T cells (Cell Resource Center, Shanghai Institute of Biological Sciences, Shanghai Academy of Chinese Sciences, Shanghai, China). Forty-eight h after transfection, the cells were lysed before determination of luciferase activity using a luciferase detection kit (K801-200; Biovision, Mountain View, CA, USA) on a Glomax20/20 luminometer fluorescence detector (Promega, Madison, WI, USA).

RNA immunoprecipitation (RIP) assay

The binding of SNHG7 and EDG4 to Argonaute2 (Ago2) was detected by RIP kit (Millipore, Temecula, CA, USA). Cells were washed with precooled PBS, and the supernatant was discarded. Cells were lysed in an ice

bath with equal volume of RIPA lysis (P0013B, Beyotime, Shanghai, China) for 5 min, and the supernatant was removed after a 10-min centrifugation at 14000 rpm and 4°C. A portion of the cell extract was taken out as input, and a portion was co-precipitated with antibody. Specifically, 50 µL magnetic beads in each co-precipitation reaction system was resuspended in 100 µL RIP wash buffer, followed by an incubation with 5 µg antibody according to the experimental grouping for binding. The magnetic bead-antibody complex was resuspended in 900 µL RIP wash buffer before overnight incubation with 100 µL cell extract at 4°C. Samples were placed on a magnetic base to collect the bead-protein complexes. Samples and Input were treated with proteinase K to extract RNA for subsequent RT-qPCR. Antibody against Ago2 (1: 50, ab32381, Abcam) was mixed at room temperature for 30 min, and anti-IgG (1: 100, ab109489, Abcam) was applied as a NC. RT-qPCR was adopted to assess SNHG7 and EDG4 expression.

Fluorescence in Situ Hybridization (FISH)

Identification of subcellular localization of RNA SNHG7 was performed in cells by FISH as per the manuals of RiboTM lncRNA FISH Probe Mix (Red) (RiboBio, Guangdong, China). Briefly, cover slides were placed in 24-well plates, and cells were cultured at 6×10^4 cells/well until the cell confluence achieved about 80%. The slides were harvested and fixed with 1 mL of 4% paraformaldehyde at room temperature. Following proteinase K (2 µg/mL), glycine, and ethylphthalide reagent treatment, 250 µL pre-hybridization solution was supplemented for a 1-h cell incubation at 42°C. After discarding of the pre-hybridization solution, 250 µL hybridization solution containing the probe (300 ng/mL) was supplemented to hybridize cells overnight at 42°C. Cell nuclei was stained with 4',6-Diamidino-2-Phenylindole (1:800, Thermo Fisher Scientific) diluted with phosphate-buffered saline/Tween-20 in the 24-well plate for 5 min. Five different visual fields were observed and photographed under a fluorescence microscope (Olympus) after cell mounting with anti-fluorescence quencher.

Statistical analysis

All measurement data were shown as mean $\pm$ standard deviation and analyzed by SPSS 21.0 software (IBM,

Armonk, NY, USA), with $p < 0.05$ as a level of statistical significance. Pairwise comparison between groups was implemented with paired t test, while comparisons among multiple groups were performed using one-way analysis of variance (ANOVA).

RESULTS

High SNHG7 expression was observed in OC tissues and cell lines

Initially, to figure out whether SNHG7 was involved in OC development, SNHG7 expression in OC was determined. RT-qPCR in 36 OC and paracancerous tissues revealed SNHG7 upregulation in OC tissues (Fig. 1A). Also, RT-qPCR showed higher SNHG7 expression in OC cell lines (A2780, OVCA433, and SKOV-3) than in normal human ovarian cell IOSE-80 with the highest expression in OVCA433 and SKOV-3 cell lines (Fig. 1B). Hence, the following experiments were implemented on OVCA433 and SKOV-3 cell lines. The silencing efficiency of SNHG7 was tested before the experiment (Fig. 1C), which found that small interfering RNA (si-RNA) SNHG7 significantly decreased SNHG7 expression in OVCA433 and SKOV-3 cells. Collectively, SNHG7 overexpression correlated to OC development.

Silencing of SNHG7 repressed OC cell migration, invasion, and EMT

To further investigate the role of SNHG7 in

OC, OVCA433 and SKOV-3 cells were transfected with si-NC or si-RNA SNHG7. As reflected by scratch test (Fig. 2A) and Transwell assay (Fig. 2B), SNHG7 silencing diminished migration distance and invasion of OVCA433 and SKOV-3 cells. The EMT-related proteins (MMP-2, MMP-9, Vimentin, and E-cadherin) were measured by Western blot analysis, demonstrating downregulated MMP-2, MMP-9, and Vimentin but upregulated E-cadherin in SNHG7-silenced OVCA433 and SKOV-3 cells (Fig. 2C). Conclusively, SNHG7 downregulation caused inhibition of OC cell migration, invasion, and EMT.

SNHG7 increased EDG4 expression by binding to miR-34a in OC cells

It was predicted through LNCIPEDIA (<https://lncipedia.org/>) that SNHG7 could target and bind to miR-34a. Bioinformatics analysis displayed that SNHG7 was localized in the cytoplasm, which was further validated through FISH experiments (Fig. 3A). As for RT-qPCR results, it was found that miR-34a expression was lower in OC tissues than in paracancerous tissue (Fig. 3B). RIP results manifested that compared with anti-IgG, SNHG7 bound to anti-Ago2 was obviously increased, and that anti-Ago2 antibody could precipitate SNHG7, indicating that SNHG7 could form a complex with Ago2 (Fig. 3C). The results suggested that the complex formed by SNHG7 and Ago2 could competitively bind to miR-34a and reduce the degree of free miR-34a.

It was predicted by TargetScan (<http://www.targetscan.org/>)

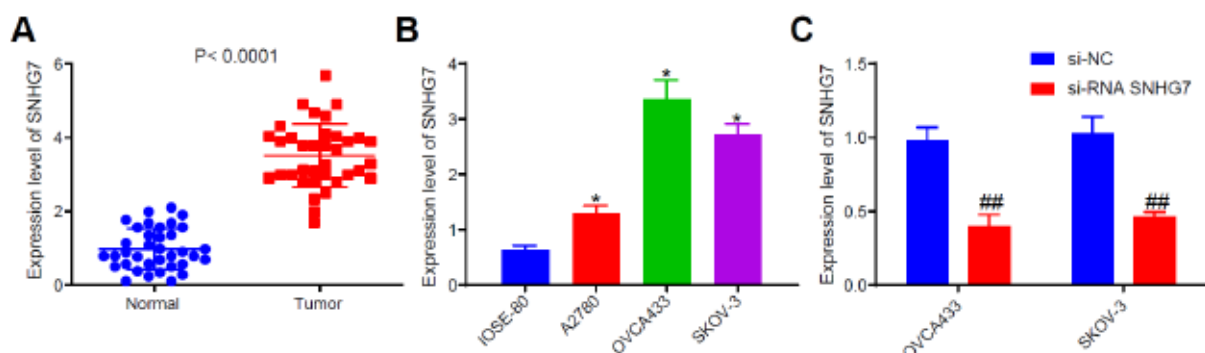


Fig. 1. SNHG7 is highly expressed in OC tissues and cells. **A:** SNHG7 expression in 36 OC and paracancerous tissues detected by RT-qPCR ($p < 0.0001$). **B:** SNHG7 expression in OC cell lines (A2780, OVCA433, and SKOV-3) and normal human ovarian cell IOSE-80 determined by RT-qPCR. **C:** SNHG7 silencing efficiency in OVCA433 and SKOV-3 cells measured by RT-qPCR. * $p < 0.05$ vs IOSE-80 cell; ## $p < 0.01$ vs OVCA433 and SKOV-3 cells transfected with si-NC.

targetscan.org/) that miR-34a could target EDG4 and bind to SNHG7 (Fig. 3D). After RT-qPCR validation of the transfection efficiency of miR-34a mimic and miR-34a inhibitor in OVCA433 and SKOV-3 cells (Fig. 3E), dual-luciferase reporter gene assay was conducted. The results described that miR-34a mimic insignificantly impacted the luciferase activity in MUT-EDG4-3'UTR or MUT-SNHG7-3'UTR but lowered the luciferase activity in WT-EDG4-3'UTR or WT-SNHG7-3'UTR (Fig. 3F), indicating that miR-34a could target EDG4 and bind to SNHG7. Based on Western blot analysis results, miR-34a mimic reduced, but miR-34a inhibitor augmented EDG4 expression in OVCA433 and SKOV-3 cells

(Fig. 3G). In addition, it was detected by RT-qPCR that EDG4 expression was enhanced in OC tissues in contrast to paracancerous tissues (Fig. 3H).

To evaluate whether SNHG7 modulated EDG4 by binding to miR-34a, RIP experiment was carried out. As documented in Fig. 3I, in comparison to anti-IgG, EDG4 bound to anti-Ago2 was potently elevated, and anti-Ago2 antibody could precipitate EDG4, illustrating that EDG4 could form a complex with Ago2. Then, OVCA433 and SKOV-3 cells were transfected with overexpression (oe)-NC + NC mimic, oe-SNHG7 + NC mimic, or oe-SNHG7 + miR-34a mimic. Western blot analysis depicted the upregulated EDG4 in SNHG7-overexpressed

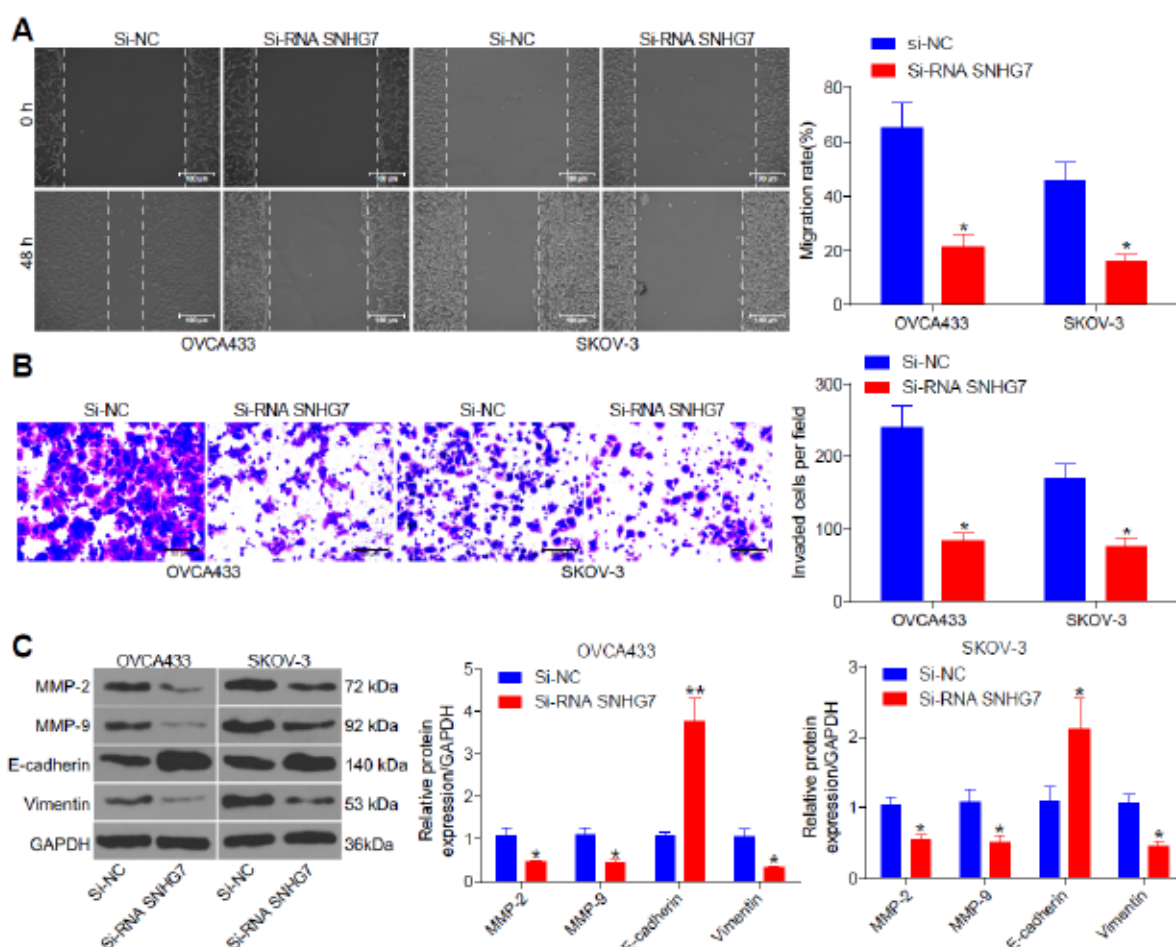


Fig. 2. OC cell migration, invasion, and EMT are suppressed by silencing SNHG7. OVCA433 and SKOV-3 cells were transfected with si-NC or si-RNA SNHG7. **A:** OVCA433 and SKOV-3 cell migration detected by Scratch test; **B:** OVCA433 and SKOV-3 cell invasion measured by Transwell assay; **C:** The EMT-related proteins (MMP-2, MMP-9, Vimentin, and E-cadherin) assessed by Western blot analysis; **p* < 0.05; ***p* < 0.01 vs OVCA433 and SKOV-3 cells transfected with si-NC.

OVCA433 and SKOV-3 cells, which was negated by additional miR-34a mimic treatment (Fig. 3J). Moreover, the overexpression efficiency of SNHG7 was verified by RT-qPCR (Fig. 3K). These results demonstrated that SNHG7 bound to miR-34a to activate EDG4 in OC cells.

SNHG7 overexpression elevated OC cell invasion, migration, and EMT by upregulating miR-34a-targeted EDG4

In order to explore whether SNHG7 modulated OC development via miR-34a and EDG4, OVCA433 and SKOV-3 cells were transfected with oe-NC +

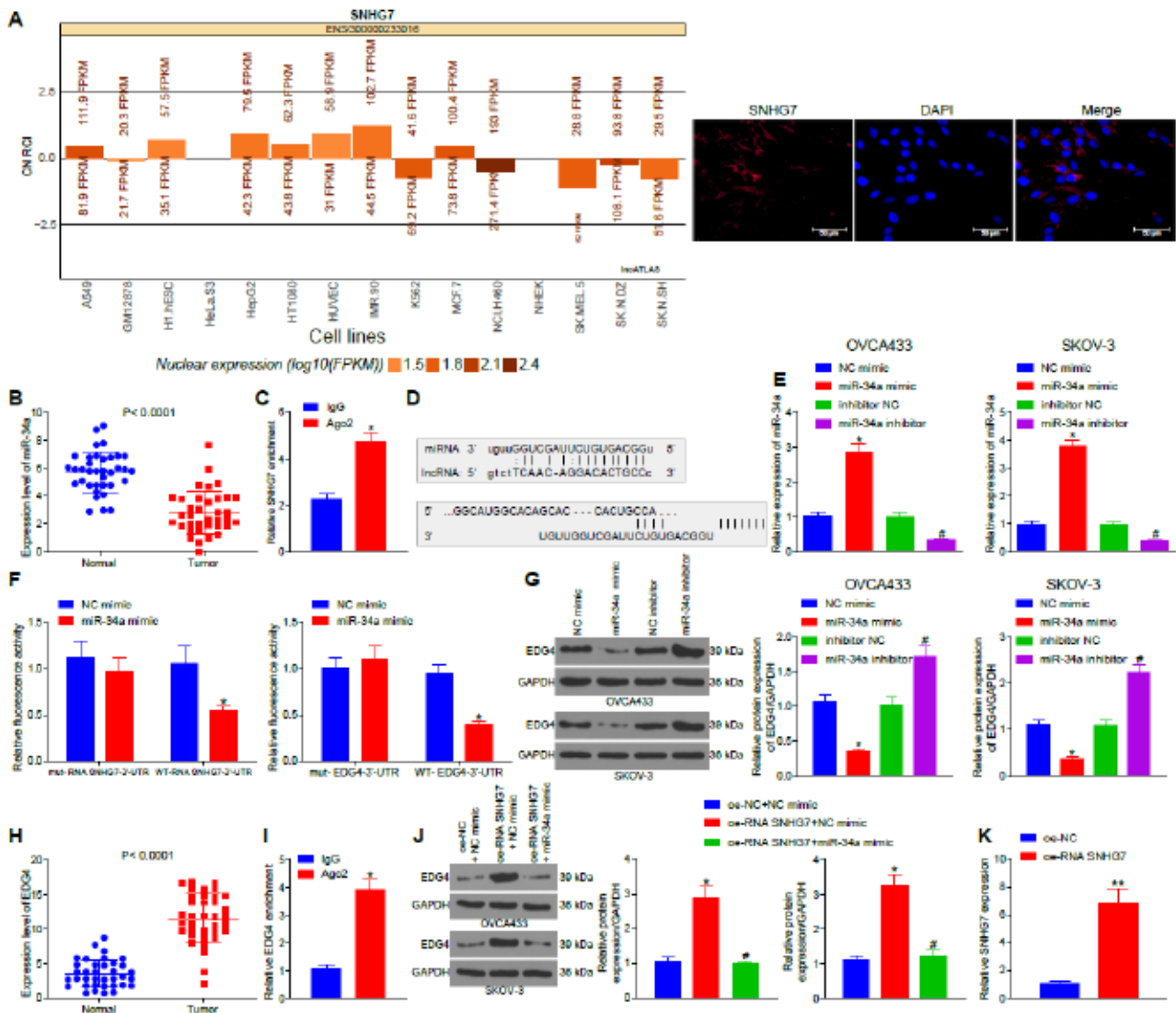


Fig. 3. SNHG7 binds to miR-34a, thereby upregulating EDG4 in OC cells. **A:** Bioinformatics analysis and FISH experiments confirming the localization of SNHG7 in the cytoplasm; **B:** RT-qPCR detection of miR-34a in 36 OC tissues and paracancerous tissues; **C:** RIP assay of binding between Ago2 and SNHG7; **D:** TargetScan prediction of targeting relationship of miR-34a with EDG4 and SNHG7; **E:** RT-qPCR detection of the transfection efficiency of miR-34a mimic and miR-34a inhibitor in OVCA433 and SKOV-3 cells; **F:** Dual-luciferase reporter gene assay in HEK-293T cells of the targeting relationship of miR-34a with EDG4 and SNHG7; **G:** Western blot analysis of the effect of miR-34a on EDG4 protein expression in OVCA433 and SKOV-3 cells; **H:** RT-qPCR analysis of EDG4 expression in OC and paracancerous tissues; **I:** RIP experiments of binding between Ago2 and EDG4; **J:** EDG4 expression in OVCA433 and SKOV-3 cells after transfection with oe-SNHG7 and miR-34a mimic detected by Western blot analysis; **K:** The overexpression efficiency of SNHG7 in OVCA433 and SKOV-3 cells measured by RT-qPCR. **p* < 0.05 vs transfection with NC mimic or NC mimic + oe-NC, or IgG; #*p* < 0.05 vs transfection with NC inhibitor or oe-SNHG7 + NC mimic.

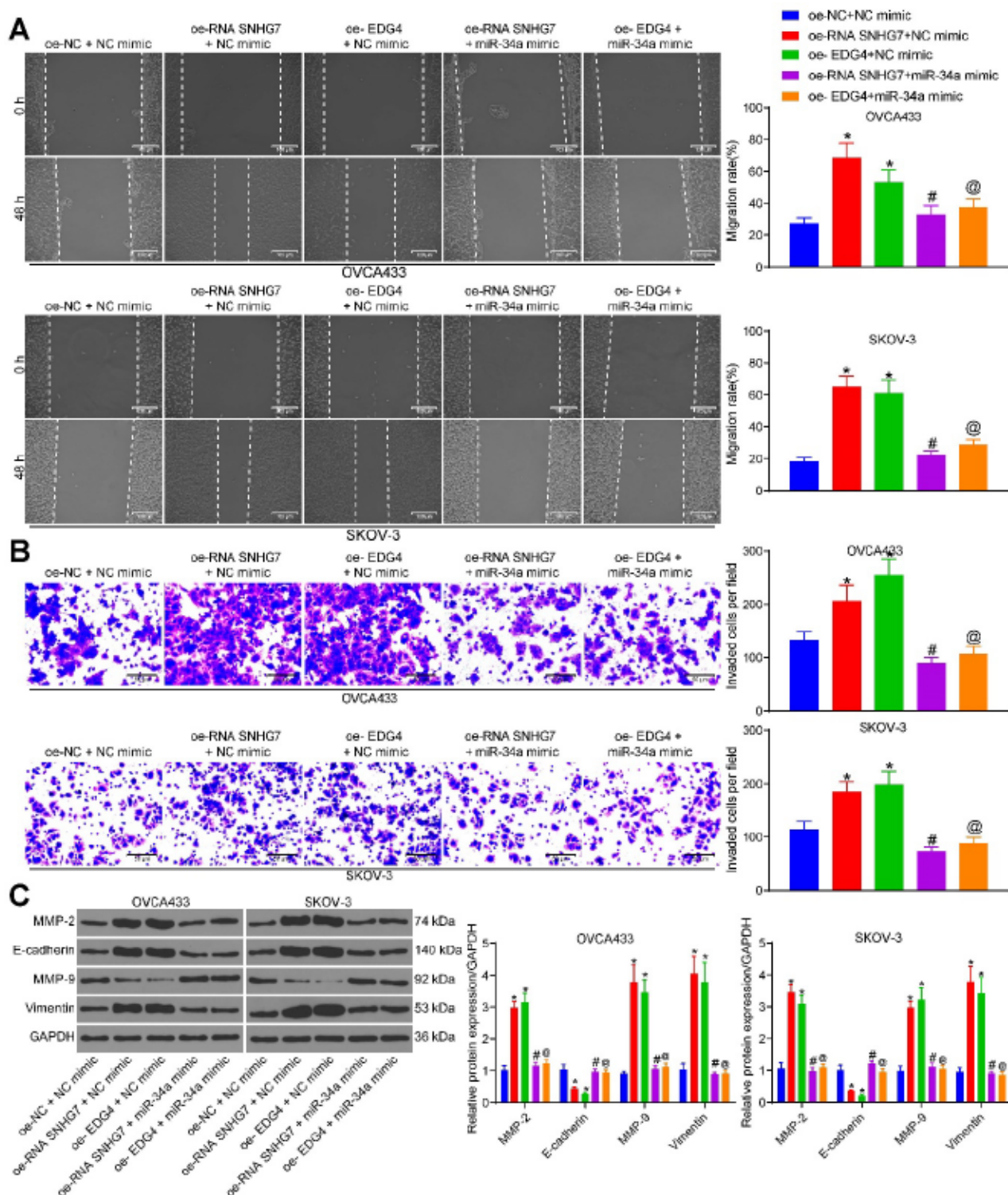


Fig. 4. SNHG7 upregulates EDG4 by binding to miR-34a to promote OC cell invasion, migration, and EMT. OVCA433 and SKOV-3 cells were transfected with oe-NC + NC mimic, oe-SNHG7 + NC mimic, oe-SNHG7 + miR-34a mimic, oe-EDG4 + NC mimic, or oe-EDG4 + miR-34a mimic. **A:** OVCA433 and SKOV-3 cell migration detected by Scratch test; **B:** OVCA433 and SKOV-3 cell invasion measured by Transwell assay; **C:** The EMT-related proteins (MMP-2, MMP-9, Vimentin, and E-cadherin) assessed by Western blot analysis. **p* < 0.05 vs transfection with NC mimic + oe-NC; #*p* < 0.05 vs transfection with oe-SNHG7 + NC mimic; @*p* < 0.05 vs transfection with oe-EDG4 + NC mimic.

NC mimic, oe-SNHG7 + NC mimic, oe-SNHG7 + miR-34a mimic, oe-EDG4 + NC mimic, or oe-EDG4 + miR-34a mimic. Scratch test (Fig. 4A) and Transwell assay (Fig. 4B) exhibited that cell migration distance and invasion of OVCA433 and SKOV-3 cells were augmented by overexpressing SNHG7 or EDG4, which was annulled by additional miR-34a mimic. As

depicted by Western blot analysis, upregulated MMP-2, MMP-9, and Vimentin but downregulated E-cadherin was found in SNHG7- or EDG4-overexpressed OVCA433 and SKOV-3 cells, whereas additional miR-34a mimic normalized these trends (Fig. 4C). In summary, SNHG7 activated miR-34a-targeted EDG4 to induce OC cell invasion, migration, and EMT.

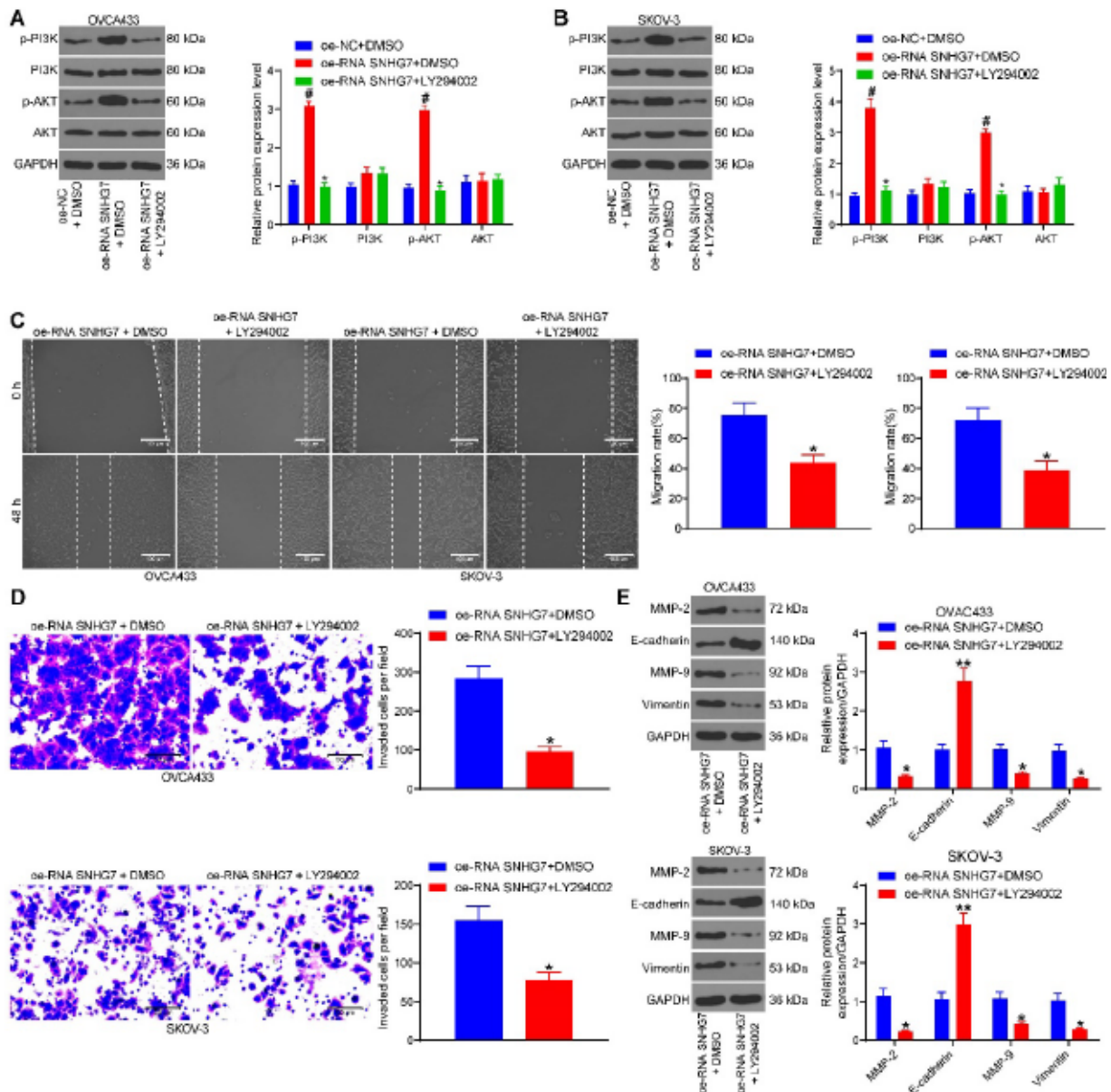


Fig. 5. SNHG7 silencing suppresses OC cell invasion, migration, and EMT via the PI3K/AKT pathway. OVCA433 and SKOV-3 cells were treated with oe-NC + DMSO, oe-SNHG7 + DMSO, or oe-SNHG7 + LY294002. **A:** Western blot analysis of AKT, PI3K, phosphorylated AKT, and phosphorylated PI3K expression in OVCA433 cells; **B:** Western blot analysis of AKT, PI3K, phosphorylated AKT, and phosphorylated PI3K expression in SKOV-3 cells; **C:** OVCA433 and SKOV-3 cell migration detected by scratch test; **D:** OVCA433 and SKOV-3 cell invasion measured by Transwell assay; **E:** The EMT-related proteins (MMP-2, MMP-9, Vimentin, and E-cadherin) assessed by Western blot analysis. **p* < 0.05, ***p* < 0.01 vs treatment with oe-SNHG7 + DMSO; #*p* < 0.05 vs treatment with oe-NC + DMSO.

SNHG7 silencing disrupted the PI3K/AKT pathway to hinder OC cell invasion, migration, and EMT

It has been reported that EDG4 activates PI3K/AKT pathway (19), which promotes EMT of OC (20). In order to investigate whether the PI3K/AKT pathway was involved in action of SNHG7 on OC, OVCA433 and SKOV-3 cells were treated with oe-NC + DMSO, oe-SNHG7 + DMSO, or oe-SNHG7 + LY294002 (an inhibitor of the PI3K/AKT pathway). As for Western blot analysis, SNHG7-overexpressed OVCA433 (Fig. 5A) and SKOV-3 (Fig. 5B) cells possessed obviously augmented phosphorylated AKT and PI3K expression and unchanged total AKT and PI3K expression, which was annulled by a PI3K/AKT pathway inhibitor, LY294002. This result suggested that silencing SNHG7 disrupted the PI3K/AKT pathway in OC cells.

As displayed by scratch test (Fig. 5C) and Transwell assay (Fig. 5D), SNHG7 overexpression led to elevation of OVCA433 and SKOV-3 cell migration distance and invasion, which was blocked by additional LY294002 treatment. Meanwhile, Western blot analysis documented enhancement in MMP-2, MMP-9, and Vimentin expression but decline in E-cadherin expression in SNHG7-overexpressed OVCA433 and SKOV-3 cells, which was counteracted by further LY294002 treatment (Fig. 5E). In conclusion, SNHG7 silencing contributed to suppression of OC cell invasion, migration, and EMT by blocking the PI3K/AKT pathway.

DISCUSSION

Most OC patients suffer from relapse after standard therapy, and the clinical benefits of cancer therapies are unsatisfactory due to drug resistance (21). It is widely recognized that non-coding RNAs, including lncRNAs and miRs are implicated in OC development (22, 23). However, the mechanism of lncRNA SNHG7 and miR-34a underlying OC remained to be further explored. Therefore, this study was designed to figure out the mechanism of SNHG7 in OC with the involvement of miR-34a. Consequently, SNHG7 silencing impaired the PI3K/AKT pathway by downregulating miR-34a-targeted EDG4, thereby hampering OC cell migration, invasion, and EMT.

Initially, data from our study unraveled

higher SNHG7 expression in OC tissues than in paracancerous tissues. SNHG16 was increased in glioma tissues and cells relative to normal brain tissues and cells, and SNHG16 expression was linked to the malignancy and poor prognosis of glioma patients (24). More specifically, the oncogenic role of SNHG7 has been well established in numerous tumors, like breast, esophageal, bladder, prostate, colorectal, and gastric cancers, in which high expression shares correlation with poor prognosis and shorter survival for patients (25). Consistent with our results, a prior study illustrated that SNHG7 expression was augmented in OC tissues (26). Moreover, it was also elaborated in our research that OC cell invasion, migration, and EMT were diminished by silencing of SNHG7. Similarly, prior research evidenced that SNHG7 depletion contributed to repressed cell migratory and invasive capacities of OC cells (9, 26). In line with our finding, under the condition of cervical cancer, cancer cell invasive and migratory capacities and EMT were accelerated by overexpressing SNHG7 (27). Besides, it was noted by that SNHG7 upregulation augmented cell invasion, migration, and EMT in colorectal cancer (28). Our study further investigated the potential mechanism of SNHG7 in OC that SNHG7 assumed a pivotal role in upregulation of EDG4 via miR-34a to orchestrate EMT in OC.

Notably, increasing evidence unveiled that SNHG7 could bind to miR-34a in multiple cancers, including breast, gastric, and liver cancers (29-31), which supported that our results of binding relationship between SNHG7 and miR-34a in OC cells. miR-34 family plays a vital part in the pathogenesis of epithelial OC, and reduced expression of miR-34b/c may be of great importance for progression to the most advanced stages (32). Interestingly, our data manifested that miR-34a was poorly expressed in OC tissues and cells, and that ectopically expressed miR-34a triggered repression of OC cell invasion, migration, and EMT. Similarly, miR-34a overexpression was also able to decrease cell invasive and migratory capacities and EMT in pancreatic cancer and OC (33, 34). More importantly, SNHG7 upregulation has been revealed to promote cell invasion, and migration, and EMT in osteosarcoma cells by lowering miR-34a expression (35).

It is widely acknowledged that miRs inhibit expression of target genes at post-transcriptional level by targeting the 3'UTR of mRNA (36). As previously reported, miR-34a targeted HDAC to exert anti-oncogenic effects on OC development (37). Moreover, in our study, TargetScan website predicted the targeting relationship between miR-34a and EDG4 3'UTR, which was then verified by dual-luciferase reporter gene assay. In the subsequent experiments, we found that the overexpression of EDG4 or SNHG7 activated the PI3K/AKT pathway to augment OC cell migration, invasion, and EMT. A prior study depicted that EDG4 upregulation elevated SGC-7901 gastric cancer cell invasion, migration, and EMT (14), which was concurred with our results. Furthermore, it was noted that SNHG7 silencing caused decline of non-small cell lung cancer cell invasive and migratory rate by disrupting the PI3K/AKT pathway (38). Importantly, another report unveiled that activation of the PI3K/AKT pathway augmented viability and EMT of OC cells (20). Also, the oncogenic role of the PI3K/AKT pathway activation has been elaborated in OC by accelerating OC cell invasion, migration and EMT (39).

In conclusion, the key findings of our study demonstrated evidence that silencing of SNHG7 enhanced miR-34a expression to downregulate EDG4 expression and the PI3K/AKT pathway activation, thereby blocking the invasion, migration, and EMT of OC cells. SNHG7-mediated miR-34a may function as a promising strategy for the treatment of OC. However, how SNHG7/miR-34a/EDG4 axis orchestrated the PI3K/AKT pathway in OC cells remains unclear. Further *in vivo* investigations are warranted to explore the specific correlation between SNHG7/miR-34a/EDG4 axis and PI3K/AKT pathway in OC.

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A comparison between the expression of tumorigenic LncRNAs in circulating exosomes and the tumor tissue in patients with the nonsmall-cell lung carcinoma: promising findings as to the novel biomarkers

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Different types of lung cancer, such as nonsmall-cell lung carcinoma (NSCLC), show the specific expression of long non-coding ribonucleic acids (LncRNAs). There is evidence showing the possible correlation between the expression of LncRNAs in tumor tissues and plasma exosomes. The present study aimed to evaluate the expression level of LncRNAs in NSCLC patients in the cancerous tissues and adjacent non-cancerous tissue and the circulating exosomes. The tumor and adjacent non-cancerous tissue samples of 142 patients with NSCLC were collected. Extracellular vesicles were isolated by the ultracentrifuge method, and the nanoparticle tracking and the flow cytometry analysis were performed to identify the exosome fraction. The relative expression of the five LncRNAs, including AFAP1-AS1, BANC1, ANRIL, CCAT1, and GAS5, between tumor tissue, adjacent non-cancerous tissue, and plasma exosomes were evaluated by quantitative real-time RT-PCR. Among 142 NSCLC samples, the expression of lncRNA-GAS5 in tumor tissue was correlated with lncRNA-ANRIL in tumor tissue ($p=0.035$, $C=0.177$), while the rest of the lncRNAs did not show a significant correlation in tumor tissue. Expression of the five different lncRNAs did not show any correlation in normal tissue and exosome content. Among the five lncRNAs, only lncRNA-BANC1 expression in ANCT was correlated with the exosomal expression ($p=0.027$, $C=0.186$). In conclusion, the present research demonstrated that a panel consisting of these five lncRNAs could be helpful in the diagnosis of NSCLC.

Lung cancer is the leading cause of cancer-related death in both genders worldwide. Moreover, the mortality rate of lung cancer has been estimated mainly due to global tobacco use. Based on the clinicopathological findings, lung cancer has two primary forms: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is a common form of lung cancer, and the prognosis is mainly related to the stage at diagnosis, and earlier

diagnosis results in more prolonged survival (1). Therefore, early detection of lung cancer, especially the NSCLC, is the main focus of research. Although various imaging modalities increased the detection rate of NSCLC in at-risk patients, the increasing rate of lung cancer mortality indicates the need for more effective screening programs and therapies. Among the lung cancer subtypes, the NSCLC is well known because of its rapid growth and poor

Keywords: Lung cancer; Exosome; LncRNAs; NSCLC

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clinical prognosis. Therefore, many efforts have been made to determine novel and valuable targets for the screening of NSCLC in recent years (1). Among various pathways and mediators responsible for the development of lung cancer, the long non-coding RNAs (lncRNAs) are considered a potential target for both detection and treatment purposes (1). The lncRNAs are RNA molecules without protein lengthening at least 200 nucleotides. These RNA molecules can regulate tumorigenesis and metastasis in various cancers, including NSCLC (1). The expression level of multiple lncRNAs is dysregulated during lung cancer development and progression in lung tissue, making these RNA molecules potential diagnostic markers (1). Abnormal expression of different lncRNAs has been associated with non-small cell lung cancer development by affecting different cellular pathways. Cellular proliferation, invasion, and metastasis and suppression of apoptosis are the mechanisms altered by abnormal expression of lncRNAs in lung cancer (2). Among the large number of lncRNAs with abnormal expression in lungs cancer, we find upregulation of lncRNA-CCAT2, lncRNA-ANRIL, and lncRNA-AFAP1-AS1 related to proliferation, invasion, and metastasis of lung cancer. On the other hand, down-regulation of lncRNA-GAS5 and lncRNA-BANCER linked to apoptosis and suppression of cell proliferation in lung cancer (2). Therefore, these five lncRNAs affecting all necessary aspects for tumor proliferation, invasion, and metastasis and down-regulation of tumor-suppressing behaviours, including cell apoptosis and suppression of proliferation, could be considered an oncogenic profile, covering the most oncogenic aspects of lung tumors.

Of note, it has been demonstrated that recent studies demonstrated that these lncRNAs could be found in extracellular vesicles (EVs), indicating the novel potential biomarker for the detection of various diseases like NSCLC (3). However, the relationship between the dysregulated expression of lncRNAs during NSCLC development in tumor tissue and EVs are not clearly understood. Therefore, the present research aimed to evaluate the relationship between the expression level of five different lncRNAs, including lncRNA-BANCER, lncRNA-Gas5, lncRNA-AFAP1-

AS1, lncRNA-ANRIL, and lncRNA-CCAT1 in tumor tissue, adjacent non-cancerous tissue (ANCT), and EVs (exosomes) in NSCLC patients.

MATERIAL AND METHODS

Patient samples

The present study has been carried out following The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the ethics committees of Shandong Provincial Qianfoshan Hospital (the ethnic code: Chia201908FS). The written informed consent was obtained from all study participants with NSCLC. The diagnosis of lung cancer subtypes was made according to the 8th lung cancer TNM classification and clinical staging system. None of these patients received any radiotherapy or chemotherapy treatment before the surgery, and patients in stage I (A or B) of lung cancer were not included in the present study. The cancerous tissue samples and the corresponding ANCTs were excised during surgery from 142 patients admitted to Shandong Provincial Qianfoshan Hospital with a definite diagnosis of NSCLC. The clinical data from patients with NSCLC is presented in Table I. Blood samples were collected in EDTA anticoagulation tubes. The plasma was separated by centrifugation at 5000 rpm for 5 min at 4°C. The clear supernatant was transferred into a labelled tube and stored at -80°C.

EV isolation from plasma

According to the manufacturer's protocol, EVs were isolated from plasma using exoRNeasy Serum/Plasma Midi Kit (QIAGEN, USA). Samples were centrifuged for 10 min at 11,000 rpm and 4°C to remove residual cells and debris. The supernatant was then transferred into a new tube and mixed adequately with isolation reagents. Finally, the supernatant was removed, and the EV pellet was recovered by re-suspending in QIAzol.

Measuring particle size of isolated EVs

Isolated EV pellets were diluted in PBS and analyzed using the Nanosight NS300 system (Malvern Instruments, UK). The distribution of the particle size was measured using Nanosight Tracking Analysis software. Size distribution profiles were averaged across three replicates or each sample to derive the representative size distribution profiles.

Flow cytometry analysis of exosome marker proteins

The flow cytometry analysis was used to validate EVs with the generally accepted exosome markers CD63 and CD81. Isolated EV pellets were diluted in PBS, then incubated with FITC (catalogue: ab102884, Abcam, US)-conjugated with Anti-CD63 antibody [TS63] (catalogue: ab59479, Abcam, US) and Anti-CD81 antibody [M38] (catalogue: ab79559, Abcam, US) diluted in PBS/0.5% BSA for 60 min at 37°C were used for the flow cytometry study. The EV samples were assayed with a Cytoflex flow cytometer (Beckman, US), and IgG incubated samples were used as a negative control.

RNA extraction

Total RNA was isolated from cancerous tissues, ANCTs, and EV samples using the TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) and the Direct-zol™ RNA MiniPrep kit (Zymo Research Corp.) according to the guidelines. The extracted RNA was supposed to be DNase I treatment to eliminate DNA contamination. Nanodrop 2000 Spectrophotometer (Thermo Scientific) and gel electrophoresis assessed the extracted RNA's quantity and quality.

cDNA synthesis and quantitative real-time reverse transcription PCR (qRT-PCR)

RNA samples were reverse-transcribed to cDNA using a reverse transcriptase kit (Applied Biosystems High-Capacity cDNA Reverse Transcription, USA). TaqMan Probe-Based Real-Time PCR Assay was performed on a rotor gene 6000 Corbett Real-Time PCR System. HPRT1 gene was selected as the reference gene. Primers and probes used for PCR were designed using the Allele ID 7 software (Premier Biosoft, Palo Alto, USA). The characteristics of primers and probes are listed in Table II. Applied Biosystems TaqMan® Universal PCR Master Mix was used to quantify lncRNAs expression. Amplifying conditions were as follows: denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 50 s, and a final extension step in 72°C for 7 min.

Western blotting

EVs were prepared by RIPA buffer supplemented with protease inhibitor (Roche). Gel electrophoresis for 25 ug protein of each sample was run in 10% SDS-PAGE gel parallel with a protein marker (Termo). Proteins were transferred to a polyvinylidene fluoride (PVDF)

Table I. The clinical and demographic data of the study population.

Variable		Number
Gender N (%)	Female	63 (44.4%)
	Male	79 (55.6%)
Mean age $\pm$ SD		62.77 $\pm$ 13.433
Subtype N (%)	Adenocarcinoma	83(58.5)
	Squamous cell carcinoma	59 (41.5)
Stage N (%)	1	27 (19)
	2	54 (38)
	3	61 (43)
Body mass index N (%)	<30	92 (64.8)
	>30	50 (35.2)
Smoking		42 (29.5)

membrane and obstructed with 5% skim milk dissolved in Tween/Tris-buffered saline (TTBS) for one hour at room temperature. Next, TSG101 (ProteinTech) and CD9 (ProteinTech) antibodies at a dilution of 1:5000 with 3% skim milk diluted in TTBS were added to incubated membranes for one hour at room temperature. Then horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. After washing final with TTBS, membranes were covered with Enhanced chemiluminescence (ECL) western blot substrate solution and visualized by the film.

Negative-staining Transmission Electron Microscopy

The EV pellets were suspended in 2% paraformaldehyde for negative-staining EM for 10 minutes. After fixation, 15 µg of the exosome suspensions were applied to formvar/carbon-coated grids; next, samples were stained on drops of 3% uranyl acetate for 5 min. Finally, excess uranyl acetate was removed by filter paper, and samples

were allowed to dry. The grids were examined under a transmission electron microscope (JEM-1400plus, JEOL Ltd., Japan, Japan) at 80 kV.

Statistical analysis

The relative expression of lncRNAs in cancerous tissues compared with ANCTs, in plasma exosomes, and tumor tissues compared with exosome samples was determined based on $\text{Ln} [\text{Efficiency}\Delta\text{CT}]$ values calculation. A Chi-square test was used to evaluate the association between lncRNAs transcript levels and patients' clinicopathological data. Pearson's correlation coefficient was used to estimate the correlation among expression levels of lncRNAs and between relative expression of lncRNAs and patients' age. Mean values of expression of genes in different tissues were compared between subgroups using the *one-way analysis* of variance (*ANOVA*) with Bonferroni post hoc. The correlations between transcript levels of lncRNAs were evaluated

Table II. The sequences of primers and probes for the studied lncRNAs.

Gene Name	Primer & Probe Sequence	length	Product length
CCAT1	F:GCCGTGTTAAGCATTGCGAA	20	168
	R:TCATGTCTCGGCACCTTTCC	20	
	FAM-ATCCATGCTGTTCTTTGAAGG-TAMRA	21	
ANRIL	F: TGCTCTATCCGCAATCAGG	20	108
	R: GCGTGCAGCGGTTTAGTTT	19	
	FAM-GAACTAAAAGCCGCTCCGC- TAMRA	19	
AFAP1-AS1	F: TCGCTCAATGGAGTGACGGCA	21	96
	R: CGGCTGAGACCGCTGAGAACTT	22	
	FAM-TCAACAGAATGACCAAGTGGA-TAMRA	21	
GAS5	F: CTGCTTGAAAGGGTCTTGCC	23	
	R: GGAGGCTGAGGATCACTTGAG	23	
	FAM- ACCCAAGCTAGAGTGCAAGTGGCCT-TAMRA	24	
BANCR	F: ACAGGACTCCATGGCAAACG	20	80
	R: ATGAAGAAAGCCTGGTGCACT	21	
	FAM- ACGCAAGTCATCCCTGGCA-TAMRA	19	
HPRT1	F: AGCCTAAGATGAGAGTTC	18	88
	R: CACAGAACTAGAACATTGATA	21	
	FAM- CATCTGGAGTCCTATTGACATCGC - TAMRA	24	

using a regression model. *P* values less than 0.05 were considered significant. Binary logistic regression was used to calculate the predictive probability of combined biomarkers for the ROC analysis. Areas under the curves (AUCs) were used to evaluate the diagnostic value of the combination of the markers and the sensitivity and specificity for each lncRNA.

RESULTS

Characterization of plasma isolated EVs

The plasma EVs isolated from subtypes of patients with NSCLC were analyzed by Nanoparticle tracking analysis and flow cytometry. The Nanoparticle tracking analysis results showed that the extracted EVs included mostly small EVs as exosomes (Fig. 1 and Table III).

The fraction of isolated EV had mainly the desirable size for exosomes, ranging from 100-200 nm. The isolated exosomes were validated by flow

cytometry, and the two generally accepted exosome markers, including CD63 and CD81, were positive (Fig. 2). Fig. 3A shows the western blotting results indicating TSG101 and CD9 bands in control, ADC, and SQCC samples. Fig. 3B indicates the exosome visualization over the electron microscope.

The relative expressions of lncRNAs

Table IV demonstrated the relative expression of lncRNAs among different tissue samples. There was a significant difference in the relative expression of lncRNAs in the tumor and ANCT tissues. The lncRNA-CCAT1, lncRNA-ANRIL, and lncRNA-AFAP1-AS1 were upregulated (relative expression = 2.936; $p < 0.001$, relative expression = 4.683; $p < 0.001$, and relative expression = 3.485; $p < 0.001$, respectively), while the lncRNA-Gas5 and lncRNA-BANCR were downregulated (relative expression = -3.697; $p < 0.001$ and relative expression = -2.176; $p < 0.001$). Moreover, the relative expression

Table III. Characterization of EV particle diameter

Sample	ADC	SQCC
Average of particle diameter (nm)	119.2	116.7
Polydispersity index (PDI) ^a	0.226	0.219
The major peak of particle diameter (nm)	163.8	143.4
Percentage of 20–200 nm diameter (%)	79.1	76.8

ADC: adenocarcinoma, **SQCC:** squamous cell carcinoma

^aPolydispersity index (PDI) is a dimensionless value that represents the distribution of particle size. PDI values of 0.08–0.7 indicate moderate dispersion system and optimum application scope of the algorithm.

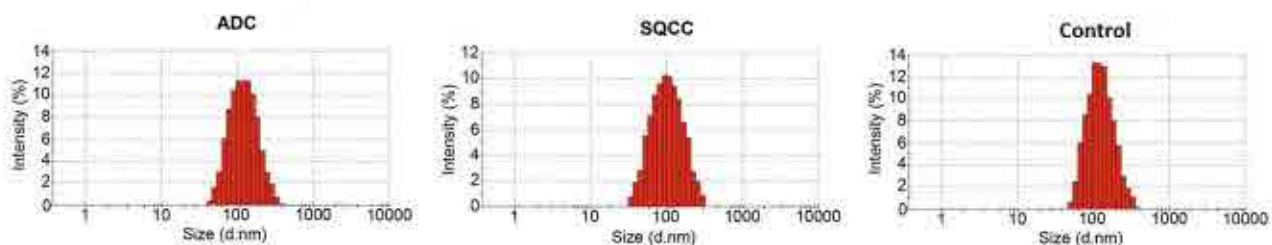


Fig. 1. The size distribution of plasma extracellular vesicles (EVs) measured by nanoparticle tracking analysis. The figure demonstrates that extracted EVs include small EVs as exosomes.

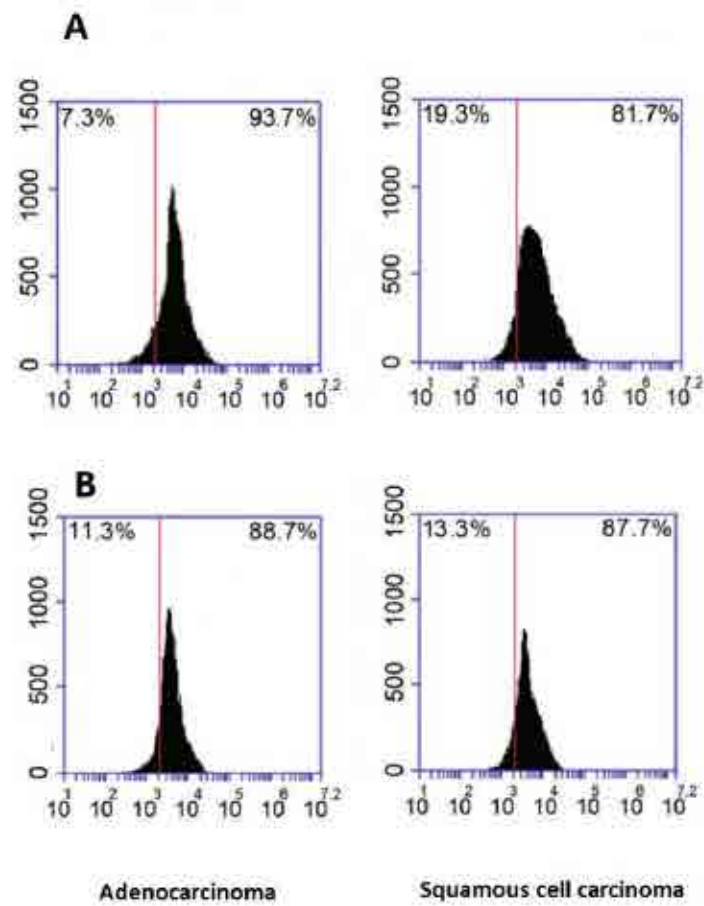


Fig. 2. Flow cytometry results for the exosomal markers antibodies. Line A illustrates the CD81, and line B illustrates the CD63.

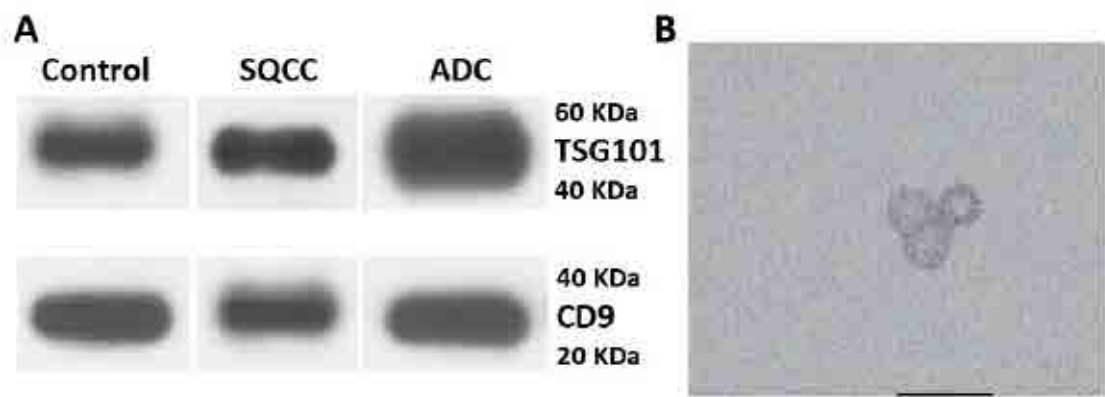


Fig. 3. Figure A shows the western blotting of TSG101 and CD9 for control, ADC, and SQCC. Figure B shows the electron microscopy of exosomes, and the black line below the figure indicates the size of 200 nm.

between lncRNAs in the tumor tissue and plasma exosomes was not significantly different except lncRNA-ANRIL and lncRNA-AFAP1-AS1 (relative expression=1.035; $p=0.006$ and relative expression=1.345; $p<0.001$).

The correlation between lncRNA expressions in different tissues and the area under curve

The Pearson correlation test revealed that, in

tumor tissue, the expression of lncRNA-GAS5 was correlated with lncRNA-ANRIL ($p=0.035$, $C=0.177$), while the rest of the lncRNAs did not show a significant correlation. The expression of the five different lncRNAs did not show any correlation in normal tissue and exosome content. Among the five lncRNAs, only the lncRNA-BANCR expression in ANCT was correlated with the exosomal expression ($p = 0.027$, $C = 0.186$). Considering a panel of these

Table IV. The comparison of relative expression of lncRNAs in different tissues.

Genes	Target sample	Compared to	Relative Expression	Std. Error	P Value	95% Confidence Interval	
						Lower Bound	Upper Bound
ccat1	tumor	normal	2.93662*	0.67101	0.000	1.3238	4.5494
		exosome	0.90141	0.67101	0.540	-0.7114	2.5142
	exosome	normal	2.03521*	0.67101	0.008	0.4224	3.6480
		tumor	-0.90141	0.67101	0.540	-2.5142	0.7114
anril	tumor	normal	4.68310*	0.33510	0.000	3.8777	5.4885
		exosome	1.03521*	0.33510	0.006	0.2298	1.8406
	exosome	normal	3.64789*	0.33510	0.000	2.8425	4.4533
		tumor	-1.03521*	0.33510	0.006	-1.8406	-0.2298
afap1-as1	tumor	normal	3.48592*	0.41162	0.000	2.4966	4.4753
		exosome	2.14085*	0.41162	0.000	1.1515	3.1302
	exosome	normal	1.34507*	0.41162	0.004	0.3557	2.3344
		tumor	-2.14085*	0.41162	0.000	-3.1302	-1.1515
gas5	tumor	normal	-3.69718*	0.42964	0.000	-4.7298	-2.6645
		exosome	0.10563	0.42964	1.000	-0.9270	1.1383
	exosome	normal	-3.80282*	0.42964	0.000	-4.8355	-2.7702
		tumor	-0.10563	0.42964	1.000	-1.1383	0.9270
bancr	tumor	normal	-2.17606*	0.28719	0.000	-2.8663	-1.4858
		exosome	0.24648	0.28719	1.000	-0.4438	0.9367
	exosome	normal	-2.42254*	0.28719	0.000	-3.1128	-1.7323
		tumor	-0.24648	0.28719	1.000	-0.9367	0.4438
*. The mean difference is significant at the 0.05 level.							

Relative expression of lncRNAs in different samples using ANOVA test with Bonferroni post hoc.

five lncRNAs demonstrated that the area under the curve (AUC) for ANCT and exosome is 0.924 ± 0.015 ($p < 0.0001$). Moreover, considering these lncRNAs in ANCT and tumor tissue demonstrated an AUC of 0.961 ± 0.009 ($p < 0.0001$) (Fig. 4 and Table 5).

The association between lncRNA expression and clinicopathological data

When considering the gene expression

differences as upregulation or downregulation, the lncRNA-CCAT1 expression in the tumor tissues and plasma exosomes was correlated with age ($p=0.047$), indicating those older than 60 years had increased level of expression in tumor tissue compared to exosome. The relative expression of the lncRNA-ANRIL and lncRNA-AFAP1-AS1 in the tumor tissue compared to plasma exosomes was significantly related to the tumor stage ($p=0.019$

Table V. The results of ROC curve analysis.

	Gene	Cut off point	AUC	PPV	NPV	Sensitivity	Specificity
Tumor compare to ANCT	AFAP1-AS1	> 3.41	0.731	76.1%	66.4%	78.2%	75.6%
	BANCR	> -2.21	0.694	72.4%	69.1%	73.4%	77.3%
	ANRIL	> 5.12	0.882	91.1%	85.5%	90.2%	82.8%
	CCAT1	> 3.47	0.653	75.3%	70.6%	76.8%	71.6%
	GAS5	> -3.56	0.713	77.5%	72.4%	81.6%	86.3%
	Combined LncRNAs		0.961	94.8%	90.3%	95.7%	94.4%
Exosome compare to ANCT	AFAP1-AS1	> 1.86	0.709	72.2%	68.1%	76.8%	74.2%
	BANCR	> -2.48	0.674	65.8%	65.2%	70.4%	68.8%
	ANRIL	> 3.74	0.843	86.1%	84.4%	90.1%	86.4%
	CCAT1	> 2.97	0.617	63.7%	61.6%	74.7%	67.7%
	GAS5	> -3.92	0.688	70.2%	67.3	80.5%	64.2%
	Combined LncRNAs		0.924	89.6%	88.7%	93.6%	90.8%

Optimal cut-off point for gene expression ($\ln(\text{ECT reference}/\text{ECT target})$), PPV: Positive Predicted Value, NPV: Negative Predictive Value.

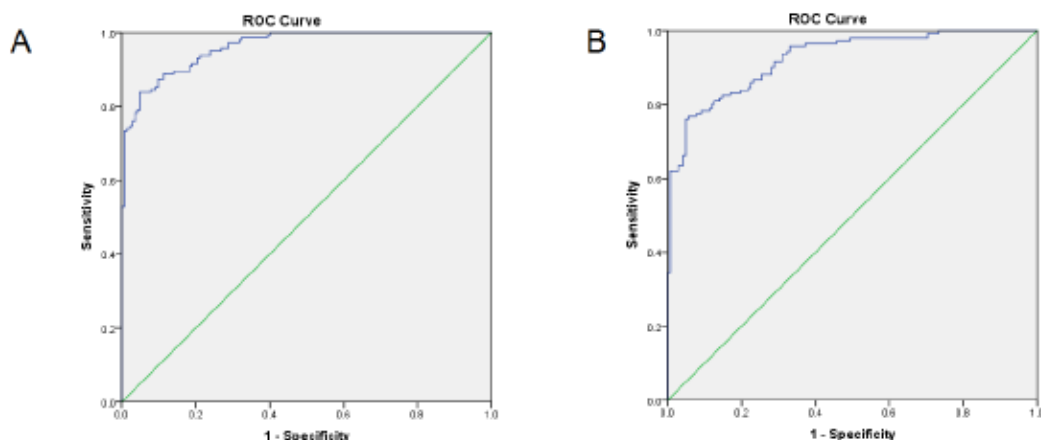


Fig. 4. The Roc curve analysis results. **A** shows the area under curve for expressing a panel of 5 lncRNAs in normal and tumor tissue; **B** shows the area under curve for expressing a panel of 5 lncRNAs in normal tissue and exosome content.

and $p=0.046$), indicating that lncRNA-AFAP1-AS1 had the highest expression in stage 2 of cancer while lncRNA-ANRIL had the highest expression in stage 3. The relative expression of the lncRNA-Gas5 and lncRNA-BANCR in the tumor tissue compared to the plasma exosomes was significantly related to the gender ($p=0.019$ and $p=0.041$).

Thus both of these lncRNAs were significantly upregulated in males and downregulated in women. The relative expression of the lncRNA-BANCR in the tumor tissue compared to plasma exosomes was significantly related to smoking ($p=0.019$), indicating that the lncRNA is upregulated in smokers (Supplementary Table I).

Supplementary table 1. *The analysis of the association between lncRNAs expression and clinicopathological data of patients.*

CCAT1									
	Tumoral:ANCT (N)			Tumoral:Exosome (N)			Exosome:ANCT (N)		
Age	Down	Up	P Value	Down	Up	P Value	Down	Up	P value
<60 years	24	36	0.385	36	24	0.047*	25	35	0.790
>60 years	27	55		35	47		36	46	
Gender									
Male	24	55	0.124	36	43	0.237	35	44	0.717
Female	27	36		35	28		26	37	
Subtype									
Adeno	28	55	0.521	40	43	0.609	34	49	0.569
SCC	23	36		31	28		27	32	
Stage									
1	14	13	0.154	17	10	0.270	14	13	0.317
2	18	36		27	27		25	29	
3	19	42		27	34		22	39	
Smoking									
Yes	13	29	0.424	22	20	0.713	17	25	0.699
No	38	62		49	51		44	56	
BMI									
<30	33	59	0.988	44	48	0.482	41	51	0.600
>30	18	32		27	23		20	30	
AFAP1-AS1									
	Tumoral:ANCT (N)			Tumoral:Exosome (N)			Exosome:ANCT (N)		
Age	Down	Up	P Value	Down	Up	P Value	Down	Up	P value
<60 years	22	60	0.843	33	46	0.952	27	33	0.475
>60 years	17	43		26	37		32	50	
Gender									
Male	19	60	0.307	23	37	0.506	30	49	0.333
Female	20	43		36	46		29	34	
Subtype									
Adeno	23	60	0.938	35	48	0.859	37	46	0.385
SCC	16	43		24	35		22	37	
Stage									
1	6	21	0.777	7	20	0.046*	13	14	0.323
2	16	38		20	34		25	29	
3	17	44		32	29		21	40	

DISCUSSION

The present study demonstrated that the lncRNAs could be used as potential biomarkers for detecting NSCLC, and studying the EVs containing specific lncRNAs can be considered a potential diagnostic

method for detecting lung cancer. We demonstrated that lncRNA-CCAT1, lncRNA-ANRIL, and lncRNA-AFAP1-AS1 were upregulated in NSCLC, while the lncRNA-Gas5 and lncRNA-BANCR were downregulated. Moreover, the expression of lncRNA-GAS5 in tumor tissue was correlated with

Smoking									
Yes	11	31	0.825	13	29	0.097	17	25	0.866
No	28	72		46	54		42	58	
BMI									
<30	21	71	0.093	37	55	0.662	38	54	0.936
>30	18	32		22	28		21	29	
GAS5									
	Tumoral:ANCT (N)			Tumoral:Exosome (N)			Exosome:ANCT (N)		
Age	Down	Up	P Value	Down	Up	P Value	Down	Up	P value
<60 years	42	18	0.206	29	31	0.844	43	17	0.219
>60 years	65	17		41	41		66	16	
Gender									
Male	62	17	0.333	48	31	0.002*	57	22	0.145
Female	45	18		22	41		52	11	
Subtype									
Adeno	59	24	0.162	42	41	0.712	60	23	0.135
SCC	48	11		28	31		49	10	
Stage									
1	21	6	0.742	15	12	0.151	21	6	0.116
2	42	12		21	33		46	8	
3	44	17		34	27		42	19	
Smoking									
Yes	30	12	0.482	21	21	0.913	33	9	0.741
No	77	23		49	51		76	24	
BMI									
<30	66	26	0.175	43	49	0.408	70	22	0.797
>30	41	9		27	23		22	11	
ANRIL									
	Tumoral:ANCT (N)			Tumoral:Exosome (N)			Exosome:ANCT (N)		
Age	Down	Up	P Value	Down	Up	P Value	Down	Up	P value
<60 years	11	49	0.309	24	36	0.370	13	66	0.127
>60 years	10	72		39	43		17	46	
Gender									
Male	10	69	0.423	32	47	0.300	16	44	0.167
Female	11	52		31	32		14	68	
Subtype									
Adeno	11	72	0.541	34	49	0.333	18	65	0.846
SCC	10	49		29	30		12	47	

Stage									
1	5	22	0.805	9	18	0.019*	3	24	0.349
2	8	46		32	22		12	42	
3	8	53		22	39		15	46	
Smoking									
Yes	9	33	0.149	21	21	0.381	10	32	0.612
No	12	88		42	58		20	80	
BMI									
<30	12	80	0.427	41	51	0.948	21	71	0.501
>30	9	41		22	28		9	41	

BANCR									
	Tumoral:ANCT(N)			Tumoral:Exosome (N)			Exosome:ANCT (N)		
Age	Down	Up	P Value	Down	Up	P Value	Down	Up	P value
<60 years	49	11	0.995	27	33	0.381	64	15	0.815
>60 years	67	15		43	39		52	11	
Gender									
Male	66	13	0.522	45	34	0.041*	53	7	0.084
Female	50	13		25	38		63	19	
Subtype									
Adeno	67	16	0.724	39	44	0.514	66	17	0.427
SCC	49	10		31	28		50	9	
Stage									
1	22	5	0.063	16	11	0.501	19	8	0.119
2	49	5		26	28		48	6	
3	45	16		28	33		49	12	
Smoking									
Yes	33	9	0.533	15	27	0.036*	34	8	0.883
No	83	17		55	45		82	18	
BMI									
<30	74	18	0.600	44	48	0.635	72	20	0.152
>30	42	8		26	24		44	6	

lncRNA-ANRIL, while the rest of the lncRNAs did not show a significant correlation in tumor tissue. The expression of the five different lncRNAs did not show any correlation in normal tissue and exosome content. Among the five lncRNAs, only lncRNA-BANCR expression in ANCT was correlated with the exosomal expression.

To the best of our knowledge, our study is the first clinical study introducing the five lncRNAs, including AFAP1-AS1, BANCR, ANRIL, CCAT1, and GAS5, as a panel for diagnosis of NSCLC. NSCLC is a common form of lung cancer with considerable mortality and morbidity. It has been demonstrated that early detection of NSCLC can result in an increased survival rate and better

prognosis. Therefore, there is a growing effort to identify promising screening tools for the early detection of NSCLC. The primary diagnostic modality in detecting NSCLC is imaging modalities, including computed tomography and chest X-ray. The suspicious findings indicating a solid mass or enlarged lymph nodes of the lungs usually raise the probability of lung cancer in a patient with specific symptoms, including respiratory disorders, weight loss, and positive history of smoking. The definite lung cancer diagnosis is then achieved by a biopsy of lung tissue (4). Therefore, a diagnostic modality reducing the diagnostic delay would help manage this type of lung cancer.

Various lncRNAs have been demonstrated to

be dysregulated in NSCLC and can be considered possible diagnostic and prognostic markers. The present study evaluated five different lncRNAs, including lncRNA-BANCR, lncRNA-Gas5, lncRNA-AFAP1-AS1, lncRNA-ANRIL, and lncRNA-CCAT1 in different tissues and found that plasma circulating exosomes can be considered as a possible diagnostic marker for NSCLC. Furthermore, our findings showed a significant difference between the relative expression of lncRNAs in the tumor and ANCT tissue.

lncRNA-CCAT1 is a well-known upregulated lncRNA in colon cancer, which promotes tumor growth. The lncRNA-CCAT1 is highly specific for detecting colorectal cancer and is upregulated even in premalignant and any other tumor stages. Regulating the Myc oncoprotein locus is one of the main functions of lncRNA-CCAT1, acting as a microRNA sponge. It has recently been demonstrated that the lncRNA-CCAT1 is upregulated in NSCLC tissue, resulting in increased tumor growth and decreased survival rate (5).

Like the lncRNA-CCAT1, the lncRNA-ANRIL is overexpressed in several tumors, including breast, colorectal, and gastric cancers. This lncRNA is located in the *CDKN2A/B* locus, coding many essential cellular cycle regulatory elements (6). The overexpression of lncRNA-ANRIL is related to tumor cell proliferation, invasion, and migration (6). It has been demonstrated that increased expression of lncRNA-ANRIL is associated with chemoresistance tumors (6). Notably, lncRNA-ANRIL has been found to promote NSCLC proliferation and inhibit apoptosis by decreasing the expression of P21 and KLF2 genes (7). Of note, our study indicated that the expression of exosomal lncRNA-ANRIL is correlated with its expression in NSCLC tissue.

In our study, another lncRNA showing a significant expression correlation between the tumor tissue and plasma exosomes was the lncRNA-AFAP1-AS1. This lncRNA plays a role in developing various cancers, including pancreatic ductal adenocarcinoma, cholangiocarcinoma, gastric, and gallbladder cancer (8). It has been also demonstrated that AFAP1-AS1 is upregulated in NSCLC (8). Furthermore, increased cellular invasion and metastasis and poor prognosis

are related to the lncRNA-AFAP1-AS1 expression (8). Moreover, it has been shown that exosomal lncRNA-AFAP1-AS1 can induce trastuzumab resistance in breast cancer (9).

The lncRNA-Gas5 inhibits cellular proliferation and mainly acts as a tumor suppressor in human cancer (10). Moreover, this lncRNA has been considered a biomarker of various cancers like colorectal cancer (10). Overexpression of lncRNA-Gas5 has been linked to inhibition of proliferation and invasion as well as an increase in apoptosis in colon cancer cells (10). In NSCLC, the suppression of lncRNA-Gas5 is related to chemoresistance and negatively correlates with the EGFR pathway (11). Like the just-discussed lncRNAs, the lncRNA-BANCR is deregulated in human cancers and induces tumorigenesis by increasing proliferation and cellular migration. Yang et al. demonstrated that the lncRNA-BANCR is downregulated in NSCLC tissue in both mouse models and humans (12). Overexpression of this lncRNA can attenuate cell viability and increase apoptosis. Therefore, restoration of this lncRNA can inhibit NSCLC (12).

One of the main limitations of the present study is the limited sample size. We used ANCT as the normal tissue, but other studies may consider normal tissue alongside ANCT. Moreover, the five lncRNAs, including AFAP1-AS1, BANCR, ANRIL, CCAT1, and GAS5, are not specific for NSCLC. Therefore, considering a panel of these lncRNAs may help determine other malignancies, and further studies may consider more lung cancer-specific lncRNAs in combination with the five lncRNAs to consider a higher diagnostic accuracy for detection NSCLC. Even more, the stage of NSCLC may affect the helpfulness of considering these five lncRNAs as a diagnostic marker while we considered NSCLC from every stage. Further studies may consider specific NSCLC stages to achieve more reliable results.

The present study demonstrated that exosomal lncRNAs could be considered as possible biomarkers of NSCLC. According to our findings, we may consider a panel of five lncRNAs, including AFAP1-AS1, BANCR, ANRIL, CCAT1, and GAS5, helpful in diagnosing NSCLC while none of these lncRNAs alone had enough AUC to be considered

as a biomarker. Therefore, considering the exosomal lncRNAs as a none invasive panel for patients suspected to have NSCLC can be a promising diagnostic modality before candidating the patients for a more invasive sampling procedure.

Authors' contributions

QZ conducted experiments. QZ collected and analyzed the data. PJ designed and supervised the project. QZ and PJ wrote the manuscript.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

The data based on the current study results obtained are accessible from the corresponding authors upon reasonable request.

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Relationship between severity of coronary artery lesions and levels of Lp-PLA2

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To the Editor,

Coronary artery disease (CAD) is a disease caused by coronary artery stenosis and occlusion, resulting in myocardial ischemia, hypoxia and necrosis. CAD mortality accounts for 30% of the world's total disease mortality in recent years. Thus, it is a common disease that endangers human health. Inflammation is the basis of atherosclerosis and plays an important role in the occurrence and development of CAD (1). Lipoprotein-related phospholipase A2 (LP-PLA2) is a newly discovered non-specific marker of vascular inflammation and is considered to be an independent cardiovascular risk factor (2).

The increased activity of LP-PLA2 is independently associated with cardiovascular disease, supporting the pro-atherogenic effect of LP-PLA2 (3). More studies focus on factors involved in atherosclerosis progression of vascular walls, especially the unstable factors of atherosclerosis promoting atherosclerosis rupture and thrombosis (4-5). However, rare studies report a correlation of LP-PLA2 and CAD progression (6). Coronary angiography (CAG) is the gold standard for assessing CAD severity by the Gensini score. Therefore, this study focused on the relationship between LP-PLA2 and Gensini score to evaluate

whether serum LP-PLA2 levels could be used as biomarkers for predicting CAD severity.

MATERIALS AND METHODS

Design and subjects

Clinical data of CAD patients with continuous CAG in the hospital were collected from March 2016 to June 2017. The history of hypertension, diabetes, CKD and smoking in each group was recorded. All subjects gave informed, signed consent to participate in the study.

Inclusion criteria: CAG showed a 50% stenosis degree of at least one coronary artery or major branch; age ≤ 75 years old. Exclusion criteria: severe hepatic and renal insufficiency (CKD 3-5); endocrine diseases (except type 2 diabetes); acute and chronic infections, tumors, severe gastrointestinal diseases and autoimmune diseases; not take folic acid, vitamin B 3-6 months ago.

According to the results of CAG Gensini score: Subjects were divided into two groups. Low-risk group: <30 points; high-risk group: ≥30 points. 69 patients were included, 46 males, 23 females, 38 cases of hypertension, 16 cases of diabetes, 5 cases of smoking, 3 cases of renal insufficiency, 34 cases in the low-risk group, 35 cases in the high-risk group. The baseline clinical characteristics of the study subjects are shown in Table I.

Key words: Lipoprotein-related phospholipase A2, LP-PLA2, Gensini scores, coronary heart disease

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Statistical Methods

The data were analyzed by SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA). LP-PLA2 level is non-normally distributed. Non-normal variables were reported as median (interquartile range [IQR]). Mann-Whitney U test was used for comparison of low-risk group and high-risk group. The area under the receiver operating characteristics curve (ROC) was used to assess whether the LP-PLA2 level was in the high-risk group. Correlation between LP-PLA2 level and Gensini score was analysed using a coefficient of Spearman. P-values <0.05 were considered significantly different.

RESULTS

Lp-PLA2 levels between the two groups

We found a statistically significant difference in Lp-PLA2 levels between the two groups, high-risk group is higher than the low-risk group, 388.0(283.0,436.0) vs 191.59(147.25,381.25) ng/ml, $Z=-3.247$, $P=0.001$ (Fig. 1).

Receiver operating characteristic (ROC) curves of Lp-PLA2

The area under the ROC of Lp-PLA2 for predicting high risk is 0.727, 95% CI (0.607~0.828). Lp-PLA2 predicting the best cut-off point of high risk is 202 ng/ml, as shown in Fig. 2. When $Lp-PLA2 \geq 202$ ng/ml, the sensitivity of Lp-PLA2 to predict high risk

was 88.57%, specificity was 55.88%, Youden index was 0.445 (Fig. 2).

Correlation between Lp-PLA2 levels and Gensini scores

Spearman's correlation was used to analyze the correlations. Correlation between Lp-PLA2 levels and Gensini scores were statistically significant ($r=0.424$, $P<0.05$) (Fig. 3).

DISCUSSION

Cardiovascular disease mortality remains high, and patients without traditional risk factors still have cardiovascular events because of the residual risk. Therefore, it is particularly important to determine new inflammatory factors to predict the occurrence of coronary heart disease and evaluate the condition. Lp-PLA2, a new type of inflammatory biomarker, could cause inflammation of blood vessel walls and atherosclerosis, which is an independent marker of poor prognosis of coronary heart disease (7). Therefore, this study chose Lp-PLA2 to study its correlation with the severity of coronary artery disease, aiming to provide a theoretical basis for preventing and treating coronary heart disease.

Lp-PLA2 is a member of the phospholipase A2 superfamily and also a calcium-independent enzyme (8). Lp-PLA2 could hydrolyze platelet activating factor (PAF), producing inflammatory factors. It has

Table I. Baseline clinical characteristics of the study subjects.

	High risk group (n=35)	Low risk group (n=34)	P value	
Age(year)	63.0(52.0,69.0)	63.0(59.0,68.5)	-0.769	0.442
Male gender (%)	11	12	0.116	0.802
Hypertension	20	18	0.123	0.811
DM 2	8	8	0.004	1.0
CKD	2	3	0.319	1.0
Smoking history	2	1	0.284	0.673

strong specificity of polar phospholipids and can hydrolyze short-chain phospholipids and oxidized phospholipids (ox-PL). In addition, Lp-PLA2 can also catalyze the oxidation of low-density lipoprotein (ox-LDL), producing lysolecithin (Lyso-PC) and oxidized non-esterified fatty acid (ox-FFA). Both Lyso-PC and ox-FFA are pro-inflammatory mediators, which can induce a chemotactic response, endothelial dysfunction and smooth muscle cell apoptosis, thereby promoting atherosclerosis (9-10). The factors that Lp-PLA2 participates in the progression of atherosclerosis in the vascular wall have become the focus of studies, especially the unstable atherosclerotic factors that promote atherosclerotic plaque rupture and thrombosis (11). At present, many studies have mentioned that Lp-PLA2 could promote the formation of atherosclerotic

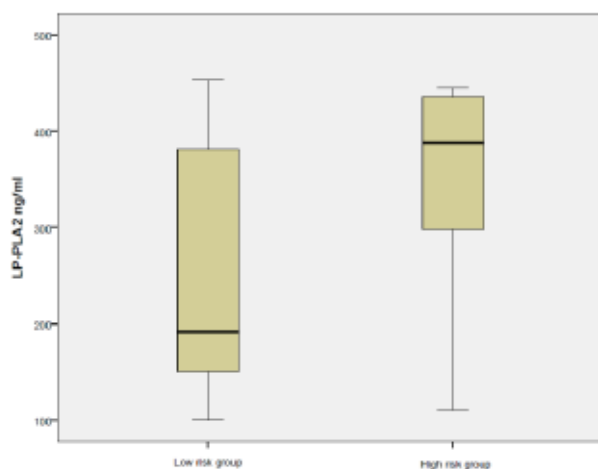


Fig. 1. *Lp-PLA2 levels between the two groups.*

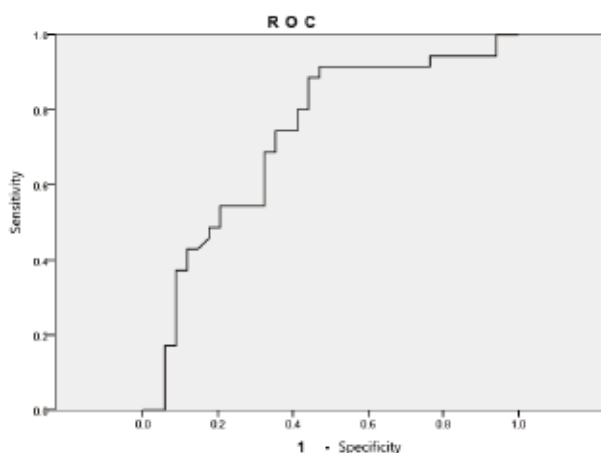


Fig. 2. *Receiver operating characteristic curves of Lp-PLA2.*

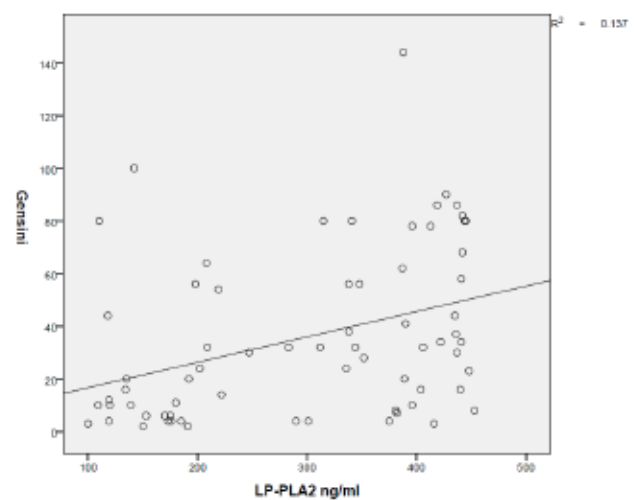


Fig. 3. *Correlation between Lp-PLA2 levels and Gensini scores*

plaques and the occurrence of atherosclerotic plaque instability (12-13). Atherosclerosis is prone to involve the coronary, causing coronary heart disease. Studies have found that patients with different types of coronary heart disease have different levels of Lp-PLA2 (14). WOCOPS first proposed that Lp-PLA2 may become an inflammatory marker for assessing cardiovascular risk, opening the door to the study of the correlation between Lp-PLA2 and coronary heart disease.

The coronary Gensini score could reflect the vasculopathy severity of each vessel in the patient. Higher severity of coronary stenosis is accompanied by a more severe disease and a higher Gensini score. This study utilized the Gensini score to evaluate the severity of coronary artery disease. The relationship between serum Lp-PLA2 and Gensini score was analyzed to assess whether serum Lp-PLA2 level could be utilized as a biomarker to predict the severity of coronary artery disease. Some studies have shown that elevated serum Lp-PLA2 levels increase the risk of restenosis after coronary stent implantation. In summary, the level of Lp-PLA2 could predict the degree of coronary artery disease, which may be another non-invasive method besides coronary angiography.

The results of this study showed that the Lp-PLA2 level of the two groups of patients increased with the severity of coronary artery disease based on the Gensini score. The Lp-PLA2 level of the high

risk group was significantly higher than that of the low-risk group ($p<0.05$). Serum Lp-PLA2 level is correlated with the risk of coronary heart disease with a high Gensini score ($p<0.05$). Therefore, it showed that the level of serum Lp-PLA2 can partly reflect the severity of coronary artery disease. The results showed that the serum Lp-PLA2 levels were significantly different only between the low-risk and high-risk groups ($p<0.05$).

In summary, there is no doubt that Lp-PLA2 is a risk factor for coronary heart disease. However, Lp-PLA2 urgently needs more in-depth research in identifying residual risks and evaluating treatment effects. This will be a huge and groundbreaking research, and a large number of clinical trials are needed for further demonstration. In conclusion, Lp-PLA2 could screen the condition and assess the severity of patients with coronary heart disease. Therefore, with the detection of serum Lp-PLA2 level, clinicians could predict the severity of patients with coronary heart disease timely and accurately in the absence of coronary angiography and then take more scientific and reasonable treatment.

All authors contributed equally to this study.

The authors declare that they have no conflict of interest.

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GAS5 rs145204276 polymorphism influences Ischemic Stroke risk via modulating transcription factor TFAP2A binding affinity

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To the editor,

Ischemic stroke (IS) is an extremely dangerous neurological condition that develops as a consequence of the obstruction of blood flow to the brain. A range of comorbidities and lifestyle-related factors, including hypertension, diabetes, obesity, and age, have been linked to IS incidence, and many associated genetic and epigenetic risk factors have additionally been identified (1).

Long noncoding RNAs (lncRNAs) over 200 nucleotides long lack coding activity yet play distinct regulatory roles in the pathogenesis of IS. For example, the lncRNA GAS5 is upregulated in IS model systems *in vitro* and *in vivo*, and the silencing of this lncRNA may suppress the apoptotic death of neurons and facilitate improved neurological recovery by preventing DNMT3b-mediated methylation of MAP4K4 (2).

The presence of single nucleotide polymorphisms (SNPs) within lncRNAs can potentially impact individual disease susceptibility. We have previously

identified the 145204276 SNP in GAS5 as associated with increased IS risk (3), but the mechanisms underlying this relationship remain defined. Genetic variations that influence the regulatory activity of transcription factors have been identified for a range of human diseases, as in the case of transcription factor AP-2 alpha (TFAP2A), which is closely linked to cerebral ischemia-reperfusion injury (4,5). Moreover, Yuan et al. reported that the GAS5 rs145204276 polymorphism may disrupt the ability of TFAP2A to interact with the GAS5 promoter, thereby contributing to the risk of glioma development (6). The present study was thus designed to expand on these prior results by exploring the potential mechanisms whereby GAS5 insertion/deletion (INDEL) mutations may contribute to the incidence of IS.

MATERIALS AND METHODS

Patient characteristics

Ninety-eight IS patients were recruited to this study;

Key words: GAS5; polymorphism; TFAP2A; glucose deprivation/reoxygenation

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the details regarding these patients were published in a prior report (3). Peripheral blood mononuclear cells (PBMCs) were isolated from donated total blood samples from these patients. All subjects provided informed consent prior to the study. All procedures were confirmed by the Ethics Committee of the Affiliated Hospital of Guangdong Medical University.

Animals and the middle cerebral artery occlusion (MCAO) model

The Animal Ethics Committee of Guangdong Medical University confirmed all animal investigations described herein, consistent with the NIH Guidelines for Care and Use of Laboratory Animals. Sprague-Dawley (SD) rats (280–320g) were anesthetized using 10% chloral hydrate, after which the left common and external carotid arteries were subjected via an endoscopic midline incision. A 4-0 nylon suture with a round tip was then inserted to occlude the middle cerebral artery for 24 h, following which the suture was removed to permit the restoration of blood flow. These same procedures were performed for sham control rats, but the middle cerebral artery was not occluded in these animals. All animals were maintained at $37\pm0.5^{\circ}\text{C}$ during surgical procedures using heating pads. At 24 h post-MCAO modeling, rats were sacrificed, and brain tissue was excised and cut into 3mm -thick sections and by employing 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, MO, USA) were meticulously stained.

Cell cultivation and oxygen -glucose deprivation/reoxygenation (OGD/R) modeling

An OGD/R cell model was prepared as in prior reports (7). Murine N2a neuroblastoma cells (ATCC, Shanghai Cell Bank) were grown in MEM (CA, USA) supplemented with the aid of 10% FBS (CA, USA) and 1% penicillin/streptomycin (Gibco, USA) in a 5% CO_2 37 °C incubator. OGD was induced by replacing culture media with glucose-free DMEM (Gibco, USA) and culturing N2a cells under 1% O_2 , 94% N_2 , and 5% CO_2 at 37 °C for 4 h, after which cells were transferred back to normoxic circumstances in standard media for 24 h to facilitate reoxygenation.

qPCR

TRIzol (Qiagen, CA, USA) was employed to extract total RNA from samples of interest, after which cDNA was provided by implementing a SuperScript II First-Strand

cDNA Synthesis Kit (Thermo Fisher Scientific, San Jose, CA, USA). SYBR Premix ExTag TMII kit (Takara, Japan) and an ABI Prism 7900 instrument (Applied Biosystems, CA, USA) were used for qPCR analyses, with relative expression of the gene being evaluated via the $2^{-\Delta\Delta\text{Ct}}$ method, with GAPDH as a normalization control. The primers were listed as follows. GAS5-F: CTTCTGGGCTCAAGTGATCCT, GAS5-R: TTGTGCCATGAGACTCCATCAG; TFAP2A-F: GGCCCAACCCTATCCTG, TFAP2A-R: CTGCTTTTGGCGCTGTT; GAPDH-F: GTCAACGGATTGGTCTGTATT, GAPDH-R: AGTCTTCTGGGTGGCAGTGAT. Bcl-2-F: CAGGTATGCACCCAGAGTGA, Bcl-2-R: TTGCCCTGTGATGTCTGAAGT; Bax-F: GGCCATCCCAACAGCTCATA, Bax-R: ATTCAGCCCCCAGTGTAACC; Caspase-3-F: TGGAAACAAATGGACCTGTTGACC, Caspase-3-R: AGGACTCAAATTCTGTTGCCACC.

Plasmid construction and dual-luciferase reporter assay

GAS5 mutant PCR products were cloned into the basic vector of pGL3 (Promega, WI, USA), and the resultant constructs were subjected to sequence validation. N2a cells were plated into the plates containing 24 wells and cotransfected with empty vector, GAS5-INS, or GAS5-DEL constructs along with TFAP2A plasmids. At 48 h post-transfection, relative luciferase activity was appraised by employing a Dual-Luciferase Reporter Assay Kit (Promega, USA).

Chromatin immunoprecipitation (ChIP)

TheChIP-ITTMmagneticChromatinImmunoprecipitation kit (ThermoFisher Scientific, Waltham, MA, USA) was used based on provided direction to conduct ChIP assays. Briefly, PBMCs were cross-linked with formaldehyde and sonicated to yield 200-1000 bp fragments. Nuclei and chromatin were then incubated with antibodies specific for TFAP2A or control IgG (Abcam) overnight at 4°C. The precipitated genomic DNA was determined by the aforementioned qPCR with GAS5 promoter primers harboring the rs145204276 polymorphism: 5'-TGTGGGCAGAGACATGACCG-3' (forward) and 5'-GCCGGAAGGAAATCACTCAG-3' (reverse).

Cell viability analysis

Cellular viability was quantified through an MTT assessment approach. In brief, N2a cells were added to

the plates containing 96 wells (1×10^4 /well) and cultured overnight, after which they were transfected with shRNA-NC or shRNA-TFAP2A and incubated for an additional 24 h prior to OGD/R treatment. Media was then exchanged for fresh media supplemented with MTT (5 mg/ml) (Sigma, CA, USA), and the incubation of the cells was executed at 37 °C for an additional 4 h. Absorbance was then assessed at 490 nm via a microplate reader (Bio-Tek, VT, USA).

Apoptosis analysis

An Annexin V-FITC/PI kit was implemented to assess cellular apoptosis. Briefly, N2a cells plated in 6-well plates were transfected with shRNA-NC or shRNA-TFAP2A constructs prior to OGD/R treatment. Cells were then resuspended in binding buffer supplemented with 5 μ L each of annexin V-FITC and PI for 15 min at ambient temperature, following which a FACSCanto II flow cytometer (Becton Dickinson and Company) was used to detect apoptotic cells.

Western blotting

The buffer of RIPA containing proteinase inhibitors was employed for the extraction of total protein from N2a

cells, after which an assay of BCA was applied for the measurement of protein concentrations in prepared lysates. Subsequently, equal protein amounts from each sample (30 μ g) were divided through 10% SDS-PAGE and transferred to PVDF membranes at 4°C overnight. Blots were subsequently probed using secondary HRP-conjugated goat anti-rabbit IgG (Abcam; 1:2000) for 90 min at ambient temperature. The detection of proteins was accomplished with the help of an improved chemiluminescence kit (Pierce, Thermo Fisher Scientific, Inc).

Statistical analysis

Data are means $\pm$ standard deviation (SD) from triplicate assessments and were scrutinized using GraphPad Prism 5.0 (GraphPad, CA, USA). Data were compared among groups using one-way ANOVAs or Student's t-tests, with $P < 0.05$ as the threshold of significance.

RESULTS

The rs145204276 DEL polymorphism is associated with increased transcription

To determine the impact of the rs145204276

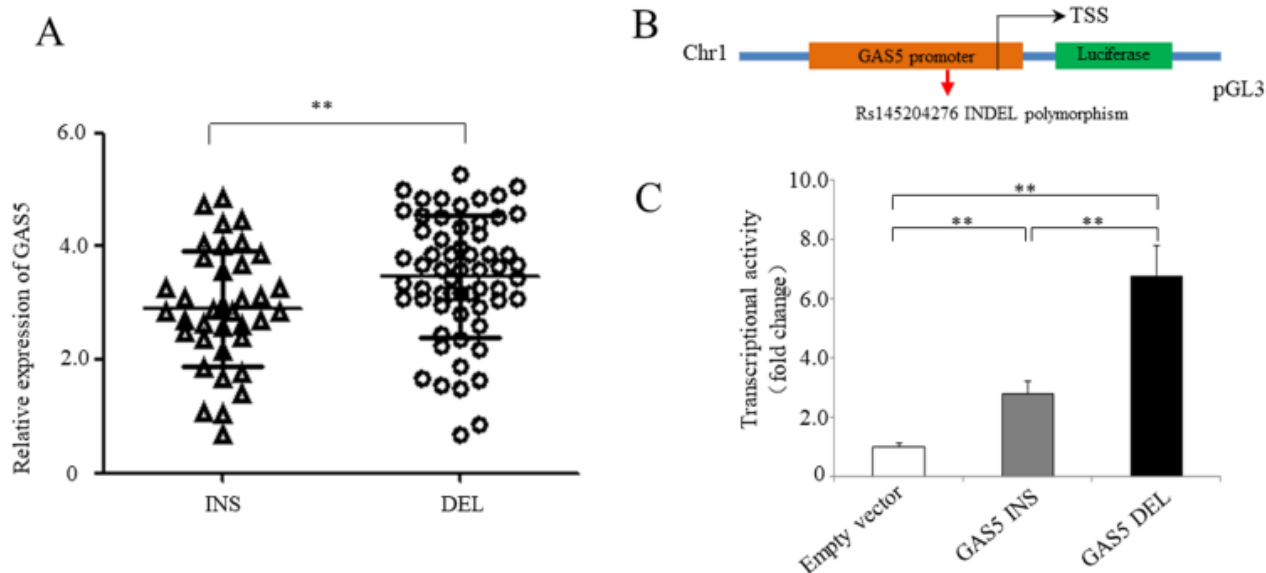


Fig. 1. The GAS5 rs145204276 DEL allele was associated with increased transcriptional activity and GAS5 expression. **A)** A quantitative real-time PCR was employed to assess the expression levels of GAS5 in 98 IS patients. Compared to rs145204276 INS carriers, rs145204276 DEL carriers had increased levels of GAS5 (** $P < 0.01$); **B)** Schematic representation of GAS5 promoter: Rs145204276 is located 268 bp upstream of the GAS5 promoter, and plasmids containing the rs145204276 INS or DEL allele were cloned into a pGL3 vector: TSS refers to the transcriptional start site; **C)** Plasmids containing the rs145204276 INS or DEL allele were transfected into N2a cells, and luciferase activity was detected 48 h post-transfection (** $P < 0.01$).

INDEL polymorphism in the GAS5 promoter on transcriptional activity, we prepared plasmids harboring the rs145204276 INS or DEL alleles and then measured luciferase activity (Fig. 1A). The rs145204276 INS and Del allele plasmids exhibited significantly increased luciferase activity relative to empty vector (** $P < 0.01$), and the rs145204276 DEL allele was related to the significant enhancement of such activity relative to the rs145204276 INS allele (** $P < 0.01$) (Fig. 1B).

The rs145204276 DEL allele increases TFAP2A affinity for the GAS5 promoter

As the rs145204276 INDEL polymorphism was

located within the region of the GAS5 promoter, we subsequently searched for candidate transcription factor binding sites in this region using PROMO and JASPAR. This analysis suggested that the rs145204276 INDEL polymorphism was likely to alter the affinity of TFAP2A for this region (TGCCTCGCGCC; Fig. 2A). To confirm this prediction, we assessed the genotype-specific binding affinity of TFAP2A for the GAS5 promoter via a ChIP assay. As shown in Fig. 2B, anti-TFAP2A was preferentially recruited to the rs145204276 DEL/DEL genotype as compared to the INS/INS genotype. We then generated plasmids in which TFAP2A and the GAS5 promoter region harboring the INS and DEL alleles were overexpressed,

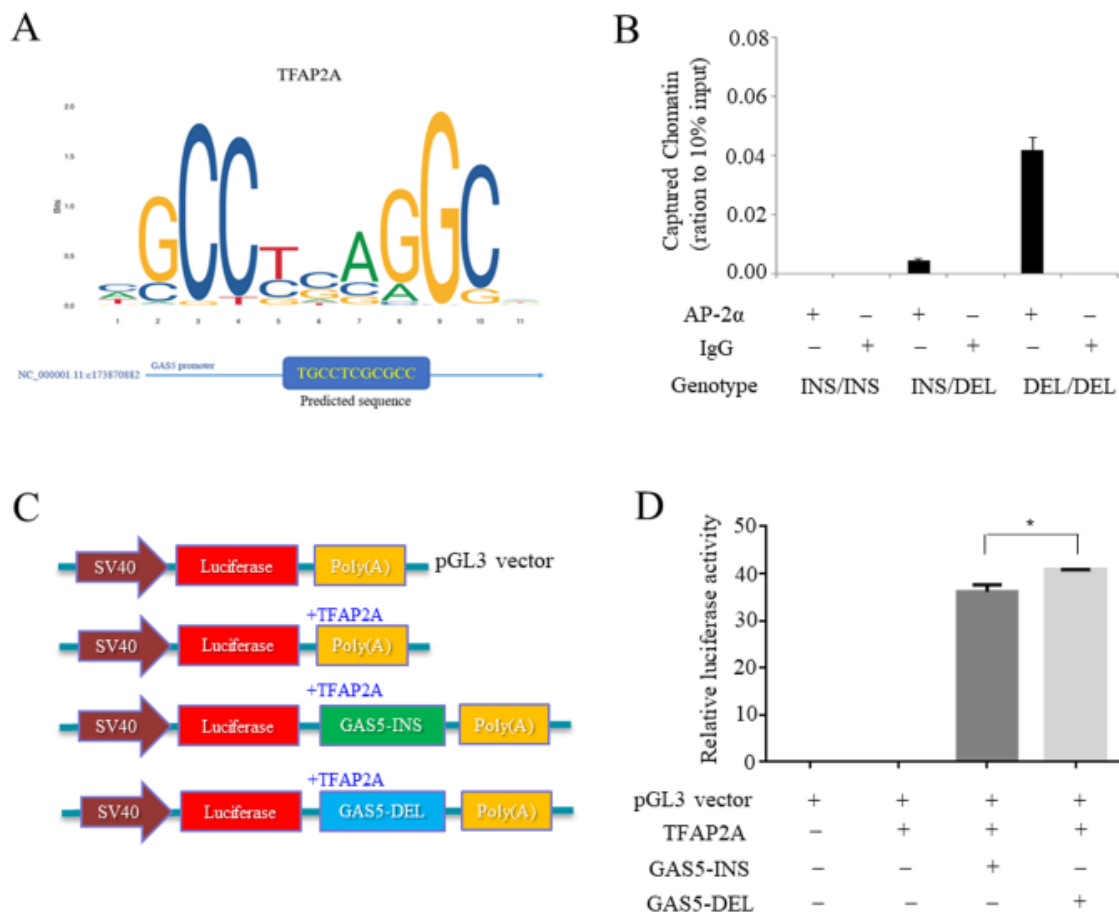


Fig. 2. GAS5 indel polymorphism may interrupt TFAP2A binding to gene promoter. **A**): Prediction of the potential binding sites of TFAP2A to rs145204276 mutation region based on the JASPAR database (<http://jaspar.genereg.net/>); **B**): Binding affinity of TFAP2A to the GAS5 rs145204276 mutation region. Results are shown as the means $\pm$ SD from three PBMCs of IS patients. **C**): Schematic representation of the plasmids harboring the rs145204276 INS and DEL allele cotransfected with TFAP2A. **D**): Luciferase activity assay showed that the GAS5 rs145204276 DEL allele might enhance the binding of GAS5 gene promoter region and transcription factor TFAP2A.

respectively (Fig. 2C). A subsequent dual-luciferase reporter assay revealed that the rs145204276 DEL allele was related to increased TFAP2A affinity as compared to the GAS5 rs145204276 INS allele (Fig. 2D).

TFAP2A is upregulated in MCAO and OGD/R model systems

We next evaluated the relative homology of human, mouse, and rat TFAP2A proteins and observed 100% homology. We then established an *in vivo* rat MCAO model system and an *in vitro* OGD/R model using N2a cells. Using these models, we found that TFAP2A was significantly upregulated under IS model conditions *in vitro* and *in vivo* (Fig. 3A-C).

TFAP2A silencing suppresses OGD/R-induced neuronal injury

To explore how TFAP2A functions in the context of ischemia-associated neuronal injury, we knocked down this transcription factor in N2a cells using a TFAP2A-specific shRNA construct (sh-TFAP2). We then transfected N2a cells with sh-TFAP2A or sh-NC and confirmed TFAP2A knockdown efficiency via qPCR and Western blotting (Fig. 4A and B). MTT assays revealed that TFAP2A knockdown

was associated with enhanced N2a cell viability in an OGD/R model context (Fig. 4C), and Annexin V-FITC/PI staining similarly indicated that TFAP2A knockdown alleviated OGD/R-induced N2a apoptosis (Fig. 4D). Subsequent qPCR analyses also revealed that TFAP2A knockdown decreased caspase-3 and Bax expression levels while promoting Bcl-2 upregulation (Fig. 4E). Moreover, TFAP2A was markedly silencing suppressed GAS5 expression following OGD/R treatment (Fig. 4F). Together, these data suggest that TFAP2A silencing may reduce the severity of ischemic neuronal injury through a mechanism that may be linked to the lncRNA GAS5.

DISCUSSION

The GAS5 rs145204276 polymorphism has been linked to increased IS risk, yet the mechanisms governing such a link are not well characterized. Herein, we found that this GAS5 INDEL polymorphism may alter TFAP2A binding to the GAS5 promoter, thereby enhancing GAS5 expression and thus increasing the risk of IS incidence.

Prior work suggests that polymorphisms within the

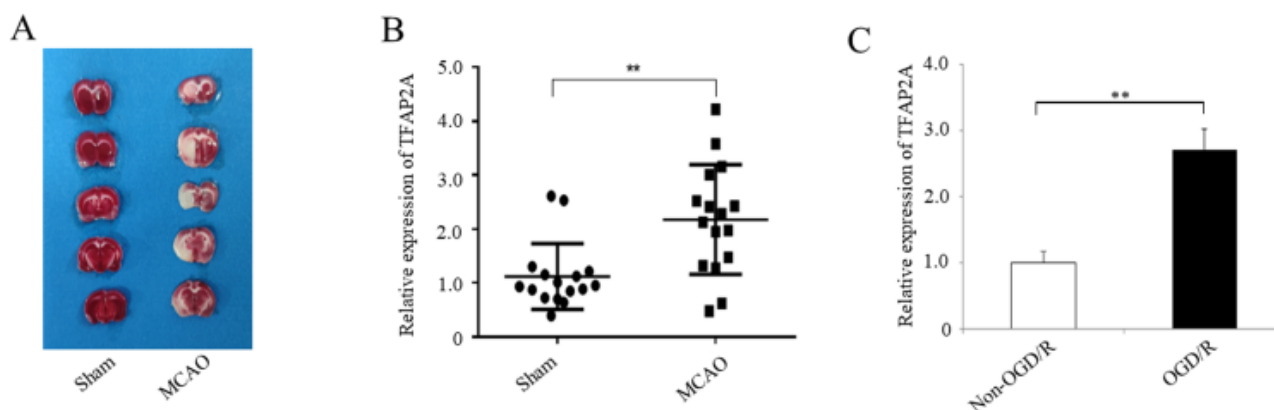


Fig. 3. A): The expression of TFAP2A was upregulated in both *in vivo* and *in vitro* models of IS. Rats were subjected to MCAO for 2 h, followed by 24 h of reperfusion, then 2, 3, 5-triphenyltetrazolium chloride (TTC) staining was used to evaluate cerebral infarct volume induced by I/R (n=16); **B):** The expression of TFAP2A was determined by quantitative real-time PCR, normalized to the expression of GAPDH in the sham and I/R group (n=16); **C):** N2a cells were exposed to OGD for 4 h and reoxygenation for 24 h. The expression of TFAP2A was measured by quantitative real-time PCR, normalized to the expression of GAPDH in the normal and OGD/R groups.

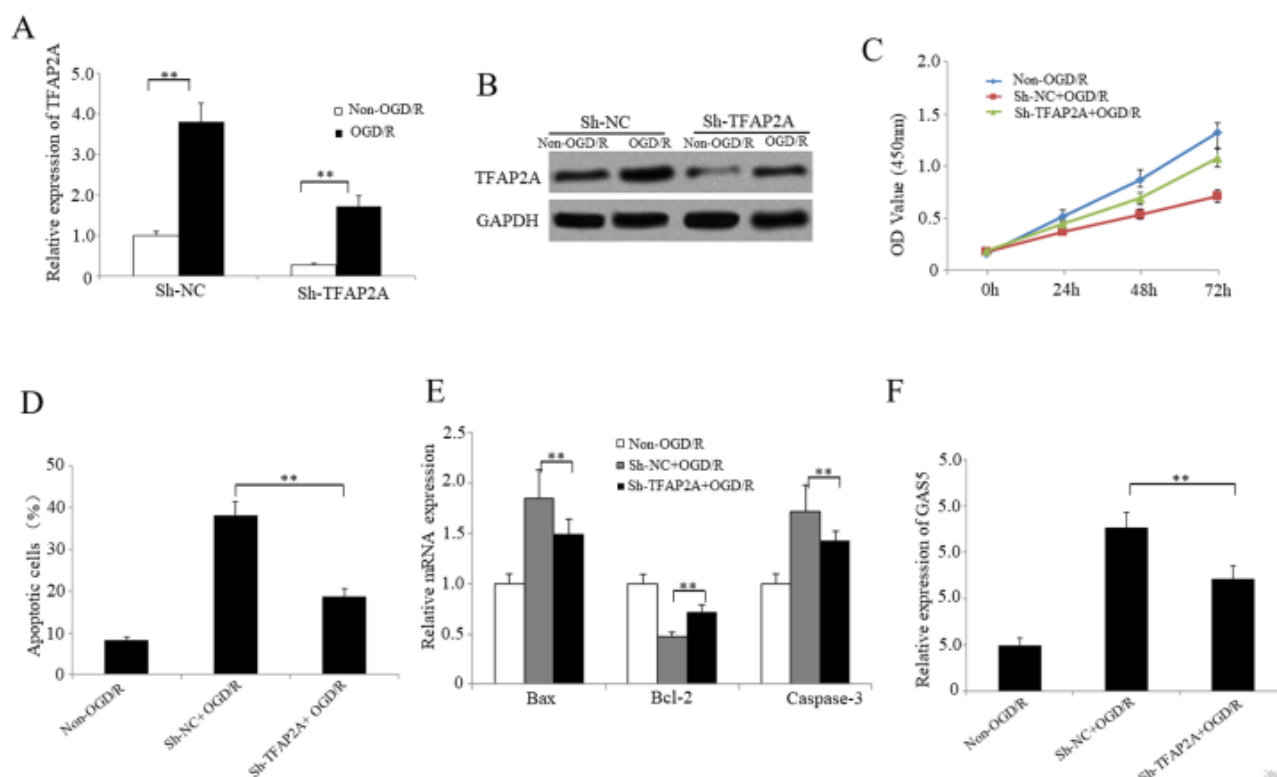


Fig. 4. Knockdown of TFAP2A inhibits the neuronal cell injury upon OGD/R treatment. The expression of TFAP2A after transfection of sh-NC and sh-TFAP2A under OGD/R and Non-OGD/R conditions were determined by RT-qPCR (** $P < 0.01$) (A) and western blot (B); C: Cell viability after transfected with sh-NC and sh-TFAP2A under OGD/R and Non-OGD/R conditions were detected using MTT assay; D: Apoptotic rate of N2a cells transfected with sh-NC and sh-TFAP2A under OGD/R and Non-OGD/R conditions were assessed using Annexin V-FITC/PI assay; E: The relative expression of Bcl-2, Bax and caspase-3 after transfected with sh-NC and sh-TFAP2A under OGD/R and Non-OGD/R conditions was determined by RT-qPCR (** $P < 0.01$); F: The relative expression of GAS5 after transfected with sh-NC and sh-TFAP2A under OGD/R and Non-OGD/R conditions was determined by RT-qPCR (** $P < 0.01$).

GAS5 promoter can interfere with transcription factor binding to alter GAS5 transcriptional activity. For example, Wang et al. determined that the rs55829688 T>C polymorphism was linked to reduced GAS5 expression and lower colorectal cancer risk through mechanisms associated with polymorphism-mediated reductions in transcription factor Yin Yang-1 binding to the GAS5 promoter region (8). Tang et al. found that the rs145204276 INDEL mutation led to increased SP1 transcription factor binding to this promoter region, thereby enhancing GAS5 transcription and increasing breast cancer risk (9). Herein, we showed that the rs145204276 DEL allele enhances TFAP2A binding to the GAS5 promoter, thereby upregulating this lncRNA and potentially thereby increasing the risk of IS.

TFAP2A has been previously identified as an important transcription factor in the context of cerebral ischemia-reperfusion injury (4). TFAP2A was shown to downregulate miR-126 via the Ikba/NF-KB pathway as a means of aggravating ischemic neuronal injury (5). Herein, we observed TFAP2A upregulation in *vitro* and in *vivo* in IS model systems, consistent with a role for this transcription factor as a modulator of cerebral ischemia-reperfusion injury through its regulatory interactions with GAS5.

In summary, these data indicate that the GAS5 DEL polymorphism may alter GAS5 transcriptional activity, and thereby further influence IS susceptibility. However, additional studies will be necessary to validate these findings.

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Conflict of interests

The authors have no actual or potential conflicts of interest related to this manuscript.

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Prognostic value and immune infiltration of ABCC5 expression in prostate adenocarcinoma

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To the Editor,

Prostate adenocarcinoma (PRAD) is one of the most common malignant cancers in males worldwide. Patients can develop castration-resistant prostate cancer, which is lethal (1). Thus, it is urgent to find novel and effective biomarkers for the prognosis and therapy of PRAD. More and more studies have shown that immune response may be associated with clinical outcomes in prostate cancer (2). Multiple genes, such as COL3A1, RAC1, FN1 and SDC2, have been proved to be associated with high infiltration immune cells in prostate cancer (3).

ABCC5 also named multidrug resistance-associated protein 5(MRP5), acts as a multiple specific organic anion pump that can transport nucleotide analogues (4). ABCC5 has been to be upregulated in prostate cancer (5, 6), which promotes tumor proliferation, metastasis, or drug resistance. However, there has been no report about the correlation between ABCC5 expression and immune infiltrates.

In this study, we analysed the expression of ABCC5 in PRAD using several public databases and assessed the relationship between ABCC5 expression with different immune cells.

MATERIALS AND METHODS

Raw data acquisition and processing

Two prostate cancer datasets were obtained from

Oncomine (<http://www.oncomine.org>) database, a web based data mining platform with RNA-seq and microarray data of human tumors. The RNA-seq data of PRAD and normal control tissues were downloaded from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). Some samples with incomplete clinical information were excluded when PRAD samples were analysed by the integrated bioinformatics. The RNA-seq data and clinical information of 492 PRAD patients were downloaded from the TCGA database.

The GEPIA database

The Kaplan-Meier analysis of the overall survival (OS) and disease-free survival (DFS) on the ABCC5 expression were obtained from the GEPIA database (<http://gepia.cancer-pku.cn/>). GEPIA is a newly developed interactive web server for analysing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects, using a standard processing pipeline.

TIMER database analysis

TIMER (<http://cistrome.dfci.harvard.edu/TIMER/>) used a previously published statistical method to analyse RNA-seq expression profile data to detect the infiltration of immune cells in tumor tissues (7). The current release of TIMER incorporated 10,009 samples across 23 cancer types

Key words: ABCC5, prostate adenocarcinoma, immune infiltration

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from the TCGA dataset to estimate the abundance of immune infiltrates.

Assessment of prognosis clinical characteristics and ABCC5 in PRAD cohort

Based on the expression level of ABCC5, 492 PRAD samples were divided into the high ABCC5 expression group and low ABCC5 expression group. Chi-square test and Spearman correlation analyses

were used to evaluate the association between ABCC5 expression and clinical characteristics in the trial cohort. P-value <0.05 was considered to have a statistically significant prognostic value.

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) preliminarily classified the genes according to their correlation with the ABCC5 expression. This

Table I. *ABCC5 expression level and clinical pathological factors in PRAD patients.*

Characteristics	No. of cases	ABCC5 expression level		p-value	spearman correlation
		Low (No. of cases)	High (No. of cases)		
Age				0.171	0.061
<60	203	109	94		
>=60	295	140	155		
Clinical T				0	0.21
T1-2	350	190	160		
T3-4	55	13	42		
Recurrent				0	0.235
No	330	190	140		
Yes	71	19	52		
No. of lymphnodes				0.012	0.113
Low	270	149	121		
High	228	100	128		
pathologic_N				0	0.238
N0	347	182	165		
N1-2	78	17	61		
pathologic_T				0	0.254
T1-2	189	125	64		
T3-4	302	121	181		

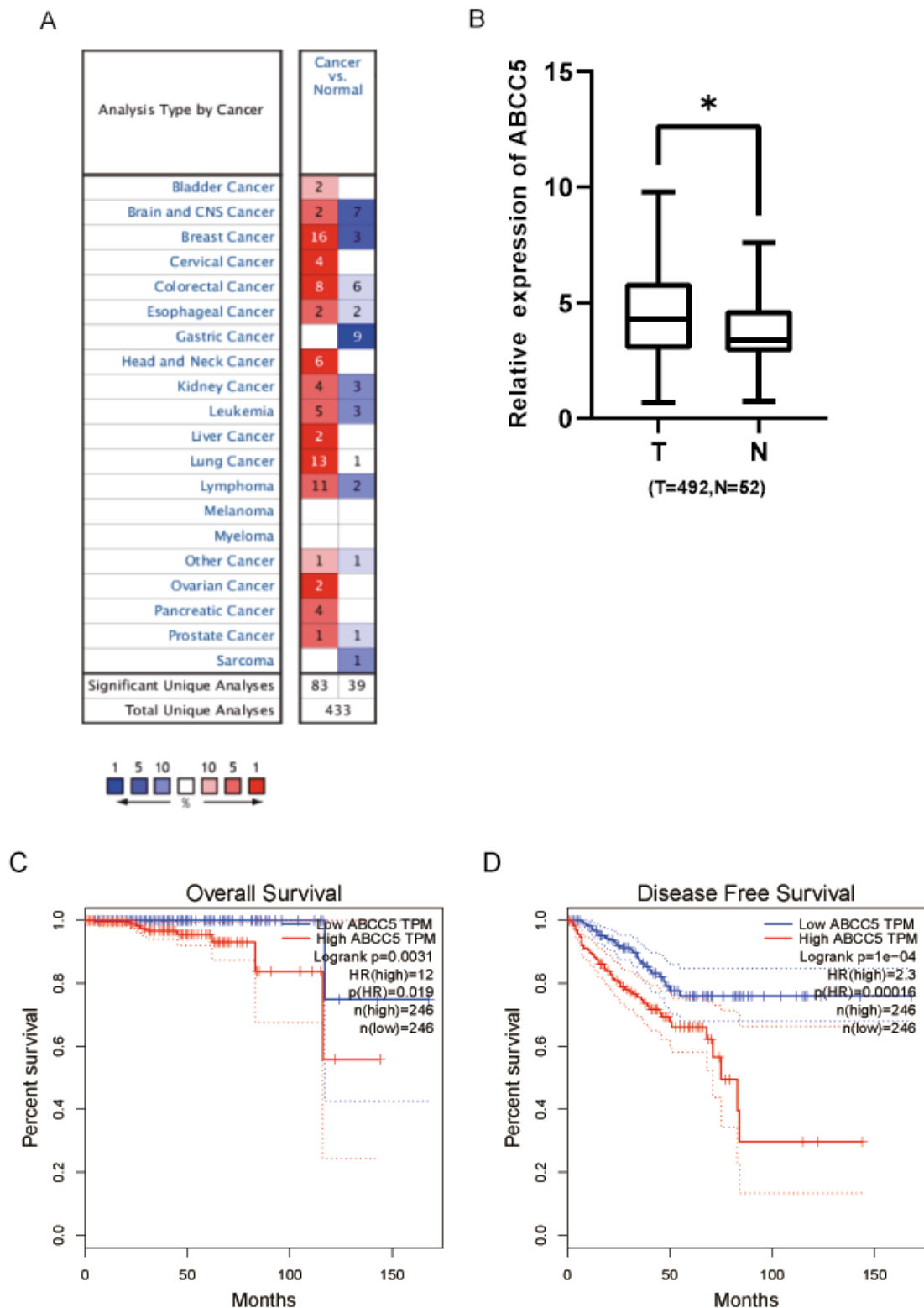


Fig. 1. High expression of ABCC5 showed poor prognosis in PRAD patients. (A): ABCC5 expression in datasets of different cancers compared with normal tissues in the Oncomine database. One dataset (showed significantly increasing of ABCC5 in PRAD; (B): ABCC5 expression level in PRAD and normal controls from TCGA ($*p<0.05$); (C): Overall survival in PRAD patients from GEPIA database; (D): Disease free survival in PRAD patients from GEPIA database.

computational method was used to perform the KEGG pathway and Gene ontology analyses, which illustrates the significant difference in survival observed between the high and low ABCC5 groups. For each analysis, we ran 1000 repetitions of gene set permutations. In addition, nominal P values and standardised enrichment scores (NES) were used to classify enrichment pathways in each phenotype (8).

Correlation analysis between ABCC5 and immune infiltration in PRAD

The composition and abundance of immune cells in the tumor microenvironment have a great influence on tumor progression and the effect of

immunotherapy. GraphPad Prism software (version 8.4.2, <https://www.graphpad.com/updates/>) was used to analyse the correlation between ABCC5 expression and TIICs, and the cut-off was p-value <0.05. The Immune Cell Abundance Identifier (ImmuCellAI) detected the abundance of immune cells, and the differences in the infiltration immune cells of 492 PRAD were analysed. ImmuneCellAI is a tool for estimating the abundance of 24 immune cells from gene expression datasets, including RNA-Seq and microarray data, of which 24 immune cells are composed of 6 immune cell types and 18 T-cell subtypes (<http://bioinfo.life.hust.edu.cn/web/ImmuCellAI/>).

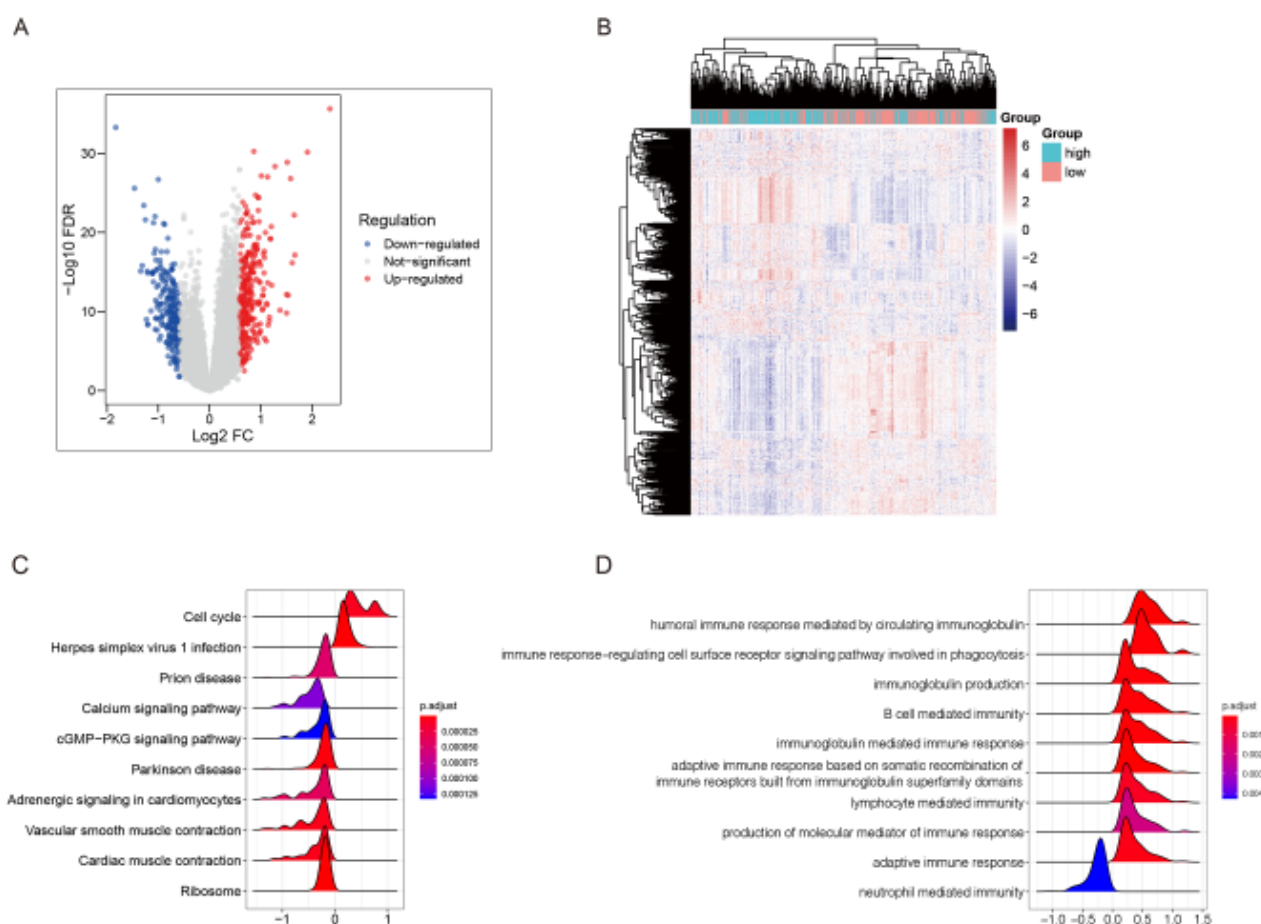


Fig. 2. The differential analysis and functional enrichment. (A): The volcano map of the genes. Red color represents upregulated genes, while blue color represents upregulated genes. (B): The heatmap showed the differentially expressed genes in ABCC5 upregulated and downregulated PRAD samples. (C): GSEA results showed differential enrichment of genes in KEGG pathway with ABCC5 expression. (D): GSEA results showed differential enrichment of genes in GO biological processes terms with ABCC5 expression. The closer the color is to the red, the smaller the p value.

RESULTS

High expression of ABCC5 showed poor prognosis in PRAD patients

In order to detect the expression of ABCC5 in prostate cancer and normal tissues, the ABCC5 mRNA levels in different tumors and normal tissues of multiple cancers were analysed by using the Oncomine database and TCGA data of prostate cancer. The results showed that ABCC5 was upregulated in tumor samples (Fig. 1A, B). Next, we performed chi-square testing to investigate the clinical value of ABCC5. The prostate cancer data were downloaded from TCGA, including 498 patients' information. As shown in Table I, we found that expression level of ABCC5 was significantly correlated with Clinical T stage ($p=0$), Recurrent ($p=0$), Number of lymph node metastases ($p=0.012$), pathologic_N ($p=0$) and pathologic_T ($p=0$). What's more, the spearman correlation analysis showed that ABCC5 expression was positively correlated with these indicators. This indicated the expression of ABCC5 plays an indicator role in clinical staging, lymph node metastasis and recurrence of prostate cancer. Otherwise, Kaplan-Meier survival analysis showed that ABCC5 expression was significant with OS ($p=0.0031$) (Fig. 1C) and DFS ($p=e^{-04}$) (Fig. 1D). This may suggest that higher expression of ABCC5 was associated with poor prognosis in PRAD patients.

Functional enrichment analysis of ABCC5 expression in PRAD

Subsequently, based on the mRNA expression profile of 498 PRAD samples from TCGA, we divided these samples into high ABCC5 expression group and low ABCC5 expression group. As shown in Fig. 2A and B, we found that 588 genes' expression model was changed. Compared to the low ABCC5 expression group, there were 257 genes upregulated and 331 genes downregulated in the high ABCC5 expression group. What's more, in order to investigate the potential biological functions of ABCC5 in PRAD, the KEGG pathway enrichment and GO term analyses were used to address our needs. By KEGG pathway analysis, we found that

the Cell cycle, Calcium signalling pathway, cGMP-PKG signaling pathway et al. were enriched (Fig. 2C). Otherwise, in enriched GO biological process terms, we found several immune-related catalogues, such as B cell-mediated immunity, lymphocyte-mediated immunity and neutrophil-mediated immunity (Fig. 2D). This may indicate that ABCC5 play an important role on the immune response.

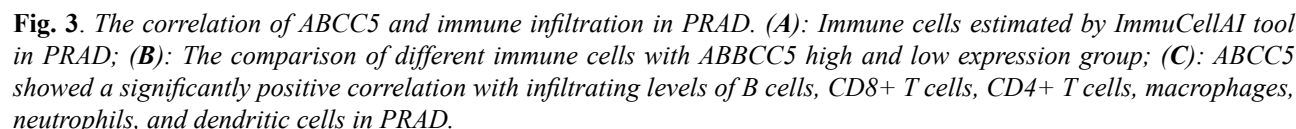
Immune infiltration of ABCC5 in PRAD

To investigate the possible effect of ABCC5 on different immune cell types in the PRAD microenvironment, the ImmuCellAI database was performed to calculate the ingredients of 24 immune cells in PRAD cases. As was shown in Fig. 3A, the immune cells varied significantly among different samples. Therefore, we compared the level of immune cells between the ABCC5 high expression group and low expression group in the above analysis (Fig. 3B). From the results, we found some immune cells were significantly correlated with ABCC5 expression, including CD8 naïve, Tex, nTreg, Th1, NKTA and Neutrophil. Subsequently, the TIMER database was utilised to investigate the association between ABCC5 and tumor immune infiltrating levels. As shown in Fig. 3C, we found that ABCC5 expression level has significantly positive correlations with infiltrating levels of B cell ($r=0.351$, $p=2.03e-13$), CD8+ T cell ($r=0.276$, $p=9.95e-09$), CD4+ T cell ($r=0.234$, $p=1.62e-06$), Macrophage ($r=0.295$, $p=8.95e-10$), Neutrophil ($r=0.378$, $p=1.68e-15$) and Dendritic cell ($r=0.318$, $p=3.30e-11$) in PRAD; In addition, ABCC5 expression level has little correlation with tumor purity in PRAD. All these results may indicate ABCC5 expression is correlated with immune infiltration level in PRAD.

DISCUSSION

Two previous studies focus on the cell function and drug resistance of ABCC5 in PRAD development (5, 6). There was no study regarding the relationship between ABCC5 expression, survival, and immune cell infiltration in PRAD. Our results proved that high expression of ABCC5 showed poor overall survival and disease-free survival. Also, high ABCC5

Targeted immunotherapy is a promising new strategy for hereditary and sporadic disease (9). Tumor immune infiltration by different kinds of immune cells is an active immune response against various tumors (10). In our study, GO enrichment suggested that *ABCC5* higher expression could mainly alter the immune response. In addition,



ABCC5 expression was positively correlated with the infiltration abundance of several immune cells. Among these cells, neutrophil has the strongest relationship with ABCC5 expression. Tumor-associated neutrophils (TANs) were highlighted to have a pivotal role in modulating the antitumor immune response (11). Neutrophils are mediators of metastatic prostate cancer progression in bone (12). This may indicate ABCC5 could regulate and recruit neutrophils in PRAD. However, to understand the relationship more accurately between ABCC5 and neutrophil *in vivo* and *in vitro*, controlled and multicenter clinical trials are further needed.

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Conflict of interest

The authors declare that there is no conflict of interest.

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OTUD7B suppresses the progression of melanoma via inhibiting the activation of NF- κ B signalingYQ. Liu¹, YZ. Xie¹, F. Wu¹, B. Ma², ZX. Ren³, Y. Tian^{1*}¹*Department of Plastic Surgery, Affiliated Hospital of Hebei Engineering University, Handan, Hebei, China;* ²*Department of Pediatrics, No.2 Hospital of Handan, Handan, Hebei, China;*³*Department of Orthopedic Surgery, Handan Central Hospital, Handan, Hebei, China**Received December 3, 2020 – Accepted November 26, 2021*

To the Editor,

Melanoma is an aggressive malignancy that originates from the melanocytes (1). The annual morbidity of cutaneous melanoma is increasing at a faster rate worldwide than other types of cancer (2). Therefore, there is a need for a better understanding of the molecular mechanism of melanoma to improve the treatment and the prognosis of the patients.

OTU deubiquitinase 7B (OTUD7B, also known as Cezanne) is a member of the ovarian tumor deubiquitinase superfamily, a family of proteins, all of which possess deubiquitinase activity (3). Some studies report that OTUD7B contributes to the progression of some malignancies. For instance, OTUD7B catalyzes the deubiquitination of the epidermal growth factor receptor (EGFR), thereby enhancing breast cancer cell proliferation and survival (4). OTUD7B inhibits NSCLC progression by serving as a negative regulator of NF- κ B signaling (5). In hepatocellular carcinoma, OTUD7B underexpression implies poor prognosis of the patients (6). However, the role of OTUD7B in melanoma has not been clarified.

In this work, we report that OTUD7B is lowly expressed in melanoma, and knockdown of OTUD7B in melanoma cells enhances the malignant biological

behaviors of melanoma cells. Mechanistically, OTUD7B exerts its tumor-suppressive effects by repressing the NF- κ B pathway. Our findings provide new insights into the role of OTUD7B in the development of melanoma and are likely to provide a theoretical basis for the treatment of melanoma.

MATERIALS AND METHODS*Tissue samples*

The research was performed according to the Declaration of Helsinki and was endorsed by the Ethics Committee of Affiliated Hospital of Hebei Engineering University. All subjects signed informed consent. Cancer tissue specimens and corresponding paracancerous tissue specimens were available from 76 subjects with melanoma diagnosed by pathologists at our hospital during February 2015 and February 2019. All subjects in the research had not been previously treated with radiotherapy, chemotherapy, immunotherapy before the sampling. Tissue specimens were immediately frozen in liquid nitrogen and preserved at -80°C after removal.

Immunohistochemistry (IHC)

All specimens were fixed in 10% formaldehyde and then embedded in paraffin. After the tissues were sliced,

*Key words: OTUD7B, NF- κ B, melanoma, proliferation, migration***Corresponding Author:*

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the sections were dewaxed and rehydrated, and then antigen retrieval was performed in citrate buffer (pH 6.0, Sigma-Aldrich, St. Louis, MO, USA). After the sections were washed by phosphate buffer saline (PBS) for 3 times, 3% H₂O₂ was supplemented to eliminate the endogenous catalase. Then the sections were blocked with 3% bull serum albumin (BSA) at room temperature for 1 h. After the sections were washed by PBS rinsed again, anti-OTUD7B antibody (1:100, ab238678, Abcam) was supplemented and the sections were incubated overnight at 4°C in a wet box, and then the sections were rinsed 3 times with PBS. Next, horseradish peroxidase-labeled goat anti-rabbit IgG (1:5000, ab205718, Abcam) was supplemented, and then the sections were incubated at room temperature for 1 h. DAB (GMS40008, Baomanbio, Shanghai, China) was supplemented after 3 washes with PBS for color development. Then the expression of OTUD7B was evaluated by the following criteria: the staining intensity: no staining, 0; light staining, 1; brown, 2; dark brown, 3. The percentage of stained cells: no positive cells, 0; 1% to 10% of the cells were stained, 1; 10% to 50% of the cells were stained, 2; 50% to 100% of the cells were stained, 3. The score of staining intensity and the score of the percentage of stained cells were added up, to obtain the value S. If $S \leq 4$, it was negative expression (low expression); if $S \geq 4$, it was positive expression (high expression).

Cell culture and transfection

The melanoma cell lines HMCB, G-361, A375 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The human normal melanocytes (HEMa-LP) were available from Cascade Biologics (UK). The cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% inactivated fetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences, Little Chalfont, UK), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Waltham, MA, USA) at 37°C with 5% CO₂. The cells in logarithmic growth phase were selected for the subsequent experiments. pcDNA3.1-OTUD7B overexpression vector (pcDNA-OTUD7B), pcDNA3.1 empty vector (NC), OTUD7B small interference RNAs (si-OTUD7B) and scrambled negative control siRNA (si-NC) were obtained from RiboBio Co. Ltd. (Guangzhou, China). The cells were transfected with Lipofectamine®2000 (Invitrogen,

Carlsbad, CA, USA) according to the manufacturer's instructions.

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

Total RNA was isolated from melanoma tissues or cells using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) and cDNA was synthesized using a reverse transcriptase kit (Takara Biotechnology Co., Ltd., Dalian, China). The qRT-PCR experiments were executed using a SYBR-Green Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China). GAPDH was the internal control. RNA expression was calculated using 2^{-ΔΔCT} method. The primers were designed and synthesized by Genepharma (Shanghai, China). The primer sequences:

OTUD7B forward: 5'-AGCAGACACAGCAGAATA-3';
reverse: 5'-TCAGTTCATTCCACTCCTT-3'.

GAPDH forward: 5'-CTTAGCACCCCTGGCCAAG-3';
reverse: 5'-CTTAGCACCCCTGGCCAAG-3'.

Cell counting kit-8 (CCK-8) experiment

The proliferation of melanoma cell lines was detected using the CCK-8 kit (YEASENBio, Shanghai, China). The transfected cells were inoculated into 96-well plates and cultured for 12, 24, 48, 72, and 96 h. Subsequently, CCK-8 reagent was added, and the cells were incubated for 2 h. A microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was utilized to measure the absorbance of the cells at 450 nm wavelength, and finally the proliferation curve was plotted after 96 h.

Colony formation assay

The transfected melanoma cells were collected and washed by PBS, and then resuspended in medium to prepare single-cell suspension. Then the cells were inoculated into a 6-well plate (1000 cells per well), and cultured for 2 weeks. Subsequently, the medium was discarded, the colonies were fixed by 70% ethanol, and stained by 0.1% crystal violet solution. After the colonies were washed by PBS, the visible colonies were counted by naked eyes.

Transwell experiment

Matrigel (1:10; BD Biosciences, Franklin Lakes, NJ, USA) was diluted with pre-chilled RPMI-1640 medium and then it was used to cover the filter of Transwell® chambers (pore size, 8 µm; Corning Incorporated,

Corning, NY, USA). Following that, 100 μ L of cell suspension containing 1×10^5 cells (suspended in serum-free medium) was supplemented to the upper compartment, and 600 μ L of complete medium was supplemented to the lower compartment and then the cells were cultured for 24 h. Next, the cells, which remained on the upper surface of the filter, were removed with cotton swabs. After the cells on the below surface of the filter were fixed with 4% paraformaldehyde, the cells were stained with crystal violet solution. The number of cells passing through the filter was recorded under a microscope (Leica, Wetzlar, Germany). The migration assay did not use Matrigel, and the other procedures were the same as the invasion assay.

Flow cytometry

The Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, Louis, MO, USA) was employed to detect cell apoptosis. Briefly, the transfected cells were collected, and mixed with 1 mL of pre-cooled 70% ethanol and fixed at 4°C overnight. After centrifugation, the cells were resuspended with 1 mL of pre-cooled binding buffer. Then 10 μ L of propidium iodide (PI) staining solution and 10 μ L of Annexin V-FITC staining solution were added. Then the cells were incubated at 37°C in the dark for 30 min. Finally, the apoptotic cells were detected using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot

The cells were trypsinized with RIPA lysis buffer (Beyotime, Shanghai, China) and collected by centrifugation ($1\,000 \times g$, 4 °C for 5 min), and the total cellular protein was extracted. After quantification, the protein samples were separated by electrophoresis, and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room temperature, and then incubated with the diluted anti-OTUD7B antibody (rabbit polyclonal, 1:100016605-1-AP, Proteintech) for 1 h at room temperature. After the membranes were rinsed, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (1:5000, Beyotime, Shanghai, China) for 1 h at room temperature. At last, the enhanced chemiluminescence kit (Promega, Madison, WI, USA) was used for the development of protein bands. GAPDH was used as the internal reference.

Statistical analysis

The experiments were performed in triplicate, and the experimental data were analyzed using SPSS 20.0 software. The Kolmogorov-Smirnov test was adopted to determine the normality of the data distribution. The data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). The data were analyzed using the student's *t*-test or one-way ANOVA. $P \leq 0.05$ signified the statistical significance.

RESULTS

OTUD7B was under-expressed in melanoma

First of all, OTUD7B expression in melanomas tissues was evaluated by IHC. The data implied that OTUD7B expression was remarkably lower in poorly differentiated tissues compared with that in the well-differentiated tissues (Fig. 1A). Besides, qRT-PCR showed that OTUD7B expression was markedly lower in cancerous tissues than in paracancerous tissues (Fig. 1B). Subsequently, the melanoma patients were divided into high OTUD7B expression group and low OTUD7B expression group based on the median value of OTUD7B mRNA expression (Fig. 1C). Then the association between OTUD7B expression and clinicopathological parameters was analyzed, and it was revealed that low OTUD7B expression was associated with patients' pTNM stage, and depth of invasion (Table I). OTUD7B expression was substantially decreased in 61.8% (47/76) of the specimens (Fig. 1D); additionally, qRT-PCR and Western blot showed that OTUD7B expression was markedly reduced at the mRNA and protein levels in melanoma cell lines compared with human normal melanocytes (HEMa-LP) (Fig. 1E). Moreover, the overall survival of patients with OTUD7B under-expression was remarkably shorter than that of patients with OTUD7B high expression (Fig. 1F). The findings indicated that OTUD7B was lowly expressed in melanoma, and it may be tumor-suppressive in melanoma progression.

OTUD7B suppressed melanoma cell proliferation, migration and invasion, and promotes apoptosis

To investigate the biological function of OTUD7B in melanoma cells, the melanoma cell lines HMCB and A375 were transfected with si-

OTUD7B and OTUD7B overexpression plasmids, respectively, and successful transfection was validated by qRT-PCR and Western blot (Fig. 2A).

CCK-8 and colony formation experiments showed that OTUD7B overexpression remarkably repressed the proliferation of A375 cells compared with the

Table I. Association of OTUD7B expression with clinicopathological characteristics of melanoma patients

Variables	Number	Expression of OTUD7B		P-value
		Low	High	
Age (years)	76	38(50%)	38(50%)	0.3450
≤60	47	26(56%)	21(44%)	
>60	29	12(41%)	17(59%)	
Gender	76	38(50%)	38(50%)	0.8178
Male	42	20(48%)	22(52%)	
Female	34	18(53%)	16(47%)	
pTNM stage	76	38(50%)	38(50%)	0.0044
I	15	5(33%)	10(67%)	
II	21	6(29%)	15(71%)	
III	28	18(64%)	10(36%)	
IV	12	10(83%)	2(17%)	
Depth of invasion	76	38(50%)	38(50%)	0.0013
T1	18	5(28%)	13(72%)	
T2	15	4(27%)	11(73%)	
T3	36	25(69%)	11(31%)	
T4	7	6(86%)	1(14%)	

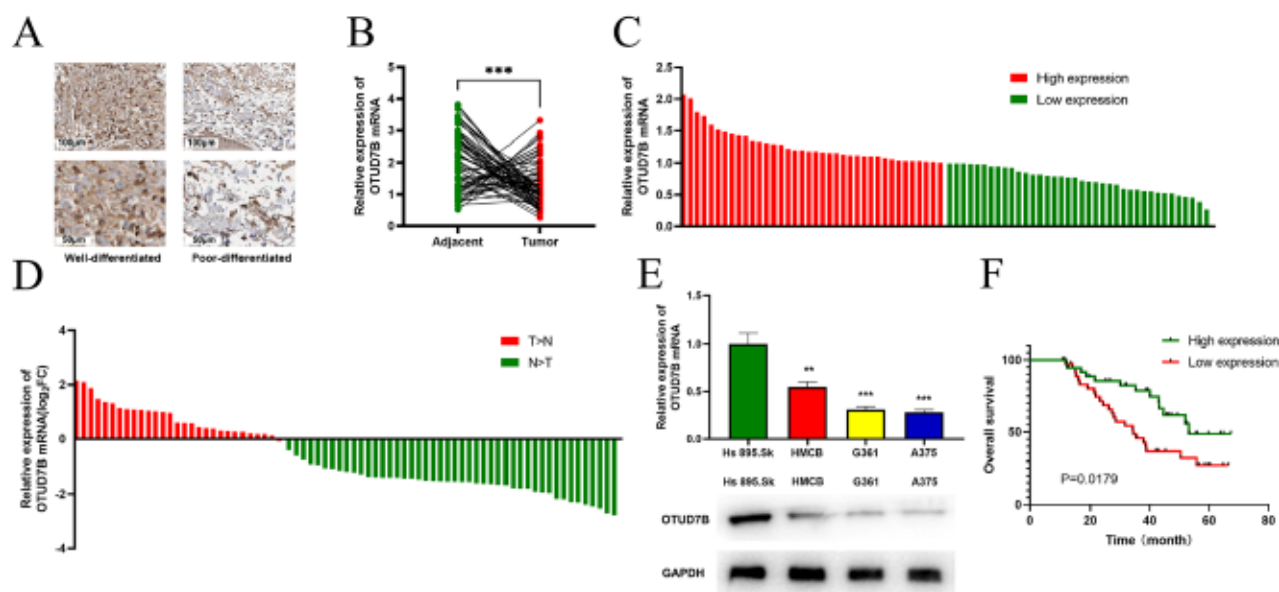


Fig. 1. OTUD7B expression was remarkably under-expressed in melanoma. **A)** IHC was performed to examine OTUD7B expression in melanoma tissues with different degrees of differentiation. **B)** OTUD7B expression in melanoma specimens and adjacent normal tissues was examined by qRT-PCR. **C)** Patients were divided into a high expression group ($n=38$) and a low expression group ($n=38$) based on the median value of OTUD7B mRNA expression. **D)** OTUD7B expression was lower in 61.8% of the melanoma tissues, compared with that in the paracancerous tissues. **E)** OTUD7B expression in melanoma cell lines was examined by qRT-PCR (upper) and Western blot (Below), respectively. **F)** Kaplan-Meier method was used to analyze the relationship between overall survival time of the patients and OTUD7B expression.

** $P < 0.01$, *** $P < 0.001$.

control group (Fig. 2B-C). Transwell experiments suggested that OTUD7B overexpression remarkably suppressed the migration and invasion of A375 cells (Fig. 2D). Flow cytometry implied that OTUD7B overexpression enhanced the apoptosis of A375 cells (Fig. 2E). Conversely, knockdown of OTUD7B markedly facilitated the proliferation, migration and invasion of HMCB cells and suppressed apoptosis (Fig. 2B-E).

OTUD7B repressed NF- κ B signaling pathway

Next, NF- κ B expression and activity were examined in melanoma cell lines with OTUD7B knockdown or overexpression. It was revealed that, knocking down OTUD7B up-modulate the expression levels of phosphorylated NF- κ B (p-NF- κ B), phosphorylated Inhibitors of kappa B kinase- α (p-IKK- α), phosphorylated Inhibitors of kappa B kinase- β (p-IKK- β) and phosphorylated Inhibitors

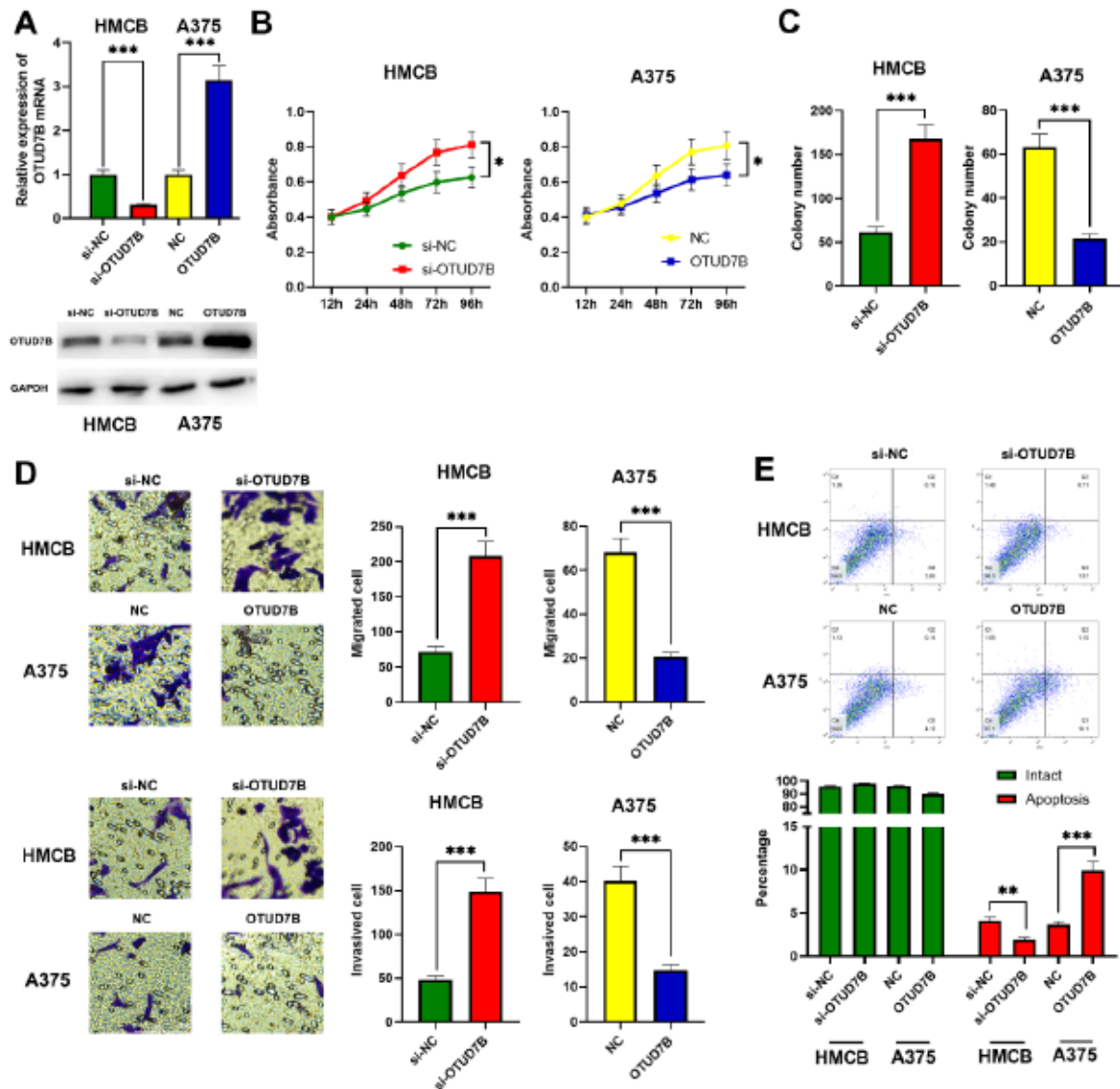


Fig. 2. OTUD7B suppressed the malignant phenotype of melanoma. A) qRT-PCR (upper) and Western blot (below) were adopted to examine OTUD7B expression in the melanoma cell line after transfection. B-C) The proliferation of melanoma cell lines was examined using CCK-8 assay (B) and colony formation assay (C). D) Transwell experiment was utilized to examine migration and invasion of melanoma cell lines after transfection. E) Flow cytometry was executed to detect the apoptosis of melanoma cells after transfection. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of kappa B- α (p-I κ B- α) expression, while OTUD7B overexpression in A375 cells reduced these proteins' expression level (Fig. 3A-B). Furthermore, different concentrations of the NF- κ B inhibitor PDTC substantially impeded the phosphorylation of NF- κ B in a dose-dependent manner, but not the expression of OTUD7B (Fig. 3C). The data indicated that OTUD7B was the upstream of the NF- κ B pathway and OTUD7B overexpression impeded the activation of NF- κ B pathway.

DISCUSSION

OTUD7B contains the ubiquitin-associated (UBA) domain, ovarian tumor (OTU) domain, cytidhesin P30/P32 domain, and A20-like zinc finger domain (7). The OTU domain exerts the deubiquitin function; the cytidhesin P30/P32 domain

is implicated in cell adhesion; and the A20-like zinc finger domain participates in ubiquitin chain binding (8). Reportedly, OTUD7B targets the K48- and K63- polyubiquitin chains of intracellular proteins and enhances deubiquitination of target proteins to modulate cell signaling (9). OTUD7B is reported to induce K63-deubiquitination of TRAF6 under hypoxic condition to modulate NF- κ B activation (10). In this work, it was revealed that OTUD7B was remarkably under-expressed in melanoma tissues and cell lines, and its low expression predicts poor prognosis of the patients. Additionally, its depletion enhances the malignant phenotypes of cancer cells via modulating NF- κ B signaling. Our findings suggest that OTUD7B is a tumor suppressor in melanoma, which is consistent with its role in lung cancer and hepatocellular carcinoma (5, 6).

For the first time, this study investigated the

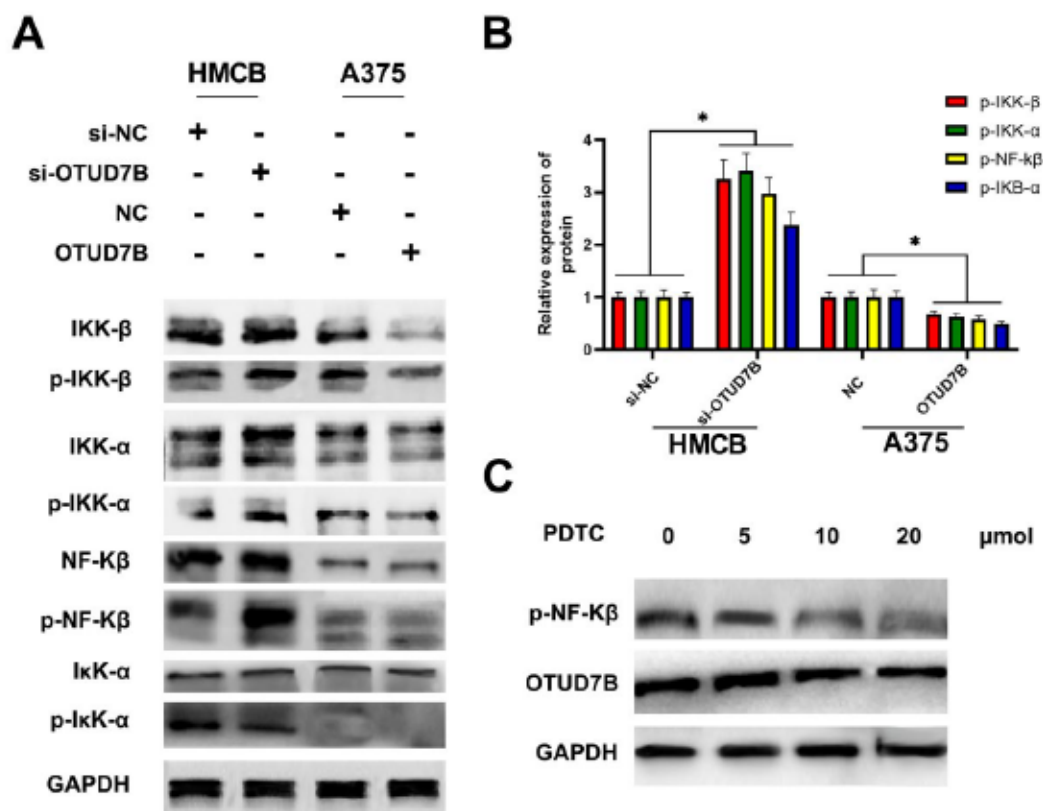


Fig. 3. OTUD7B repressed NF- κ B signaling pathway. A-B) After melanoma cells were transfected with OTUD7B siRNA or overexpression plasmid, the expression level of NF- κ B pathway-related protein in melanoma cell lines was examined by Western blot. C) Western blot was used to detect the effect of different concentrations of PDTC on the expression level of NF- κ B pathway-related proteins and OTUD7B. * $P < 0.05$.

expression characteristics, biological function and underlying mechanism of OTUD7B in melanoma. Our data suggest that OTUD7B may be a promising target for melanoma therapy, and it has the potential to be a biomarker to predict the prognosis of the patients. Nonetheless, in the present work, only *in vitro* experiments were performed, and in the following studies, *in vivo* model is required to further validate the tumor-suppressive role of OTUD7B in melanoma. Additionally, more tissue samples from multiple medical centers is helpful to further evaluate the prognostic value of OTUD7B in melanoma.

Ethics approval and consent to participate

Our study was approved by the Ethics Review Board of the Affiliated Hospital Of Hebei Engineering University.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and material

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: YT; Performed the experiments: YL, YX, FW, YT; Statistical analysis: BM; Wrote the paper: YL, BM, ZR, YT.

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Role of the hemostasis system in COVID-19

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To the Editor,

SARS-CoV-2 has become a new reality in the modern world. The spread of acute respiratory infection COVID-19 poses a severe threat of decompensation due to chronic illness. In Russia, 4.08 million cases of COVID-19 were registered, and 110 million worldwide. It comes with high mortality of 80,587 people at present, and 2.44 million respectively in Russia and worldwide. The most vulnerable categories for complications are obesity, chronic obstructive pulmonary disease, arterial hypertension, coronary heart disease, diabetes mellitus, and chronic kidney disease (1, 2).

The pathogenesis of COVID-19 has been little studied, and its aspects are in an active search process (3). After the SARS-CoV-2 invasion of the alveolar epithelium cells with receptors for the angiotensin-converting enzyme type II, type 2 transmembrane serine protease binds the virus to angiotensin-converting enzyme using the S-protein. It leads to diffuse alveolar damage, catastrophic activation of the immune system, cascade failure of the cytokine storm, endotheliopathy (damage to the vascular endothelium), cellular hypoxia, multiple organ failure, and acute respiratory distress syndrome. Hypercoagulation and suppression of anticoagulation have accompanied these changes. The result is the disruption of the processes of controlling the

inflammation resolution and the progression of the disease (4-6). Therefore, the current research aimed to study the hemostasis system's pathogenetic role in COVID-19 progression.

MATERIALS AND METHODS

The prospective with continuous sample research has been conducted (the evidence level: II). An analysis of 80 patients with COVID-19 of varying severity who were undergoing conservative treatment in the therapeutic department and intensive care unit of the state hospital in Perm, Russia, was carried out. All actions were made according to the ethical principles of the Declaration of Helsinki (7).

Selection criteria

The inclusion criteria were:

A verified COVID-19 diagnosis by clinical, laboratory, and instrumental methods.

Severity assessment in time of clinical observation.

Signed-personal (to patients) informed consent.

Age (from 18 to 75 years old) both male and female.

Mild and severe severity (according to the ARASN-II system); and

Mild comorbidities.

The exclusion criteria were:

Age under 18 and over 75 years old.

Keywords: COVID-19, endotoxemia, microcirculation, hemostasis, pathogenesis, TEG

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Patient's refusal.

Patient's violation of the study protocol.

Surgical operations.

Moderate severity (in ARASN-II system).

Presence of severe concomitant diseases (malignant and psychological).

Pregnancy and lactation period.

Patients grouped by disease severity

Patients were divided into two groups according to the disease severity degree. Group I (n=40) compounded patients with mild severity, with an average age of 51.5 ± 5.37 years, 18 men (45.0%) and 22 women (55.0%). Group II (n=40) compounded patients with a severe form of COVID-19, with an average age of 47.4 ± 6.51 years; 16 men (40.0%) and 24 (60.0%) women. Thirty conditionally healthy persons of both men, 16 (53.3%) and women 14 (46.7%), were observed to compare the experimental data with the reference values. The age of the reference group was 23-58 years.

Assessment of the disease severity (according to the ARACNE-II-point scale)

The scores obtained were 4.1 ± 0.11 and 15.3 ± 0.49 , respectively, for Group I and Group II. The score was based on such range of disorders development: 1-4 points the mild severity, 5-8 points moderately severe, and 9 or more – severe (8). The observed patients underwent standard therapy with including hormonal, anticoagulant, antiviral, infusion-detoxification, desensitizing, antibacterial, antihistamines, and other components. Such comorbidities were registered in most of the patients: cardiovascular diseases (arterial hypertension, coronary artery disease), gastrointestinal pathologies (gastritis, peptic ulcer), respiratory complications (bronchitis, tonsillitis), and excretory system pathologies (urolithiasis). In group I, the concomitant pathologies have been registered in 65.0% of patients (40.0; 25.0; and 15.0% for each one). For the group II – 55.0% of patients (35.0; 20.0; and 10.0%).

Further data and statistics

Determination of endogenous intoxication was carried out according to the values of hydrophobic metabolites (effective albumin concentration (ECA, g/L), total albumin concentration (OCA), albumin binding reserve (PCA, conventional units)), and hydrophilic middle-mass molecule (MSM, $\lambda=254$ nm). The microcirculatory status was assessed using the LAKK-02 apparatus (NPP

Lazma, Russia) using the area of the posterior surface of the left forearm at a point located along the midline 4 cm above the base of the styloid processes of the ulna and radius. The following parameters were determined: microcirculation rate (MR, pf. units) and microcirculation efficiency index (IEM, %). Functional assessment of the coagulate-lytic blood system was carried out according to the value of such biochemical parameters as reactive time (RT, min), the clot lysis index (CLI, %), and the TEG®5000 thromboelastograph apparatus (USA). The control was performed on the 1st, 3rd, 6th, and 10th days.

Experimental data statistical analysis was carried out with the Statistica 12.0 software and Microsoft Excel 2013. Student's criteria and Hardy-Weinberg test were determined.

RESULTS

Blood is the tissue that connects all body parts, and its hemostatic function has great importance in homeostasis providing (9). COVID-19 is characterized by the syndrome of endogenous intoxication development. It is accompanied by the production and accumulation of excessive concentrations of toxic metabolites in the body and disrupts normal homeostatic metabolism (10).

In the early stages of coronavirus infection, the development of an endotoxic phenomenon was noted. A decrease in the values of hydrophobic metabolites (OCA and PCA) and MSM were already observed during the early hospitalization of patients in the comparison group by 29.6, 31.4, and 48.4% ($p < 0.05$) (Table I).

In group I, endotoxic signs continued on the 3rd-6th days: OCA and PCA decreased compared to the norm by 32.1 and 20.7 and 34.1 and 17.7% ($p < 0.05$), and the level of MSM increased by 54.6 and 36.2% ($p < 0.05$). The parameters of endotoxicosis were close to normal at the final observation period (10th day). In severe COVID-19 flowing, on the first day in patients of group II OCA and PCA were lower than the reference by 48.6 and 45.5 ($p < 0.05$), respectively, but the content of MSM was increased by 66.7% ($p < 0.05$).

On the 3rd-6th day, the level of OCA and PCA relatively increased but remained below the normal parameter by 46.5 and 39.6; 43.0 and 34.1% ($p < 0.05$),

respectively. But the value of the MSM in blood plasma was increased by 78.7 and 54.3% ($p < 0.05$). At the end day of the study period (10th day), OCA and PCA were reduced by 34.6 and 25.3% ($p < 0.05$) in patients with severe disease flowing, and MSM has been increased by 30.3% ($p < 0.05$). According to literature data, the imbalance of the renin-angiotensin system, pro-inflammatory vasoconstriction, and endotheliopathy has been recorded in the early period of COVID-19 flowing. These contribute to the disruption of regional microcirculation and blood circulation in various organs (11).

Table I shows significant microcirculatory disorders (MCD) have been developed against the background of the endotoxiosis formation, and their severity has been compared with the disease severity level. Mild COVID-19 degree has the least severity of MCD, the value of the microcirculation index (reflecting the level of vascular perfusion) and the

index of microcirculation efficiency (characterizing the ratio of active and passive mechanisms of regulation of blood flow through microvessels) were reduced by 29.3 and 34.1% ($p < 0.05$). On the 3rd and 6th days, according to the level of the studied parameters, restoration of microcirculation disorder was established. At the same time, the microcirculation rate (MR) and IEM remained below 33.2 and 25.3 and 24.5 and 19.8% ($p < 0.05$). In the last control stage of the study, the value of the above parameters approached close to the normal level.

In group II, severe MCDs were significantly pronounced (Table I). On the 1st day, the level of MR and IEM was reduced by 49.2 and 48.4% ($p < 0.05$). On the 3rd day, MCD continued to increase: MR became lower by 46.0% ($p < 0.05$) and IEM – by 45.8% ($p < 0.05$). On the 6th and 10th days, the level of the studied parameters increased while maintaining a reduced level compared with the norm by 40.4,

Table I. Homeostasis system parameters in COVID-19 disease

Parameters	Control	Study groups	Observation period, days			
			1 st	3 rd	6 th	10 th
Tacrolimus	36.1±2.15	I	25.4±1.56	24.5±1.74	28.6±1.37	33.1±1.92
		II	18.6±0.35*	19.3±0.45*	21.8±0.88*	23.6±0.89*
Erythrocytes	0.79±0.06	I	0.54±0.03	0.52±0.04	0.65±0.05	0.72±0.04
		II	0.43±0.03*	0.45±0.04*	0.51±0.04*	0.059±0.03*
AMW	0.33±0.01	I	0.49±0.06	0.51±0.05	0.45±0.03	0.36±0.01
		II	0.55±0.05*	0.59±0.06*	0.51±0.04*	0.43±0.03*
Microcirculation Rate	1.26±0.03	I	0.89±0.03	0.84±0.02	0.94±0.02	1.12±0.07
		II	0.64±0.03*	0.68±0.04*	0.75±0.08*	0.91±0.03*
MEI	5.98±0.53	I	3.94±0.29	4.52±0.33	4.79±0.39	5.56±0.44
		II	3.08±0.16*	3.24±0.24*	3.57±0.28*	3.69±0.32*
Reactive time	4.13±0.25	I	2.91±0.12	3.14±0.17	3.37±0.19	3.78±0.22
		II	5.56±0.61*	5.66±0.59*	5.32±0.51*	5.27±0.46*
Clot lysis index	1.46±0.23	I	1.35±0.21	1.37±0.22	1.40±0.23	1.41±0.20
		II	2.21±0.27*	2.35±0.38*	2.24±0.41*	2.15±0.32*

HMM: Hydrophilic Middle-Mass Molecule; MEI: Microcirculation Efficiency Index bold type: the reliability of the difference from the norm group data at $p < 0.05$ *: the reliability of the difference from the first group data at $p < 0.05$

27.8, 40.3, and 38.2% ($p < 0.05$), respectively.

Pathogenic processes of homeostatic insufficiency, formed in COVID-19 flowing, lead to significant changes in the coagulation system that plays a primary role in the progression of infection and serious complications development (2, 4, 10, 12). COVID-19 is characterized by significant disorders of the hemostasis system according to disease severity. In the mild form (group I) an increase in the coagulation potential of the blood was recorded while maintaining the active process of dissolution of blood clots. The value of reactive time on the 1st day was crushed by 29.5% ($p < 0.05$), on the 3rd – by 24.4% ($p < 0.05$). On the 6th day, an extension of the reaction time was observed, maintaining a reduced level relative to the outcome by 18.4% ($p < 0.05$). On the 10th day, the RT (reaction time) value returned to normal (Table I and Fig. 1).

In patients with severe infection (group II), hypercoagulation manifestations and hypofibrinolytic reactions were established. A decrease in reactive time has been noted, representing the coagulation ability of the hemostatic blood system, by 29.5% ($p < 0.05$) on the 1st day. On the 2nd day, blood coagulation activity was reduced by 24.2% ($p < 0.05$). On the 3rd day, there was a tendency for the restoration of coagulation processes. However, the RT was

decreased compared to the norm by 18.2% ($p < 0.05$). On the 10th day the RT level came to the initial.

The clot lysis process index CLI, reflecting the hemostasis fibrinolytic ability, in patients of the I group was within the normal range throughout the entire observation period. Coagulation disorders in the I group have been the more significant. On the 1st day, RT was lengthened in relation to the norm by 34.6% ($p < 0.05$) in patients of the I group. Table I demonstrates that the hypocoagulation state has been recorded at subsequent stages: RT was lower than the outcome by 37.0, 29.1, and 27.6% ($p < 0.05$). Analyzing of the fibrinolysis system activity in patients of the II group demonstrated the increase in the time of CLI: on the 1st day by 51.3% ($p < 0.05$), on the 3rd day by 60.9% ($p < 0.05$), on the 6th by 53.4% ($p < 0.05$), on the 10th day by 47.2% ($p < 0.05$).

DISCUSSION

Data on the intensity of homeostatic changes, including the hemostatic system in COVID-19, have been associated with different levels of severity (4-6). Research results confirmed this: OCA and ECA levels as indicators of intoxication syndrome were significantly lower in patients of the main group than in the comparison group at all study stages by 27.1-28.7

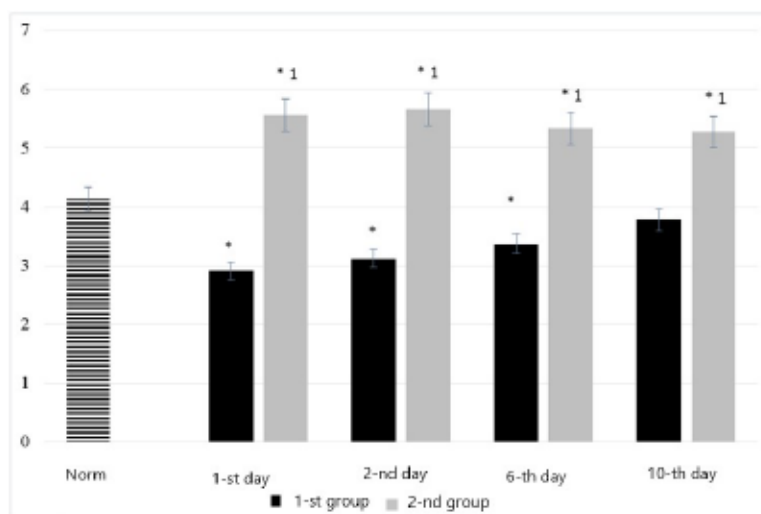


Fig. 1. Dynamics of BP index in coronavirus infection. *: reliability of difference from the data of the normal group at $p < 0.05$; 1: significance of difference from the data of the comparison group at $p < 0.05$.

Table II. Correlation between homeostasis indicators in case of mild coronavirus infection

Characteristics	Tacrolimus	Erythrocytes	HMM	Microcirculation rate	MEI
Reactive time	0.789	0.715	0.815	0.789	0.719
Clot lysis index	0.717	0.846	0.833	0.797	0.783

MEI: Microcirculation Efficiency Index, HMM: Hydrophilic Middle-Mass Molecule

Table III. Correlation between homeostasis indicators in case of severe coronavirus infection

Characteristics	Tacrolimus	Eritrocites	HMM	Microcirculation rate	MEI
Reactive time	0.812	0.905	0.934	0.885	0.778
Clot lysis index	0.789	0.821	0.719	0.766	0.691

MEI: Microcirculation Efficiency Index, HMM: Hydrophilic Middle-Mass Molecule

and 20.3-18.0% ($p < 0.05$), and MSM has been higher by 15.6-19.4% ($p < 0.05$). Table I demonstrated that MR and IEM in group II were lower than group I by 19.0-20.2 and 21.8-25.4% ($p < 0.05$). In addition, the reactive coagulation time and CLI in group II were 58.1-39.4 and 71.5-52.4% lower than group I ($p < 0.05$).

Table II and Table III show there is correlation dependence between indicators of the homeostasis and hemostasis systems almost in all parameters. The correlation between markers of coagulation activity of the coagulation system, its fibrinolytic activity, indices of endogenous intoxication (total albumin concentration, albumin binding reserve, and middle-mass molecule), and microcirculation (microcirculation rate together with microcirculation efficiency index) were revealed in group 1 of patients ($p = 0.01$, Table II) and group 2 ($p = 0.01$, Table III).

In the early period, pronounced manifestations of endotoxiosis syndrome and disorders in the microcirculation system were developed in patients with COVID-19. This is associated with a violation of the hemostatic system (blood coagulation and fibrinolytic activity). And the severity of deviations has been associated with the disease flowing. Homeostatic disorders can be reversed and were less pronounced in the case of mild COVID-19 form. The

change of the studied parameters was large in severe disease cases. The record of the hemostatic disorders has depended on the disease form: was fixed increased coagulation activity of the blood while maintaining the intensification of the fibrinolytic system (in the mild form of COVID-19). In severe cases, modulation of the hemostasis system was noted in the form of suppression of blood coagulation and hypofibrinolysis.

Declaration of conflict of interest

The presented research has no actual conflict of interest. The research has not been externally sponsored.

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Clinical efficacy of propofol and midazolam in cerebral hemorrhage surgery

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To the Editor,

Cerebral hemorrhage is a serious type of stroke. It is easy to cause cerebral multi-site hematoma (1). The mortality rate varies from high to low depending on the bleeding site and amount of hemorrhage (2,3). Surgical treatment is mainly introduced for this kind of disease clinically. The smooth operation depends on the selection of anesthetic drugs. Efficient and reliable anesthetic drugs not only could reduce the operation risk, but also effectively reduce the occurrence probability of complications on patients (4).

Propofol, as an ultra-short-acting opioid intravenous anesthetic, has the advantages of fast onset, superior sedative and hypnotic properties, down-regulating intracranial pressure, protecting the integrity of mitochondrial sodium pump function in cells, reducing cerebral edema caused by cytotoxicity and brain protection (5,6). Midazolam is a potent sleep-inducing drug commonly used in clinic, which has the effects of anti-anxiety, sedation, hypnosis and muscle relaxation (7). Nitric oxide (NO), a fat-soluble gas that is difficult to dissolve in water, belongs to a free radical whose molecular structure and electrons are not matched. It plays an important regulatory role in physiological pathology and related clinical diseases of systemic multiple systems such as respiration, circulation, nerve and immunity, and

it is commonly used to monitor anesthesia quality (8, 9). In this study, propofol and midazolam were used for anesthesia respectively. Combined with the effect of drugs on NO expression, the clinical efficacy and regulation mechanism of the two anesthetic drugs in the operation process were systematically analyzed.

MATERIALS AND METHODS

Medical records

120 patients with cerebral hemorrhage were strictly selected for statistical analysis of relevant clinical data. They were treated by surgical operation from January 2016 to February 2019 in our hospital. All patients were treated with intravenous inhalation combined anesthesia and minimally invasive removal of intracranial hematoma. All patients were informed and signed consent to participate in the study. The patients were divided into groups according to the application mode of surgical anesthesia. 67 patients with propofol of 6 mg/(kg·h) were set as GA, and 53 patients with midazolam of 0.2 mg/(kg·h) were set as GB.

Inclusion and exclusion criteria

Inclusion criteria: Patients who were diagnosed as cerebral hemorrhage by CT examination; Patients who met the surgical indications for cerebral hemorrhage and the scope of surgical anesthesia; Patients knew all

Keywords: propofol, midazolam, cerebral hemorrhage surgery, clinical efficacy, NO level

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operation steps, duration and general methods.

Exclusion criteria: Patients that had undergone previous cerebral hemorrhage surgery; Patients that developed severe disturbance of consciousness; Patients that had had major organ diseases such as liver and kidney. This experiment was carried out with the approval of the ethics committee association of our hospital, and the patients and their families all knew and supported the experimental conditions.

MATERIALS AND METHODS

The experimental patients fasted and deprived of water for 12h before operation, and all of them received intravenous inhalation combined anesthesia. After entering the operating room, the patients were given routine venous channel, and routine heart rate (HR), mean arterial pressure (MAP), hemoglobin (Hb), blood oxygen saturation (SaO₂) and other indicators were monitored. Patients were given 1 mg/kg of pethidine (Shenyang No.1 Pharmaceutical Factory of Northeast Pharmaceutical Group Company, SFDA Approval No. H21022413), 0.2 mg/kg of diazepam (Tianjin Jinyao Pharmaceutical Co., Ltd., SFDA Approval No. H12020957), 0.15 mg/kg of vecuronium (Chengdu Tiantai Mountain Pharmaceutical Co., Ltd., SFDA Approval No. H20063411) and 0.1 mg/kg of droperidol (Shandong Hualu Pharmaceutical Co., Ltd., batch number: H37022102). In GA, patients were given 6 mg/(kg·h) of propofol (Jiangsu Enhua Pharmaceutical Co., Ltd., approval number: SFDA Approval No. H20031037) based on the above narcotic drugs, while patients in GB were given 0.2 mg/(kg·h) of midazolam (Yichang Humanwell Pharmaceutical Co., Ltd., SFDA Approval No. H20067040).

Isoflurane (Maruishi Phaceutical, Japan) with a concentration of 1.5%-2% throughout the operation was inhaled in a continuous low-flow manner and propofol/midazolam was applied to maintain the anesthetic effect and ensure stable anesthesia depth state of patients. The patient's blood pressure status was closely monitored to adjust the infusion speed. When the patient's blood pressure was out of control, nitroglycerin (Henan Runhong Pharmaceutical Co., Ltd., SFDA Approval No. H20057216) was used.

After a 3 h operation, blood samples were routinely collected from patients. After centrifugation and

precipitation, the supernatant was collected and tested for NO level by nitrate reductase kit (Shanghai Xinfan Biotechnology Co., Ltd.).

Observation indexes

Anesthetic effect: After anesthesia, if the patient stays still during the operation, it was considered to be excellent. If there was body movement but with no interference to the operation, it was considered good. If the patient's body movement affected the operation, it was considered poor (10).

Clinical indicators: HR, MAP, Hb, SaO₂ and other indexes of patients in the two groups were detected and observed respectively before and after operation.

The oxygen extraction ratio (CEO₂) of patients was observed and compared in the two groups before, during and after operation.

The analgesic and sedative effects in the two groups were compared by using the Intensive Care Pain Observation Scale (CPOT) and the Restlessness-Sedation Score (RASS). The higher the score, the worse the analgesic and sedative effect (11,12).

The occurrence of anesthesia complications and NO level of patients were observed in the two groups.

Statistical methods

Statistical analysis was performed by using SPSS19.0. The measurement data were expressed as mean number $\pm$ standard deviation and tested by t test. The enumeration data were expressed in % and tested by χ^2 . The difference was statistically significant when $p < 0.05$.

RESULTS

After the completion of patient grouping, statistical software was used to analyze the distribution of baseline data between the two groups of samples, showing that there was no statistically significant difference between the data ($p > 0.05$), which could be compared (Table I).

The clinical anesthesia effect of GA was significantly better than that of GB ($p < 0.05$). After anesthesia, the improvement degree of clinical indicators (HR, MAP, Hb, SaO₂) in GA was significantly better than that in GB ($p < 0.05$) (Table II).

There was no significant difference in CEO₂

between the two groups before operation ($p>0.05$). CEO_2 in GA was significantly higher than that in GB during operation and after operation for 10 min ($p<0.05$), and CEO_2 basically returned to normal level at 30 min after operation. The incidence of anesthesia complications in GA was lower than that in GB ($p<0.05$). There were no deaths (Table III).

The pain CPOT score of patients with cerebral hemorrhage in GA was significantly lower than that in GB ($p<0.05$) (Fig. 1).

The restlessness RASS score of cerebral hemorrhage patients in GA was significantly lower than that in the GB ($p<0.05$) (Fig. 2).

The level of NO in patients with cerebral hemorrhage in GA was significantly higher than that in GB ($p<0.05$). (Fig. 3).

DISCUSSION

The two drugs selected in this study have definite curative effects in patients undergoing cerebral hemorrhage surgery, but there are few reports on the oxidation state and vascular regulation of the body during the application of the two drugs. The clinical anesthesia effect and clinical indexes (HR, MAP, Hb and SAO_2) in GA were significantly better than that in GB. Propofol is superior to midazolam in anesthetic effect and blood pressure control, which may be related to oxidation reaction through analysis. Therefore, CEO_2 was further studied between the two groups. There was no significant difference in CEO_2 between the two groups before operation. CEO_2 in GA was significantly higher than that in GB

Table I. Comparison of baseline data between the two groups.

Grouping (cases)	GA (n=67)	GB (n=53)	t / X^2	p
Gender (c)			0.020	0.886
Male	38 (56.72)	31 (58.49)		
Female	29 (43.28)	22 (41.51)		
Age/years old	64.52±4.34	64.73±4.48	0.260	0.796
Hematoma focus (c)			0.028	0.986
Epidural	22 (32.84)	18 (33.96)		
Subdural	19 (28.36)	15 (28.30)		
Intracerebral	26 (38.81)	20 (37.74)		
Comorbid with cerebral infarction (c)			0.189	0.663
Yes	9 (13.43)	6 (11.32)		
No	58 (86.57)	47 (88.68)		
Comorbid with diabetes mellitus (c)			0.117	0.733
Yes	14 (20.90)	12 (22.64)		
No	53 (79.10)	41 (77.36)		
Comorbid with coronary heart disease (cases)			0.038	0.845
Yes	11 (16.42)	8 (15.09)		
No	56 (83.58)	45 (84.91)		
Gastrointestinal diseases (cases)			0.149	0.700
Yes	10 (14.93)	9 (16.98)		
No	57 (85.07)	44 (83.02)		
Pulmonary disease (cases)			0.110	0.741
Yes	17 (25.37)	12 (22.64)		
No	50 (74.63)	41 (77.36)		

Table II. Influence of two anesthesia methods on anesthesia effect and clinical indexes of surgical anesthesia in cerebral hemorrhage patients [n (%)].

Grouping	GA (n=67)	GB (n=53)	X ²	p
Excellent	39 (58.21)	26 (49.06)	-	-
Good	25 (37.31)	15 (28.30)	-	-
Poor	3 (4.48)	12 (22.64)	-	-
Excellent and good rate	64 (95.52)	41 (77.36)	15.461	<0.001
Grouping	GA (n=67)	GB (n=53)	t	p
HR (times/min)	89.45±11.32	64.64±10.73	12.20	<0.001
MAP (mmHg)	143.43±10.73	167.54±10.94	12.00	<0.001
Hb (g/L)	126.34±6.37	112.53±5.93	12.16	<0.001
SaO ₂ (%)	0.74±0.13	0.64±0.10	4.621	<0.001

Table III. Comparison of CEO₂ and anesthesia complications between the two groups before and after operation [n (%)].

Grouping	GA (n=67)	GB (n=53)	t	p
Before operation	24.53±3.53	24.57±3.45	0.062	0.951
During operation	38.56±5.63	29.37±6.02	8.612	<0.001
After operation for 10 min	40.34±4.82	32.13±4.62	9.436	<0.001
After operation for 30 min	44.52±4.94	41.32±5.01	3.502	<0.001
Grouping	GA (n=67)	GB (n=53)	X ²	p
Stress ulcer	1 (1.49)	3 (5.66)	-	-
Hydrocephalus	0	1 (1.89)	-	-
Nausea and vomiting	2 (2.86)	5 (9.43)	-	-
Abnormal blood pressure	1 (1.49)	3 (5.66)	-	-
Cough	1 (1.49)	2 (3.77)	-	-
Incidence of anesthesia complications	5 (7.46)	14 (26.42)	13.101	<0.001

during operation and after operation for 10 min, and CEO_2 basically returned to normal at 30 min after operation. Propofol could improve the perfusion level of cerebral edema secondary to cerebral hematoma and slightly reduce intracranial pressure and cerebral blood flow. Brain CEO_2 also decreased, but midazolam was not ideal in this aspect. Propofol was far better than midazolam in the anesthesia

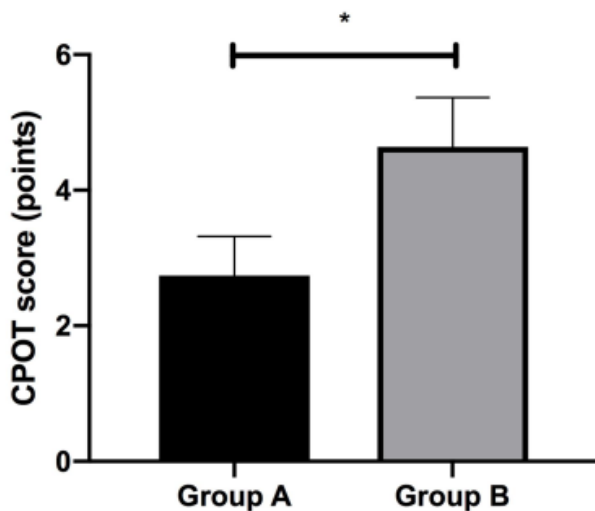


Fig. 1. Comparison of pain scores of cerebral hemorrhage patients between the two groups. The pain CPOT score of patients with cerebral hemorrhage in GA was significantly lower than that in GB. Note: * represents the comparison between the two groups, $p < 0.05$.

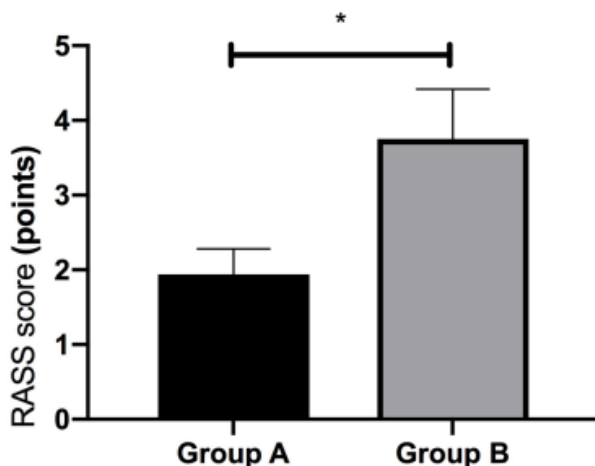


Fig. 2. Comparison of restlessness scores cerebral hemorrhage patients between the two groups. The restlessness RASS score of cerebral hemorrhage patients in GA was significantly lower than that in GB. Note: * represents the comparison between the two groups, $p < 0.05$.

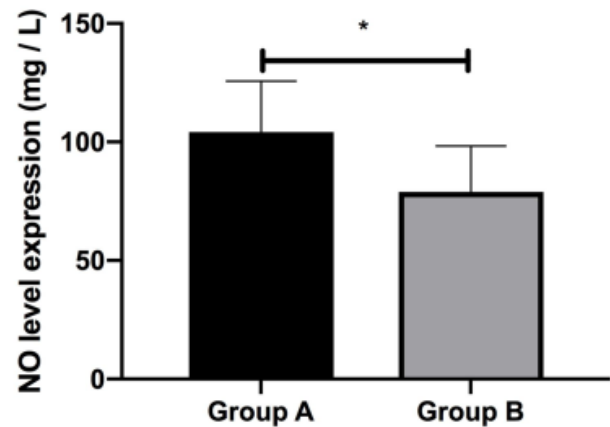


Fig. 3. Comparison of NO level of cerebral hemorrhage patients between the two groups. The NO level of cerebral hemorrhage patients in GA was significantly higher than that in GB. Note: * represents the comparison between the two groups, $p < 0.05$.

application of cerebral hemorrhage surgery, which was not difficult to conclude from the degree of pain, restlessness, and anesthesia complications in this experiment. Propofol anesthesia was beneficial to realize the dynamic balance of NO/ET after cerebral hemorrhage, and it had better efficacy in regulating vasomotor function and anticoagulation. To sum up, propofol has better anesthesia effect than midazolam in patients with cerebral hemorrhage, reduces cerebral metabolic rate, improves cerebral oxygenation, increases NO expression, and stabilizes cerebral blood flow dynamic balance.

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On Water Pollution in Agriculture and COVID 19

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To the Editor,

Water is an essential element for human health and well-being as well as for sustainable development. Polluted water, primarily when used for irrigation, is the leading source of contamination of fresh vegetables in the world (1). It is among the causes of several diseases and a leading cause of death for children, the elderly, and people with a compromised immune system (2).

The quality of existing water resources is threatened by pollution, such as direct discharges of municipal and industrial wastewater, uncontrolled solid waste disposal, leaching of pesticides, fertilizers from agricultural lands, and seawater intrusion along the coast, resulting from pollution overexploiting groundwater. In addition, farmers generally irrigate their produce with water from nearby rivers, streams, ponds, and wells that do not meet the required irrigation standards in many cultivated areas worldwide. (3). Therefore, fresh produce is being contaminated, resulting in bacterial infection for those who consume them. As a result, people can become sick when exposed to such bacteria and suffer from a weak immune system. In addition, taking immunosuppressive medication can make people more susceptible to developing a severe bacterial infection (4). Research on intestinal diseases and microbiome and their impact on the body's digestive system has taken off in recent years, spreading the macro theme of intestinal health (5).

Recent studies reported that bacteria in the gut are correlated with signature molecules that they may produce, which may impact high blood pressure, depression, cardiovascular system, metabolism, hormones, and the nervous system. Moreover, researchers have found that antibiotics may also weaken the immune system's ability to fight against infection (6).

In addition, regarding the recent outbreak of the novel coronavirus diseases (Covid-19), analysis of patients' data from both China and the United States has shown that individuals with high blood pressure exhibit a higher risk of COVID-19 infections and complications (7); therefore it is imperative to investigate the factors that would lead to an increased percentage of the population suffering from high blood pressure due to water and food pollution.

Within this context, the study aims to review the use of polluted irrigated water and its impact on food consumption. All these existing health risks are due to bacterial infection and highlight the rate of infected adults in COVID-19 in Lebanon. It also highlights the COVID-19 mortality rate, considering Lebanon as a use-case country since its agriculture system suffers from heavily polluted irrigation water.

Polluted water in agriculture and its effect on human health

Agriculture and water quality

Agriculture is by far the largest consumer of water

Key words: Covid-19, polluted water, gut microbiome, fatality rate, immune system

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in Lebanon (as elsewhere in the region), followed by domestic and industrial uses, and it represents around 80% of total water consumption in Lebanon.

Industrial, agricultural, and domestic wastewaters adversely affect the water quality used for irrigation in Lebanon. For instance, the leaching of pesticides and fertilizers from agriculture causes ground and surface water pollution. At the same time, Industrial activity releases a wide range of chemical effluents into watercourses, especially surface and coastal waters (8). Contaminated water may contain various types of viruses and bacteria. For instance, pathogenic viruses, bacteria, protozoa, *Salmonella*, and helminths may survive in raw municipal wastewater for an extended period. Pathogenic microscopic organisms are available in wastewater at much lower levels than the coliform gathering of microbes, which are more straightforward to distinguish and specify (as complete coliforms/100ml). *Escherichia coli* are the most generally embraced sign of waste contamination. They can also be isolated and identified fairly simply, with their numbers usually being given in the form of fecal coliforms (FC)/100 ml of wastewater. The composition of typical raw wastewater depends on the types and number of industrial and commercial units and the social life of the local communities, such as the global demographic and the implications for health protection and environment and wastewater governance approaches. Partially treated or untreated wastewater is discharged to reservoirs, rivers, and canals that supply irrigated water to agriculture. Wastewater contains microbes and chemicals that pose a risk to human and environmental health.

Wastewater governance refers to the guidelines, regulations, policies, and laws developed to guide wastewater use for agricultural and other uses and minimize the risk of public health and the environment (9).

Bacterial Water Contamination

Several studies and experiments were carried out on freshwater (well water) and treated wastewater from different wastewater treatment plants (WWTP) located in the Bekaa Valley of Lebanon, considering different crops and vegetables such as spinach,

eggplants, lettuce, and grapes for the presence of bacterial and chemical contamination in fresh and treated wastewater (1).

According to the World Health Organization (WHO), water samples were taken at the discharge of the WWTP and from the hydrants in the fields and proposed to Lebanese guidelines (FAO) for reuse of treated wastewater in irrigated agriculture. It noted that the total *coliforms* were highly present in both fresh and wastewater while the Faecal coliforms (3.5×10^3 CFU/100 mL) were present in treated water, exceeding the proposed limit value. Furthermore, *Salmonella* and *Escherichia coli* (*E. coli*) were detected in treated water and freshwater (10). The studies also observed that pathogens could enter plants and become internalized. Other studies showed that *E. coli* could be transported into the edible part of lettuce from the soil through the root system. Likewise, *salmonella* can be transported from contaminated roots to the aerial parts of lettuce seedlings (10).

Based on these studies, we can conclude that poor water quality is a significant concern affecting human health in Lebanon. Studies have shown that, in general, waterborne diseases, especially diarrheal diseases, are some of the leading causes of mortality and morbidity among children less than five years old. Moreover, we also noted that health issues from exposure to water pollutants often result in health care expenditures. In addition to the impact on health and the body's immune system, poor water quality increases the cost of water treatment and forces people to buy more bottled water instead of having free governmental access to good quality drinking water that is found in nature.

Food contamination and health

The chemical contamination of food has emerged as a serious concern with potential health hazards in their wake. The majority of food contamination occurs naturally through toxins and environmental pollutants like water and soil. Also, during the processing, packaging, preparing, storage, and transportation of food, the primary source of contamination of these vegetables is from irrigated water. Therefore, vegetables could act as a reservoir

for several microorganisms from which they will be colonized inside these vegetables and infect susceptible hosts (11). The symptoms of infection typically include diarrhea (which can range from mild to severe) to abdominal pain and, in some cases, nausea and vomiting. Some *E. coli* links to heart and renal disorder and outbreaks are quite severe and have even caused deaths in rare instances (12).

The gastrointestinal system is controlled by the enteric nervous system ENS, located in its tract, and it is part of the peripheral nervous system. The ENS is directly correlated with the central nervous system through vagal, mesenteric, pelvic innervation, and consequently, the immune system. The pollutants of the food chain (glyphosate, mycotoxins) and their toxicity modify and alter the intestinal flora (microbiota), both in quantitative and qualitative terms. The result is a pathological situation of intestinal permeability (dysbiosis) and the passage of toxins from the intestinal content into the blood, key elements in the pathogenesis of many chronic inflammatory and autoimmune diseases. These toxins prefer the nervous tissue and cause brain fog due to their toxicity (brain fog) and consequently block the central control unit. (astrocyte, glia, and mast cell). The result is a mast cell degranulation that releases cytokines, a highly inflammatory substance.

If this mechanism is not identified at early stages and adequately treated, it undoubtedly can lead to a weak

immune system and, therefore, to its ineffectiveness and appearance of autoimmune diseases (13).

Bacterial infection and the body's immune system

Excessive use of antibiotics in a severe bacterial infection through food contamination has common and less common side effects in the human body such as stomach upset or other gastrointestinal diseases, including mood disorders such as depression. As the antibiotics fight the bacteria by blocking their vital processes to stop them from multiplying, they might kill the bacteria that belong in the gut, creating a state of dysbiosis; this refers to a condition where the fragile ecosystem in your gut is disrupted (14). Gut bacteria is a crucial component of the microbiota ecosystem within the human gut, which is colonized by 10¹⁴ microbes, ten times over the human cells. Gut bacteria play a crucial role in human health, like supplying essential nutrients, synthesizing antihemorrhagic factors, aiding in the digestion of cellulose, and promoting angiogenesis and enteric nerve function (15). However, they will even be potentially harmful because of the change of their composition when the gut ecosystem undergoes abnormal changes in the light of the utilization of antibiotics, illness, stress, aging, bad dietary habits, and lifestyle (12).

The gut microbiome plays a vital role in your health by helping control digestion and benefiting

Table I. The percentage among deaths COVID-19 deaths for the different age groups in Lebanon, Jordan, Tunisia, France, and Italy as of January 2021.

Age Group	Lebanon	Jordan	Tunisia	France	Italy
0-9	0.12	0.14	0.01	0.00	0.00
10-19	0.21	0.18	0.09	0.00	0.00
20-29	0.56	0.53	0.18	0.00	0.00
30-39	1.65	2.28	0.62	0.41	0.03
40-49	4.31	4.01	1.67	1.11	1.05
50-59	10.02	10.24	6.71	4.64	3.47
60-69	19.90	20.49	27.99	11.35	9.49
70 plus	63.23	62.13	62.72	82.49	85.96

your system and plenty of other aspects of health. Conversely, an imbalance of unhealthy and healthy microbes within the intestines may contribute to weight gain, high glucose, high cholesterol, hypertension, attack system, and cause other disorders (16).

Hypertension is a chronic pathological state that afflicts about a third of the entire human population (17). It is a significant risk factor for premature cardiovascular disease, coronary and peripheral atherosclerosis, cardiac hypertrophy, heart failure. More than 90% of the cases of hypertension do not have an identifiable cause and, therefore, are classified as essential or primary hypertension. Essential hypertension is thought to originate from the interaction between genetic and environmental factors.

The study tested this hypothesis in the context of hypertension, which is a severe risk factor for cardiovascular diseases. Hypertension and diabetes were highly prevalent in males aged 40-60 and even less (18). Among the Lebanese adult population, hypertension and diabetes are prevalent and multifactorial but remain incompletely recognized, leading to insufficient control. Yet, measures to

reduce the number of individuals suffering from such diseases are paramount to decreasing the percentage of individuals with weak immune systems.

Coronavirus 2 (SARS-CoV-2) and body's immune system

Analysis of early data from both China and the US shows that top pressure is the most typically shared pre-existing condition among those hospitalized, affecting between 30% to 50% of the patients (19). Other health conditions included cancer, diabetes, or lung disease. In Italy, it noted that over 99% of individuals who had died from the virus had one in all these conditions - and 76% of them had high vital signs. Other research shows that individuals with high vital signs also are slightly more likely to die from coronavirus. Their risk is about twice as high as that of the population (19).

COVID-19 was confirmed to own reached Lebanon in February 2020, and the general public infected with the COVID-19 virus will experience mild to moderate disease and recover without requiring special treatment. However, older people and people with underlying medical problems like disorders, diabetes, chronic disease, and cancer are

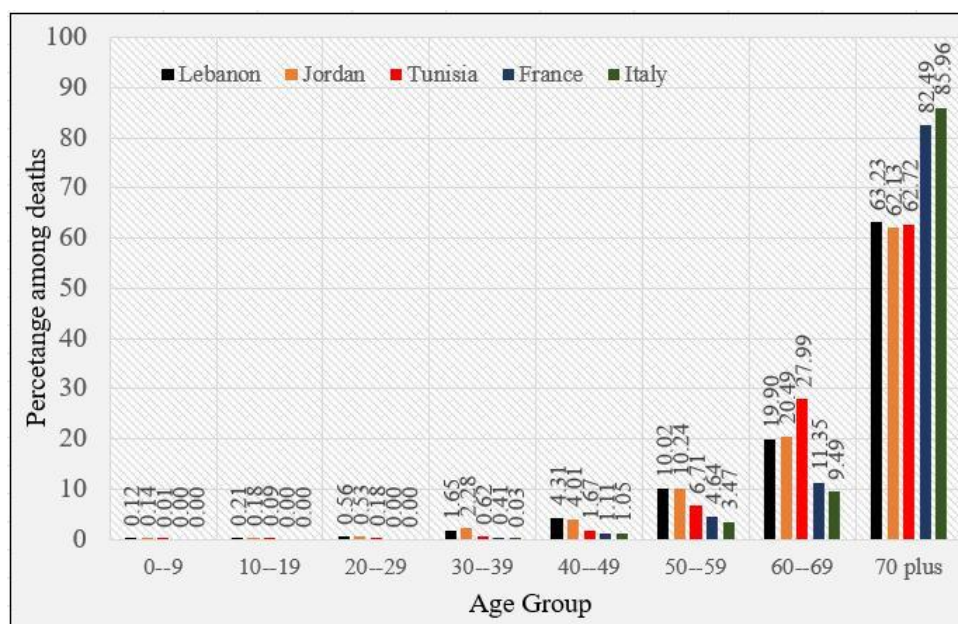


Fig. 1. the percentage among deaths COVID-19 deaths for the different age groups in Lebanon, Jordan, Tunisia, France, and Italy as of January 2021.

more likely to develop serious illnesses (20). The number of infected people in Lebanon was very high, and the government announced a full lockdown after detecting its highest daily number of coronavirus cases to stem a rise in COVID-19 infections that threaten to overwhelm hospitals in a country already facing financial meltdown, with full occupancy in beds and ICUs.

According to the latest statistics of the Lebanese Ministry of Health at the beginning of the year 2021, the age-specific cumulative incidence rate (1/100000), from 0 to 60 years plus, the highest rate incidences are those with age group 40-49 and the age group 50-59 and 54% of them are males (20).

Like other countries in the Mediterranean areas, Lebanon is characterized by a relatively high number of persons within the 40 – 60 group age that are susceptible to get diseases as diabetes, hypertension, and cardiovascular disease; this makes these individuals more exposed to a serious infection of COVID-19 in comparison with other countries.

Many studies shall be conducted to find out the causes of the high number fatality among young people in Lebanon, while in other countries, the number of fatalities is most probably due to elderly people.

The difference in terms of percentage of COVID-19 deaths between Lebanon and other countries (Fig. 1) reports the percentage among deaths due to COVID-19 per age group considering, in addition to Lebanon, Jordan, Tunisia, France, and Italy. The figure highlights how the percentage of COVID-19 deaths in relatively young age groups is higher in Lebanon than in other countries. In particular, we have considered Jordan a Middle Eastern country that follows a similar dietary habit as Lebanon and suffers from a similar situation in its agricultural system. Moreover, we have considered France and Italy due to their high suffering from COVID-19 death tolls; however, not suffered from similar problems that Lebanon suffers in its agricultural system. Moreover, we introduced Tunisia to compare the percentage of COVID-19 deaths per age group to non-European countries.

Reported statistics in Fig. 1 and Table I show that Lebanon and Jordan suffer a much higher mortality rate in relatively young age groups (20) than France

and Italy; specifically, in age groups between 0 and 29 years (21, 22) both Lebanon and Jordan suffer a rate of almost 1% (23), while the rate for the same age group in Tunisia is up to 0.28% (24) and France and Italy is 0% (21-22). In age groups between 30 and 60, the mortality rate in both Lebanon and Jordan is around 17%, much higher than in Tunisia (around 9%), France (around 6%), and Italy (around 5%) (25). More specifically, for the age group 40-49, both Lebanon and Jordan have a percentage of deaths higher than 4%, 4 times that of France and Italy (each around 1%), which further shows how both Lebanon and Jordan heavily suffer from deaths of relatively young age groups. On the contrary, for the oldest age groups, e.g., for 70-plus, Lebanon and Jordan show a relatively lower percentage of deaths (around 60%) than France and Italy, which show the highest percentage of deaths for the 70-plus age group being close to 85%. These figures show that the deaths in France and Italy are mainly concentrated in elderly patients (70 years and older) and that deaths barely occur for younger patients, while in Lebanon and Jordan, relatively younger patients (patients aged between 40 and 60 years) make up around 15% of overall deaths.

DISCUSSION

Lebanon's water resources are severely polluted, domestic and industrial sewage goes largely untreated and unsustainable agricultural practices worsen. Lebanon's major rivers have very high levels of bacterial contamination because of the discharge of raw domestic sewage. However, irrigating with untreated wastewater poses serious public health risks, as sewage may be a major source of excreted pathogens, the bacteria, viruses, protozoa that cause gastrointestinal infections in human beings; and it's reported that microorganisms within the human gut play a crucial role in digestive health. However, recent research indicates that gut bacteria may relate to wider aspects of health, including gastrointestinal, obesity, and metabolic health (26), and influence the system. Immune tissues within the digestive tract constitute the most important and most fraction of the human system. The intestinal mucosa could be

a large surface lining the intestine and is exposed to pathogenic (disease-causing) and non-pathogenic environmental antigens (substances that trigger the system to supply antibodies). Within the gut lumen, microorganisms play a critical role in developing a balanced and robust system (27).

The researchers, who presented their findings recently at the European Society of Cardiology, revealed a link between higher levels of 11 bacteria (estimated from genetic data) that were associated with a total of 28 health and disease outcomes, including chronic obstructive pulmonary disease (COPD), high blood pressure, high cholesterol, and BMI (Body Mass Index). In one example, they found that the higher the level of *Ruminococcus bacteria*, the greater the risk of high blood pressure (28).

CONCLUSION

Because of the lack of control and monitoring the food safety and agricultural practices in Lebanon, it is necessary to oblige the people to apply the preventive measures to protect themselves from COVID- 19 infection, especially since the fatality rate was very high, involving people with diabetes, cardiovascular disease, hypertension, kidney failure, and cancer. A proposal of the “Farm to Fork” policy should be applied to accelerate the transition to a sustainable food system that should positively impact food safety and public health. Many studies shall be conducted to determine the causes of the high number of fatalities among young people in Lebanon, while in other countries, the number of fatalities is in elderly people.

Conflicts of interest

All authors report no conflicts of interest relevant to this article.

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High-density lipoprotein-cholesterol may be independently correlated with IL-1 β in patients with multiple myeloma

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To the Editor,

Multiple myeloma (MM) is a disease due to the proliferation of a single slurry cell line in the bone marrow caused by immunoglobulin dysfunction in serum or urine, which leads to bone injury, anemia, renal dysfunction, hypercalcemia, and amyloidosis (1). The occurrence and development of multiple myeloma are inseparable from the inflammatory process, inflammatory cells, and related cytokines. Interleukin-1 β (IL-1 β) is part of the original member of the IL-1 family, responding to the rapid production and release of inflammatory signals from a variety of immunized and non-immunized cells to help hosts against infection and reduce damage (2). Meanwhile, Interleukin-1 β causes organ and tissue damage and promotes the development of tumor cells as a pro-inflammatory cytokine, as does IL-18. As a member of the IL-1 family, it has been demonstrated that the level of IL-18 increased was associated with poorer survival, disease progression, and increased concentrations of angiogenic cytokines in MM patients (3). Furthermore, a recent study by Hofbauer et al. has found that β 2-microglobulin triggers NLRP3 inflammasome activation in tumor-associated

macrophages, resulting in lysosomal rupture and production of active IL-1 β and IL-18, promoting MM progression (4). IL-1 β also increased the secretion of MM-related IL-6 (5), and the level of IL-22 in MM was positively correlated with IL-1 β (6).

MATERIALS AND METHODS

Patients

A total of 50 newly diagnosed patients with multiple myeloma in the Department of Hematology in Changzheng Hospital, Shanghai, from early August 2020 to the end of March 2021 were selected as the experimental group, including 23 males and 27 females, aged 37-79 (63.46 $\pm$ 10.40) years old. Besides, 34 healthy individuals without any disease who visited Changzheng hospital for routine physical examinations were recruited as the control group, including 16 males and 18 females, aged 38-75 (58.06 $\pm$ 8.00) years. The ethics committee approved this study of Changzheng Hospital, and all of the subjects gave their signed informed consent when they were in the hospital.

Clinical data collection

Clinical characteristics of all subjects were obtained

Key words: interleukin-1 β , multiple myeloma, influencing factors, laboratory indicators, risk factors

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from the medical records. The types, stages, extramedullary invasion, related complications of MM were collected according to the final diagnosis by clinical physicians.

Sample collection and IL-1 β measurement

Blood samples were centrifuged at 3000g for 10 minutes at 4 degrees centigrade, and sera were separated into Eppendorf tubes and stored at -80°C until determination. IL-1 β levels were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Shenzhen Dakowei Bioengineering Co., LTD) following the manufacturer's instructions. In brief, the serum samples were applied to the plate that was precoated with monoclonal antibodies to human IL-1 β . The biotinylated antibody was added to the plate, and it was incubated for 3 h at room temperature. The plate was washed 3 times by washing buffer, and then the streptavidin-HRP conjugate was added. After washing, the reaction was stopped with TMB and the stop solution. The absorbance at 450 nm was determined in a microplate reader (Bio-Rad, California, USA). The standard curve was made by serial dilution of recombinant protein. Then the levels of IL-1 β were calculated based on the values of optical density (OD) of the samples.

Other laboratory indicators

The levels of other laboratory indicators were obtained from the records of the laboratory information system. Levels of CRP were detected by Delta special protein analyzer (SEAC, Florence, Italy). Complete blood counts were done on an XN-9000 multifunction automatic blood analyzer (Sysmex, Kobe, Japan). Levels of liver and kidney function were determined on the AU5800 automatic biochemical analyzer (Beckman Coulter, California, USA). The laboratory indicators were all determined according to the manufacturer's protocols.

Statistical analysis

The experimental data and extracted laboratory information data were processed with SPSS 26.0. Firstly, the Kolmogorov-Smirnov method was used for the normality test. The data of normal distribution was described as mean $\pm$ standard deviation, and the skewness data were expressed as the median and interquartile range (IQR). χ^2 test was used to calculate the counting data. Mann-Whitney U test was used to compare the measurement data of non-normal distribution between

the two groups, and the Kruskal-Wallis H test was used to compare multiple groups. Spearman rank correlation analysis was used to test the correlation among variables in the MM group. Multiple linear regression was used to analyze the factors affecting IL-1 β expression level, and logistic regression was used to analyze the risk factors of MM. P value < 0.05 was considered statistically significant.

RESULTS

Expression of IL-1 β in MM group and control group

As shown in Fig. 1, the concentration of IL-1 β in the MM group [19.595 (15.613-24.455) pg/ml] was higher than that of the healthy control group [18.650 (6.9625-26.490) pg/ml], but there was no statistical significance ($P > 0.05$).

Differences of other laboratory parameters between MM and control groups

There were significant differences in age, lymphocytes, eosinophils, basophils, RBC, HGB, bilirubin, albumin, sodium, uric acid, urea, and CRP between MM and the healthy control group ($P < 0.05$) (Table I).

Correlation between IL-1 β and qualitative indexes in MM group

The level of IL-1 β in MM patients with different genders did not show a significant difference ($P > 0.05$). The expression of IL-1 β was statistically different among different types of patients ($P < 0.05$).

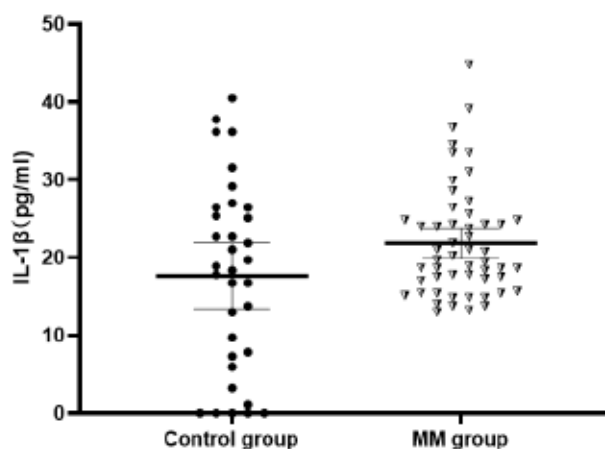


Fig. 1. Scatter plot of IL-1 β in MM and control.

Table I. Comparisons of laboratory indicators in two groups.

Parameters	MM group (n=50)	Control group (n=34)	Mann-WhitneyU /t Value	P Value
Age (years)	63.46±10.40	58.06±8.00	2.56	0.01
Lymphocyte (*10 ⁹ /L)	1.37 (0.97, 1.76)	1.68 (1.48, 2.21)	469.50	0.00
Eosinophils (*10 ⁹ /L)	0.08 (0.02, 0.13)	0.09 (0.06, 0.19)	633.00	0.048
Basophils (*10 ⁹ /L)	0.02 (0.01, 0.03)	0.03 (0.02, 0.04)	446.50	0.00
Red blood cells (*10 ¹² /L)	2.89±0.80	4.68±0.38	-13.72	0.00
Hemoglobin (g/L)	90.30±25.60	144.59±11.46	-13.18	0.00
Total bilirubin (μmol/L)	9.25 (6.33, 11.73)	12.80 (11.05, 14.93)	275.00	0.00
Direct bilirubin (μmol/L)	0.00 (0.00, 1.30)	2.25 (2.00, 2.73)	184.00	0.00
Indirect bilirubin (μmol/L)	8.50 (5.48, 11.73)	10.650 (9.05, 12.45)	478.00	0.00
Albumin (g/L)	36.33±6.08	44.27±2.37	-8.36	0.00
Sodium (μmol/L)	138.00 (135.00, 140.25)	141.000 (139.00, 142.00)	405.50	0.00
Urea (μmol/L)	7.25 (5.50, 10.68)	5.90 (4.85, 6.90)	546.50	0.01
Uric acid (μmol/L)	381.00 (307.25, 525.25)	331.50 (265.00, 373.500)	523.00	0.00
Creactive protein (mg/L)	3.54 (1.34, 8.62)	1.715 (0.88, 3.94)	581.00	0.01
White blood cells (*10 ⁹ /L)	4.95 (4.10, 6.78)	5.950 (4.58, 6.85)	721.50	0.24
Neutrophils (*10 ⁹ /L)	3.14 (2.25, 4.19)	3.26 (2.54, 4.22)	818.50	0.77
Monocytes (*10 ⁹ /L)	0.38 (0.26, 0.55)	0.36 (0.29, 0.48)	762.50	0.43
Platelets (*10 ⁹ /L)	183.00 (131.75, 233.25)	192.50 (165.00, 222.50)	747.50	0.35
Globin (g/L)	41.00 (23.00, 57.00)	30.00 (28.75, 33.00)	673.50	0.11
Potassium (μmol/L)	4.00±0.45	4.15±0.29	-1.66	0.10
Calcium (μmol/L)	2.25 (2.14, 2.39)	2.29 (2.21, 2.39)	701.00	0.25
Creatinine (μmol/L)	72.50 (58.00, 180.75)	68.00 (59.75, 73.75)	696.50	0.16
Lactate dehydrogenase (U/L)	197.50 (159.50, 381.00)	181.50 (164.75, 210.25)	661.00	0.15

The content of IL-1 β with IgG type was higher than that with IgD type, and the content of IL-1 β with light chain type was significantly higher than that with IgD type (Table II).

Correlation analysis of IL-1 β and laboratory quantitative indexes in MM group

In the MM group, IL-1 β concentration was negatively correlated with such laboratory quantitative indexes as basophils, albumin, and high-density lipoprotein cholesterol (HDL-C), with statistical significance ($P < 0.05$) (Table III).

Analysis of influencing factors of IL-1 β in MM patients

Multiple linear regression analysis was performed on the quantitative indexes related to IL-1 β , such as basophils, albumin, and HDL-C, and the regression equation was $Y = 41.396 - 20.172x$ (x was HDL-C). Therefore, the results suggested that the main index affecting IL-1 β concentration in MM patients was HDL-C. Common linear judgment indicated no common linearity, and HDL-C was an independent correlation with IL-1 β (Table IV).

Table II. Correlation between IL-1 β and qualitative indexes in MM.

Parameters	cases	IL-1 β (pg/ml)	Mann-Whitney U	P Value
Gender				
male	23	20.81 (15.41, 24.32)	304.50	0.91
female	27	18.65 (15.68, 26.49)		
Stages				
DS			79.50	0.12
II	6	17.44 (13.65, 21.76)		
III	44	20.00 (16.09, 24.86)		
ISS			1.03	0.60
I	12	18.11 (15.41, 23.71)		
II	18	18.79 (15.55, 24.25)		
III	20	20.68 (17.57, 25.48)		
R-ISS			1.50	0.47
I	9	17.57 (14.60, 26.49)		
II	16	18.79 (15.21, 23.98)		
III	8	24.19 (17.98, 26.90)		
Type				
IgA	5	17.84 (16.356, 27.70)	8.63	0.04
IgG	27	23.78 (17.84, 27.30)		
IgD	4	14.32 (13.38, 16.89)		
Light chain	13	20.27 (15.41, 21.49)		

Table III. *Correlation of IL-1 β with laboratory quantitative indicators in MM.*

Parameters	Cases	R	P value
Basophils (*10 ⁹ /L)	50	-0.31*	0.03
Albumin (*10 ⁹ /L)	50	-0.34*	0.02
High-density lipoprotein cholesterol (mmol/L)	22	-0.63**	0.00
White blood cells (*10 ⁹ /L)	50	-0.24	0.09
Lymphocyte (*10 ⁹ /L)	50	-0.20	0.17
Eosinophils (*10 ⁹ /L)	50	-0.12	0.39
Red blood cells (*10 ¹² /L)	50	-0.17	0.24
Hematocrit (%)	50	-0.18	0.21
Total bilirubin (μ mol/L)	50	-0.02	0.89
Indirect bilirubin (μ mol/L)	50	-0.03	0.81
Globin (g/L)	50	0.23	0.11
Sodium (μ mol/L)	50	-0.18	0.21
Calcium (μ mol/L)	50	0.06	0.66
Urea (μ mol/L)	50	0.11	0.47
Neutrophils (*10 ⁹ /L)	50	-0.22	0.12
Monocytes (*10 ⁹ /L)	50	-0.08	0.57
Hemoglobin (g/L)	50	-0.18	0.22
Platelets (*10 ⁹ /L)	50	0.11	0.43
Direct bilirubin (μ mol/L)	50	0.14	0.32
Potassium (μ mol/L)	50	-0.01	0.96
Creatinine (μ mol/L)	50	0.09	0.52
Uric acid (μ mol/L)	50	-0.05	0.76
Creactive protein (mg/L)	50	-0.04	0.80
Age (years)	50	0.27	0.06
Lactate dehydrogenase (U/L)	48	0.14	0.34
β 2-microglobulin (mg/L)	46	0.14	0.37
NT-proBNP (ng/L)	47	0.10	0.51
Creatine kinase (U/L)	44	0.18	0.23
Alanine transaminase (U/L)	50	-0.07	0.63
Aspartic acid transaminase (U/L)	50	0.18	0.21
Alkaline phosphatase (U/L)	45	-0.15	0.32

Risk factors of MM

The laboratory indicators with statistically significant differences between MM patients and the control group were selected for Logistic regression analysis, using Forward: LR screening for risk factors of MM, demonstrating RBC and direct bilirubin reduction increased the risk of MM (Table V).

DISCUSSION

MM is a blood cancer of monoclonal plasma cells that accumulate in the bone marrow (1). It is well known that several cytokines are promoters of MM development. IL-1 is a powerful pro-inflammatory cytokine that operates as an endogenous pyrogen.

Table IV. *Influencing factors of IL-1 β in MM patients.*

Variables	R	T	P Value	Collinearity Statistics	
				Tolerance	VIF
(Constant)	41.396	5.760	0.000		
High-density lipoprotein cholesterol	-20.172	-3.725	0.002	0.452	2.210
Basophils	60.858	1.047	0.309	0.719	1.391
Albumin	-0.070	-0.984	0.338	0.537	1.864

Table V. *Analysis of risk factors in MM.*

Variables	OR (95%CI)	P value
Red blood cells	0.01 (0.00-0.20)	0.00
Direct bilirubin	0.14 (0.04-0.60)	0.01
Age		0.28
Lymphocytes		0.42
Eosinophils		0.68
Basophils		0.94
Hemoglobin		0.36
Indirect bilirubin		0.27
Albumin		0.50
Sodium		0.64
Uric acid		0.08
Urea		0.18
C reactive protein		0.67
IL-1 β		0.09

Blockade of IL-1 results in a decrease in IL-6 activity. Treatment with a recombinant IL-1R antagonist (Anakinra) prolongs progression-free survival of patients with indolent myeloma (2). Our study showed the concentration of IL-1 β in the MM group was higher than that of the healthy control group, but there was no statistical significance, which may be due to the small sample size and the severity of MM patients.

In this study, IL-1 β level was negatively correlated with basophils, albumin, and HDL-C. Iype et al. found that IL-1 β promoted the release of IL-8 and was more potent in the presence of IL-3 because it activated the basophils signaling pathway in healthy donors (7). Recent studies have found albumin tended to decrease under inflammatory conditions and was inversely associated with CRP(8). Therefore, IL-1 β , as a pro-inflammatory factor, may be negatively correlated with albumin.

Our study demonstrated that HDL-C was an independent factor affecting IL-1 β expression in MM patients and erythrocytes, and direct bilirubin reduction may be risk factors for MM. Studies have shown that HDL inhibited serum amyloid A (SAA)-mediated reactive oxygen species production and NLRP3 inflammasomes activation (9-10). As a result, IL-1 β transcription and maturation were inhibited with HDL-C increased. Anemia has proven to be an MM factor of poor prognosis, and the severity of anemia in determining staging and prognosis of the disease was very important (11). In addition, direct bilirubin has antioxidant and anti-inflammatory activities, the decrease of which may be a risk factor for MM. In conclusion, the serum level of IL-1 β in MM patients was not significantly different from that of the healthy control. HDL-C was an independent influencing factor for serum IL-1 β level. Anemia and direct bilirubin reduction were risk factors for MM.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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A single-center retrospective analysis of clinical characteristics of 95 acute myeloid leukemia patients older than 55 years

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To the Editor,

Acute myeloid leukemia (AML) is a heterogeneous disease. Morphological and chromosomal aberrations can be detected in leukemic cells (1). The median age of AML is 67 years, and patients over 65 years, at diagnosis, account for 54%. The proportion of patients over 75 years is about 35% (2). The therapeutic regime of leukemia in the older age group is difficult, and the prognosis is adverse. Five-year OS is adverse and estimated to be as low as 3-8% in patients ≥ 60 years (3). The patient-related and biological disease-related factors both restrict the therapy effects in older patients (4).

Cytogenetic and molecular markers are pivotal in evaluating patients with AML and can guide the treatment elections. Chromosome 5 and 7 abnormalities are progressively increasing in the elderly, with a complex karyotype prevalence of 28% in the older group (5). According to the *Common Knowledge of Chinese Experts*, in the AML patients aged over 60, the proportion of better cytogenetics and molecular biology markers is only 3.6% (6). Tsai reported that older patients with AML had more mutations regarding *DNMT3A*, *ASXL1*, *PTPN11*, *RUNX1*, *TET2*, *NPM1*, and *TP53* genes, but fewer

WT1 mutations. *DNMT3A* and *TP53* mutations were found to be independent adverse prognostic factors in older patients (7). Epigenetic modifier genes like *IDH2*, *TET2*, and *DNMT3A*, were found more frequently in older patients with AML and considered to be related to age-related clonal hematopoiesis and poor survival.

The prognosis of younger patients with AML has improved with new drugs and better treatment, but it is still unsatisfactory in older adults.

In this study, we analyzed the clinical characteristics and the prognosis in older patients with AML to clarify the factors affecting the prognosis of these patients to find the independent adverse prognostic factors affecting survival.

MATERIALS AND METHODS

Patients and treatments

A total of 95 older patients (>55 years) diagnosed with AML were enrolled in this study between October 2012 and July 2019. The ethics committee of Soochow University approved the study (approval number: nct03715621). Patients and their families provided informed consent. We divided the patients into two groups (<60 years vs. ≥ 60

Key words: AML; older; cytogenetics; prognosis factors

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years). The patients in relatively good general conditions received a standard first-line induction chemotherapy regimen consisting of an IA-like regimen comprising Idarubicin 8–10 mg/m² (days 1–3) and cytarabine 100 mg/m² (days 1–7), patients in poor performance conditions could receive the treatment based on Decitabine + AAG (aclacinomycin, Ara-C and G-CSF) or best supportive care. Decitabine + AAG: Decitabine 20mg/m²/d (days 1–3); aclacinomycin 20 mg/d (days 1–7); Ara-C 40 mg/m²/d (days 1–14, q12h); G-CSF 300 ug/d (stop while white blood cells >20*10⁹/L). Patients received allo-HSCT or chemotherapy as consolidation after complete remission.

Cytogenetic and molecular analyses

Bone marrow was taken from patients with AML and was tested using standard unstimulated cultures of 24 hours. At the same time, traditional R-banding tests were conducted for karyotyping. A minimum of 20 bone marrow-derived cell metaphases was analyzed for each sample. Genomic DNA was extracted from these mononuclear cells using the Purelink TM Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) according to the standard operating method. Acute leukemia-associated genes, including *DNMT3A*, *NPM1*, *CEBPA*, and *FLT3-ITD*, were evaluated. PCR amplification of entire or partial coding regions of the DNA was performed to detect gene mutations, and then direct bidirectional DNA sequencing was conducted.

Moreover, as described previously (8), we performed next-generation sequencing using a panel of approximately 700 tumor-associated genes to explore potential leukemic mechanisms in older patients with AML. According to the manufacturer's protocols, illumina HiSeq 4000 was used for 75×75 paired-end sequencing (80–100 per flow cells). The whole genome was sequenced using DNA-Seq and RNA-Seq. The average sequencing depth of DNA-Seq was 300 times, and the reads of RNA-Seq were 50 M.

Statistical analyses

Statistical Product and Service Solutions (SPSS) software (version 22.0; IBM, USA) was employed for data analysis. Student's t-test and χ^2 test were utilized to compare the counting data in different groups, and to analyze the correlation of different gene mutations, respectively. Two definitions were used in this study: OS and DFS. OS and DFS indicate the time from the date

of disease diagnosis to the date of death or last follow-up and to the first relapse of complete remission (CR) or death, respectively. Complete remission (CR) means bone marrow blasts $\leq 5\%$. The differences in survival rates among groups were compared by the log-rank test. Survival curves were plotted using the Kaplan–Meier method. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of 95 older patients with AML

The study recruited 95 older patients with AML who were diagnosed from October 2010 to July 2017. The median age was 60 years (56–74 years), with 63 males and 32 females. On the basis of the French–American–British (FAB) criteria, three (3.2%) patients were classified as M0, while there were 14 (14.7%) M1 patients, 29 (30.5%) M2 patients, 13 (13.7%) M4 patients, 20 (21.1%) M5 patients, six (6.3%) M6 patients, one (1.1%) M7 patient, and nine (9.5%) unclassified AML patients. The median bone marrow blast was 58.0%, and the median white cell count was 9.1 (0.5–441.5)*10⁹/L. We divided the patients into two groups (<60 years vs. ≥ 60 years) and found that older patients had lower hemoglobin counts ($P=0.046$). Based on the FAB criteria, obvious differences were found between the two groups ($P=0.033$). Besides, differences were also seen in disease status ($P=0.005$). The clinical characteristics of the 95 patients are shown in Table I.

Cytogenetic and molecular analyses of 95 older patients

In chromosomal analyses, of the 95 patients, 11 with complex karyotypes, 3 with chromosome 7 abnormalities, 1 with chromosome 5 aberrations, 2 with t(v;11q23.3), while 6 patients had t(8;21). In the routine mutation analyses, *FLT3-ITD*^{mut} and *NPM1*^{mut} were both detected in 20 patients, among which *FLT3-ITD*+/*NPM1*+ *FLT3-ITD*+/*NPM1*–, *FLT3-ITD*–/*NPM1*+ were detected in 10 patients, respectively. *CEBPA*^{mut} was detected in 14 patients, *DNMT3A*^{mut} in eight patients, *IDH2*^{mut} in five patients, *GATA2*^{mut} in two patients, and *TET2*^{mut} in one patient. Based on the 2017 ELN risk classification, 20

patients with AML were classified into the low-risk group, 57 into the intermediate-risk group, and 18 into the high-risk group.

Genetic aberrations using next-generation sequencing
We tested seven *de novo* samples for genetic aberrations using next-generation sequencing

Table I. Clinical characteristics of 95 elderly AML patients

	Total patients (n=95)	<60 years (n=58)	≥60 years (n=37)	p-value
Sex				
Male	63	36	27	0.374
Female	32	22	10	
Disease status				
De novo	87	57	30	0.005
Secondary	8	1	7	
FAB classification				
M0	3	2	1	0.033
M1	14	8	6	
M2	29	11	18	
M4	13	10	3	
M5	20	15	5	
M6	6	6	0	
M7	1	1	0	
Unknown	9	5	4	
White blood cells counts (*10 ⁹ /L)	9.1(0.5-441.5)	14.6(0.7-441.5)	6.3(0.5-240)	0.362
Hemoglobin (g/L)	89(40-140)	103(40-140)	83(48-122)	0.046
Platelet counts (*10 ⁹ /L)	48(6-229)	50(6-229)	40(8-196)	0.422
BM blast (%)	58(20-96)	58(20-96)	56.5(20-94)	0.658
PB blast (%)	38(10-91)	45(12-91)	32(10-85)	0.225

BM: Bone marrow; **PB:** Peripheral blood.

Table II. Gene aberrations with next-generation sequencing of 7 elderly AML patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age	58	57	56	58	57	59	56
HSCT	HSCT	HSCT	HSCT	HSCT	HSCT	HSCT	HSCT
Relapse	-	-	Relapse	-	Relapse	Relapse	-
Death	-	Death	Death	Death	Death	Death	-
Gene aberrations							
Biallelic <i>CEBPA</i> mutation	+						
<i>FLT3-ITD</i>		+	+				
<i>DNMT3A</i>		+	+				
<i>NPM1</i>			+				
<i>IDH2</i>			+	+	+		
<i>KRAS</i>		+				+	
<i>NRAS</i>			+				
<i>RUNX1</i>				+	+		
<i>PHF6</i>					+		
<i>PTPN11</i>			+				
<i>GNAS</i>				+			
<i>U2AF1</i>				+			
<i>WT1</i>					+		
<i>KMT2C</i>		+					
<i>KMT2A-MLLT4</i>							+
<i>KMT2A-MLLT10</i>						+	

HSCT: Hematopoietic stem cell transplantation.

technology with a panel of 700 tumor-associated genes to investigate potential mechanisms of leukemogenesis in older patients with AML. As shown in Table II, *IDH2* (3/7, 42.9%) was the most frequent mutation in older patients, and *FLT3-ITD* (2/7, 28.6%), *DNMT3A* (2/7, 28.6%), *KRAS* (2/7, 28.6%), and *RUNX1* (2/7, 28.6%) were also frequent mutations. The distribution of other gene

mutations was *CEBPA* (1/7, 14.3%), *NPM1* (1/7, 14.3%), *PTPN11* (1/7, 14.3%), *NRAS* (1/7, 14.3%), *PHF6* (1/12, 8.3%), *U2AF1* (1/12, 8.3%), *WT1* (1/7, 14.3%), *KMT2C* (1/7, 14.3%), *KMT2A-MLLT4* (1/12, 8.3%) and *KMT2A-MLLT10* (1/12, 8.3%).

Clinical outcome of older AML patients

95 patients received either a standard first-

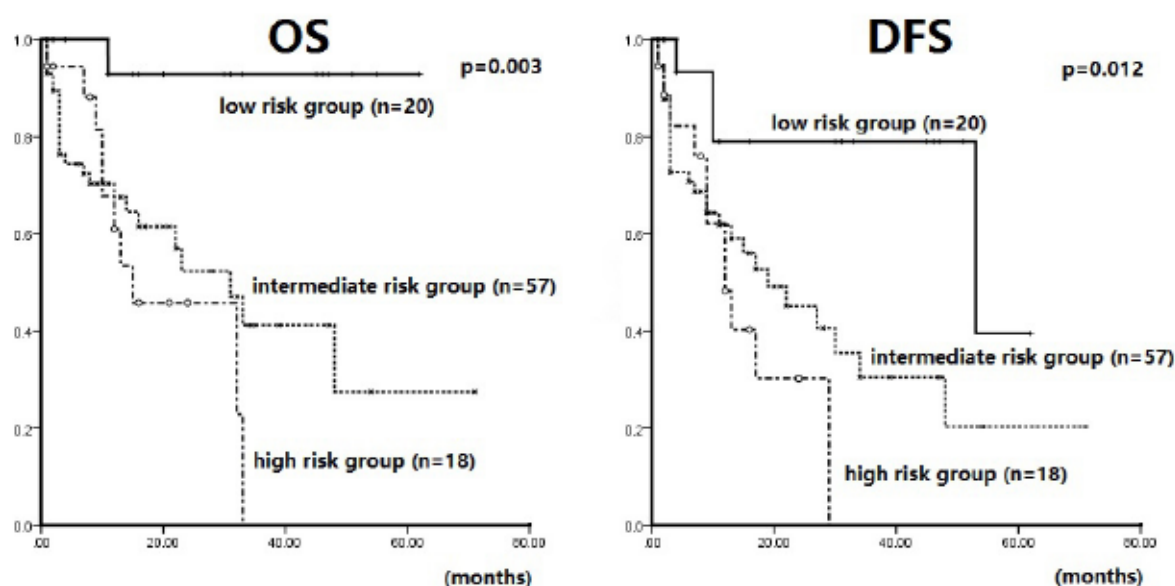


Fig. 1. The overall survival and disease-free survival of elderly AML patients with different risk classifications

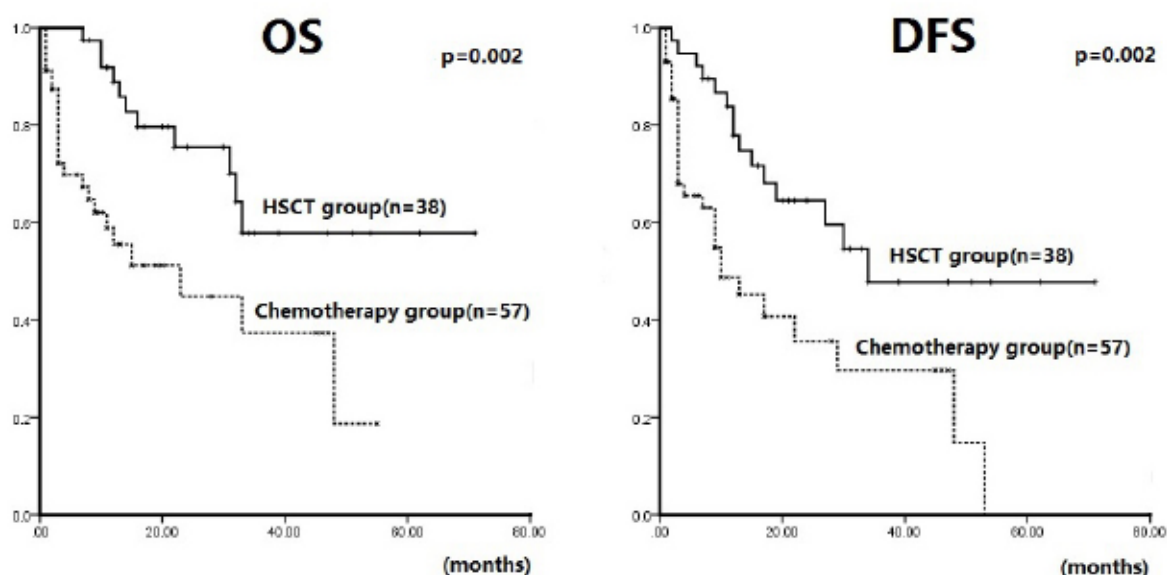


Fig. 2. The overall survival and disease-free survival of elderly AML patients receiving HSCT and chemotherapy

line induction chemotherapy regimen consisting of an IA-like regimen, or the treatment based on Decitabine + AAG, or best supportive care. Among them, 41 patients used Decitabine, and the CR rate was 56.10% (23/41). The median follow-up time of

the 95 patients was 32 months (range: 1-71 months), and the 3-year OS was 46.4%. Based on 2017 ELN risk classification, it could be concluded that there were obvious differences in 3-year OS (92.9% vs. 41.2% vs. 0.0%, $P = 0.003$) and 3-year DFS (79.0%

Table III. Comparison of dysphagia Stooler grading of patients in the two studied groups

Stooler grading	Combination group n=58	¹²⁵ I group n=58	P-value
Preoperative			0.673
0	0 (0%)	0 (0%)	
1	5 (8.7%)	8 (13.8%)	
2	23 (39.7%)	19 (32.8%)	
3	25 (43.1%)	27 (46.6%)	
4	6 (10.3%)	4 (6.9%)	
1 w postoperative			0.819
0	14 (24.1%)	11 (19.0%)	
1	19 (32.8%)	22 (37.9%)	
2	17 (29.3%)	15 (25.9%)	
3	8 (13.8%)	10 (17.2%)	
4	0 (0%)	0 (0%)	
1 m postoperative			0.781
0	33 (56.9%)	29 (50.0%)	
1	19 (32.8%)	21 (36.2%)	
2	5 (8.7%)	6 (10.3%)	
3	1 (1.7%)	2 (3.4%)	
4	0 (0%)	0 (0%)	

Table IV. Results of Multivariate Analyses for OS and DFS in elderly AML patients

	OS		DFS	
	p-value	HR(95%CI)	p-value	HR(95%CI)
Gene mutations (favorable vs. intermediate vs. unfavorable)	0.000	2.72(1.60-4.62)	0.001	2.32(1.42-3.78)
Treatment (allo-HSCT vs. chemotherapy)	0.000	3.69(1.77-7.68)	0.001	3.10(1.63-5.91)

OS: Overall survival; **DFS:** Disease-free survival; **HR:** Hazard ratio; **CI:** Confidence interval; Gene mutations (favorable vs. intermediate vs. unfavorable): based on the 2017 ELN classification.

vs. 30.4% vs. 0.0 %, $P = 0.012$) among the low-risk group ($n = 20$), the intermediate-risk group ($n = 57$) and the high-risk group ($n = 18$). (Fig. 1).

Among the 95 patients, 38 patients received allo-HSCT as consolidation, and 57 received chemotherapy consolidation. Based on the ELN classification, among the 38 HSCT patients, 23 were in the intermediate-risk group, 12 were in the high-risk group, and there were also 3 low-risk patients, 2 with CR2, and 1 with CR3. Dramatic differences also occurred in 3-year OS (57.8% vs. 37.3%, $P = 0.002$) [Hazard ratio (HR) (95% Confidence Interval (95% CI): 2.940 (1.422-6.080)] and 3-year DFS (47.7% vs. 29.7%, $P = 0.002$) [HR (95% CI): 2.594 (1.374-4.898)] between the two groups. (Fig. 2).

Outcomes of multivariate analyses in older AML patients

Univariate and multivariate analyses were performed in 95 patients with AML at the endpoint OS and DFS (Table III, IV). The analysis items include sex, age, disease status, white blood cell counts, hemoglobin counts, platelet counts, gene mutations, and treatment. In univariate analyses, *de novo* AML ($P=0.008$), favorable gene aberrations ($P=0.002$), and allo-HSCT ($P=0.004$) were predictors of prolonged OS. In contrast, male patients ($P=0.029$), favorable gene aberrations ($P=0.005$), and allo-HSCT ($P=0.003$) were predictors of prolonged DFS. In the multivariate analyses, allo-HSCT and favorable gene mutations were significantly and independently associated with prolonged OS ($P=0.000$, 0.000 , respectively) and DFS ($P=0.001$, 0.001 , respectively).

DISCUSSION

The study retrospectively analyzed the clinical features and outcomes of 95 older AML patients (>55 years of age) in our center in China. In this study, among the clinical characteristics in two groups (<60 years vs. ≥ 60 years), some differences were observed in disease status, FAB classification, and hemoglobin counts. In addition, we found older patients had lower hemoglobin counts. The results also revealed that secondary AML was more common

in older patients. Still, the number of secondary AML patients was too small, and more data is necessary to make a conclusion.

Creutzig reported that with aging, favorable cytogenetics would decrease to a low rate. In older patients (70+ years), the percentage was $<5\%$, while the ratio of unfavorable cytogenetics increased to 36% in patients over 85 years (5). In chromosomal analyses of the 95 patients, we found that the rate of unfavorable risk cytogenetics was 17.89%, while the percentage of favorable cytogenetics accounted for 6.3%. Prassek reported that ELN adverse-risk categories like *RUNX1*, *ASXL1* and *TP53* mutations are common in very old patients (9). In our study, we found older AML patients carried not only adverse molecular events, including *FLT3-ITD*, *DNMT3A* mutations, *KMT2A* rearrangements, but also favorable ones, including *NPM1* and biallelic *CEBPA*. In 7 patients using next-generation sequencing technology, adverse cytogenetics like *IDH2*, *FLT3-ITD*, *DNMT3A*, and *RUNX1* were frequent mutations in older patients. Consistent with Tsai's research (7), we also found older patients had fewer *WT1* mutations.

We also observed that AML in older people was associated with inferior survival. According to ELN risk classification, the prognosis of the high-risk group was worse than that of the intermediate and low-risk group. Meanwhile, the prognosis of the chemotherapy group was worse than that of the group receiving allo-HSCT. Legar found that the overall survival was much higher in the chemotherapy group than in the untreated group, and induction chemotherapy was an important factor affecting prognosis (10). In the multivariate analyses, we found the choice of therapy and gene mutations were significantly and independently associated with the prognosis. Indeed, nowadays, transplantation is an independent influencer compared to chemotherapy.

In conclusion, the study helped to reveal some actual data and information about older AML patients in China. And it suggested that the inferior cytogenetic and molecular abnormalities in older AML patients could lead to poor outcomes, and allo-HSCT could improve the prognosis.

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki (as was revised in 2013). The study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (NO.nct03715621). Written informed consents were obtained from all patients and their families.

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Efficacy of ^{125}I Seed Stent Implantation Combined with Transcatheter Arterial Infusion in the Treatment of Advanced Esophageal Cancer

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To the editor,

Esophageal cancer is a common malignant tumor in clinic with very high mortality and morbidity (1). Due to atypical early clinical symptoms, most patients with esophageal cancer have been in the late stage when diagnosed. Based on the epidemiological statistics, the 5-year survival rate for patients with esophageal cancer is about 10-15% (2). The palliative treatment for advanced esophageal cancer needs to not only improve dysphagia but also prolong the survival time of patients as far as possible. Chemoradiotherapy is the preferred treatment for advanced esophageal cancer patients who are unable to tolerate surgery or receive difficult surgery (3, 4). ^{125}I seed stent is produced by ^{125}I radioactive seeds sealed in a metal stent shell, and the particle source produced releases γ -rays to conduct radiotherapy for tumor cells, and effectively alleviate dysphagia and reduce the incidence rate of restenosis (5, 6). Stent implantation combined with transcatheter arterial infusion (TAI) can better control the development of tumor.

In this study, the clinical efficacy and safety of ^{125}I seed stent implantation combined with TAI and ^{125}I seed stent implantation alone were compared in the treatment of advanced esophageal cancer, so as to provide a strong basis for selecting the clinical therapeutic regimen for such patients.

MATERIALS AND METHODS

Objects of study

The clinical data of 116 patients with advanced esophageal cancer were collected. Inclusion criteria included: 1) patients meeting the diagnostic criteria for primary esophageal cancer according to gastroscopy, tissue biopsy and imaging examination, 2) those with inoperable esophageal cancer and dysphagia, 3) those with an estimated survival time >3 months, 4) those with a Karnofsky performance scale (KPS) score ≥ 60 points, and 5) those with normal liver and kidney functions. Exclusion criteria included: 1) patients with severe heart or lung disease, or unable to tolerate endoscopy and treatment, 2) those with severe coagulation disorders, or 3) those with severe liver or kidney dysfunction. Among all patients, there were 67 males and 49 females aged 39-77 years old with an average of (59.23 ± 10.15) years old. It was pathologically confirmed that there were 95 cases of squamous cell carcinoma, 12 cases of adenosquamous carcinoma, and 9 cases of adenocarcinoma. In terms of clinical stage, 77 cases were in stage III and 39 cases in stage IV. The baseline data (age, gender, pathological type, degree of tumor differentiation, tumor stage and KPS score) had no statistically significant differences between the two groups ($P > 0.05$), and they were comparable (Table I). This study was approved by the Ethics Committee

Key words: esophageal cancer; ^{125}I seed; transcatheter arterial infusion; stent implantation

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of Yantai Yuhuangding Hospital Affiliated to Qingdao University. Signed written informed consents were obtained from all participants before the study.

Treatment methods

The procedures of ^{125}I seed stent implantation were described below: Before operation, the lesion site, tumor diameter and surrounding invasion were determined via esophageal angiography, gastroscopy and CT. The CT image was imported into the treatment planning system

(TPS), and the seed dose and the number of layer and spacing of seeds were determined, followed by complete source distribution combined with corresponding slice thickness and area in CT image. The ^{125}I radioactive seed-covered stent (diameter: 18-20 mm, length: 6-18 cm) (Nanjing Micro-Tech Co., Ltd., Nanjing, China) was used. After anesthesia via lidocaine, the 5FKM catheter was delivered into the esophagus using the loach guide wire under the subtraction angiography system. The guide wire was withdrawn, and the stenotic region was marked

Table I. Demographics and general clinical data of all studied patients

Parameters	Combination group n=58	^{125}I group n=58	P-value
Gender (Male/Female)	36/22	31/27	0.452
Age (years)	60.34±10.18	58.92±10.29	0.457
Tumor location			0.842
Upper third	13 (22.4%)	11 (19.0%)	
Middle third	30 (51.7%)	33 (56.9%)	
Lower third	15 (25.9%)	14 (24.1%)	
Tumor differentiation degree			0.684
High	10 (17.2%)	9 (15.5%)	
Moderate	20 (34.5%)	24 (41.4%)	
Poorly	27 (46.6%)	25 (43.1%)	
Undifferentiated	1 (1.7%)	0 (0%)	
Pathologic type			0.764
Squamous cell carcinoma	46 (79.3%)	49 (84.5%)	
Adenosquamous carcinoma	7 (12.1%)	5 (8.6%)	
Adenocarcinoma	5 (8.6%)	4 (6.9%)	
TNM staging			0.326
III	41 (70.7%)	36 (62.1%)	
IV	17 (29.3%)	22 (37.9%)	
Karnofsky Score			0.373
70-90	19 (32.8%)	15 (25.9%)	
60-70	39 (67.2%)	43 (74.1%)	

Notes: TNM: Tumor, lymph node, metastasis.

and positioned. The guide wire and catheter were sent into the gastric cavity, and then was withdrawn and replaced with the loach guide wire. Subsequently, the 5F catheter was withdrawn. The stent introducer was slowly inserted along the guide wire with the position of implantation determined under the guidance of X-ray (2 cm beyond the upper and lower ends of stenosis segment), and then the stent was released. In severe cases, balloon dilatation was performed before stent implantation. After operation, the contrast agent was taken orally again, and whether the stent was released smoothly was observed under fluoroscopy. Whether ^{125}I seeds are displaced or lost was also observed. The safety line was retained at the upper end of stent, and its external end was wound around the root of ear and fixed with adhesive tape.

After operation, the antibacterial drugs were orally taken, and the symptomatic treatment (acid suppression therapy and enhancement of gastric motility) was performed. At 48-72 h and 1 month after operation, esophageal angiography was performed to detect the expansion and adherence of the stent and whether the stent was displaced, and the stent was adjusted if necessary.

Transcatheter arterial infusion: At 1 week after stent implantation, local infusion chemotherapy was performed. The femoral artery was punctured using Seldinger technique, the catheter was inserted, and the tumor site was determined, followed by selective or super selective intubation angiography for the possible supplying vessels of tumor. The chemotherapy drugs were infused into tumor vessels: cisplatin (DDP, 80-100 mg/m²) + fluorouracil (FU, 750-1,000 mg/m²) + pirarubicin (THP, 14-25 mg/m²). Infusion chemotherapy was performed for 4 cycles at an interval of 4-6 weeks.

Observation indexes

Evaluation of clinical efficacy: CT examination was performed at 2 months after operation, and the short-term efficacy was evaluated based on the Response Evaluation Criteria In Solid Tumors (RECIST) of WHO. Complete response (CR): The esophageal cancer lesions or enlarged lymph nodes completely disappear, and none of them or only strip-shaped shadow can be seen in imaging examination. Partial response (PR): The lesions shrink >50%. Stable disease (SD): The lesions shrink <50 % or expand <25%. Progressive disease (PD): The lesions or lymph nodes expand

≥25%, or there are new metastatic lymph nodes. In the case of treatment of multiple lymph nodes, the worst efficacy will prevail. Objective effective rate= (CR + PR)/total cases × 100%, clinical benefit rate= (CR + PR + SD)/total cases × 100%.

Before operation and at 1 week and 1 month after operation, the dysphagia grade was evaluated and compared in both groups using Stooler grading: Grade 0: no symptoms, and normal eating, grade I: able to take soft diet, grade II: able to take semi-liquid diet, grade III: able to take liquid diet only, and grade IV: unable to take food and drink (7). The postoperative complications were observed and recorded in the two groups, and symptomatic treatment was performed promptly.

The postoperative survival time (duration from stent implantation to death or deadline of follow-up) of patients was recorded via follow-up. Before and after operation, the behavioral status was evaluated using the KPS score, and the quality of life was assessed using EORTC QLQ-C30 and QLQ-OES18. The higher the score is, the lower the quality of life will be (8, 9).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$), and t-test was performed for intergroup comparison. Enumeration data were expressed as rate (%), and χ^2 test or Fisher exact probability test was performed for comparison. t-test was used for the intragroup comparison of paired data of immunological indexes, and two-way analysis of variance for the intergroup comparison. The survival curves were plotted using the Kaplan-Meier method, and log-rank test was performed. $P < 0.05$ suggested the statistically significant difference.

RESULTS

Comparison of clinical efficacy after treatment between the two groups

In the Combination group, there were 14 cases of CR, 28 cases of PR, 10 cases of SD, and 6 cases of PD. The total effective rate was 72.4% (42/58), and the clinical benefit rate was 89.7% (52/58). In the ^{125}I

group, there were 8 cases of CR, 21 cases of PR, 13 cases of SD, and 16 cases of PD. The total effective rate was 50.0% (29/58), and the clinical benefit rate was 70.7% (41/58). The total effective rate and

clinical benefit rate were significantly superior in Combination group to those in ^{125}I group, and the differences were statistically significant ($P=0.013$, $P=0.010$) (Table II).

Table II. Clinical effective rates of the two studied groups

	Combination group n=58	^{125}I group n=58	P-value
CR	14 (10.3%)	8 (19.1%)	
PR	28 (44.1%)	21 (52.9%)	
SD	10 (30.9%)	13 (23.5%)	
PD	6 (14.7%)	16 (4.4%)	
ORR	42 (72.4%)	29 (50.0%)	0.013
CBR	52 (89.7%)	41 (70.7%)	0.010

Notes: CR: Complete Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; ORR: Overall response rate; CBR: Clinical benefit rate.

Table III. Comparison of dysphagia Stooler grading of patients in the two studied groups

Stooler grading	Combination group n=58	^{125}I group n=58	P-value
Preoperative			0.673
0	0 (0%)	0 (0%)	
1	5 (8.7%)	8 (13.8%)	
2	23 (39.7%)	19 (32.8%)	
3	25 (43.1%)	27 (46.6%)	
4	6 (10.3%)	4 (6.9%)	
1 w postoperative			0.819
0	14 (24.1%)	11 (19.0%)	
1	19 (32.8%)	22 (37.9%)	
2	17 (29.3%)	15 (25.9%)	
3	8 (13.8%)	10 (17.2%)	
4	0 (0%)	0 (0%)	
1 m postoperative			0.781
0	33 (56.9%)	29 (50.0%)	
1	19 (32.8%)	21 (36.2%)	
2	5 (8.7%)	6 (10.3%)	
3	1 (1.7%)	2 (3.4%)	
4	0 (0%)	0 (0%)	

Comparison of dysphagia grade before and after operation between the two groups

The Stooler grade significantly declined in both groups at 1 week and 1 month after operation compared with that before operation ($Z=42.9$, $P<0.001$, $Z=67.8$, $P<0.001$), while it also significantly declined at 1 month after operation compared with that at 1 week after operation ($Z=183.1$, $P<0.001$). There was no statistically significant difference in the Stooler grade between the two groups before operation and at 1 week and 1 month after operation ($P=0.673$, $P=0.819$, $P=0.781$) (Table III).

Comparison of adverse reactions between the two groups

No massive hemorrhage, asphyxia, perforation, fistula, radiation pneumonia and pulmonary fibrosis occurred in patients. In Combination group, there were 17 cases of substernal pain, 7 cases of cough and expectoration, with blood streak occasionally, and 9 cases of foreign body sensation, and these symptoms were spontaneously relieved without

treatment after 1 week. After chemotherapy, nausea, vomiting, abdominal distension and fatigue occurred in 15 cases, and they were tolerable. 7 cases had bone marrow suppression above degree III that was improved after treatment with granulocyte colony-stimulating factor. In ^{125}I group, there were 15 cases of substernal pain, 11 cases of cough, 12 cases of foreign body sensation, 1 case of stent displacement, and 2 cases of postoperative bleeding. The incidence rate of stent implantation-related local complications was 46.6% (27/58) and 41.3% (24/58), respectively, in Combination group and ^{125}I group, and there was no statistically significant difference ($P>0.05$). In Combination group, adverse reactions were all tolerable and improved after symptomatic treatment.

Comparison of KPS score before and after operation between the two groups

Before operation, the KPS score was (69.79 ± 7.88) points and (68.80 ± 8.06) points, respectively, in Combination group and ^{125}I group, and the difference was not statistically significant ($p=0.505$). At 1

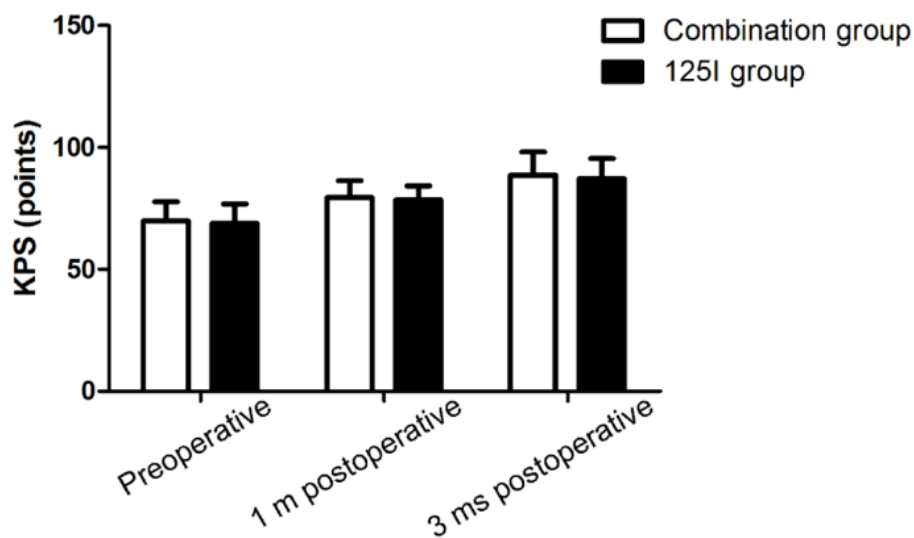


Fig. 1. Comparison of KPS score of patients in the two groups. Preoperative KPS score of patients had no significant difference between Combination group and ^{125}I group ($P=0.505$). 1 month Postoperative KPS score of patients in both Combination group and ^{125}I group significantly increased compared with preoperative KPS score ($P<0.05$). Three months Postoperative KPS score of patients in both Combination group and ^{125}I group significantly increased compared with 1 month Postoperative KPS score ($P<0.05$). Both 1 month and 3 months Postoperative KPS score of patients had no significant difference between Combination group and ^{125}I group ($P=0.373$, $P=0.396$).

month after operation, the KPS score obviously rose to (79.42 ± 6.90) points and (78.36 ± 5.83) points, respectively, in Combination group and ^{125}I group compared with that before operation ($P < 0.05$). At 3 months after operation, the KPS score obviously rose to (88.57 ± 9.56) points and (87.15 ± 8.34) points, respectively, in the two groups compared with that before operation and at 1 month after operation ($P < 0.05$). There was no statistically significant difference in the KPS score between the two groups at 1 month and 3 months after operation ($P = 0.373$, $P = 0.396$) (Fig. 1).

Comparison of quality of life before and after operation in two groups of patients

Before operation, no statistically significant differences were found in the EORTC QLQ-C30 and QLQ-OES18 scores between the two groups ($P = 0.221$, $P = 0.512$). In the two groups, the EORTC QLQ-C30 and QLQ-OES18 scores were evidently lower at 1 month after operation than those before operation ($P < 0.05$), while they were also evidently lower at 3 months after operation than those at 1 month after operation ($P < 0.05$). There were no

statistically significant differences in the EORTC QLQ-C30 and QLQ-OES18 scores between the two groups at 1 month and 3 months after operation ($P > 0.05$) (Table IV).

Follow-up results of patients' survival

All patients were followed up for 5-24 months, with a median of 18.3 months. The 1-year overall survival (OS) was 70.7% (41/58) and 53.4% (31/58), and the 2-year OS was 37.9% (22/58) and 27.6% (16/58), respectively, in Combination group and ^{125}I group. The survival curves were plotted using the Kaplan-Meier method (Fig. 2). It was confirmed by the log-rank test that the OS had no statistically significant difference between the two groups ($P = 0.167$).

DISCUSSION

In this study, ^{125}I radioactive seed stent implantation in the esophagus combined with TAI could significantly improve the Stooler grade for dysphagia of patients with advanced esophageal cancer, and greatly ameliorate the diet of patients,

Table IV. Comparison of preoperative and postoperative EORTC-QLQ-C30 and EORTC-QLQ-OES18 scale scores of the studied patients in two different groups

Complications	Combination group n=58	^{125}I group n=58	P-value
QLQ-C30			
Preoperative	32.69 $\pm$ 5.40	31.48 $\pm$ 5.18	0.221
1 m postoperative	25.02 $\pm$ 4.71	23.78 $\pm$ 4.19	0.137
3 ms postoperative	19.22 $\pm$ 3.55	18.38 $\pm$ 3.07	0.176
QLQ-OES18			
Preoperative	26.34 $\pm$ 3.48	25.89 $\pm$ 3.87	0.512
1 m postoperative	21.86 $\pm$ 3.35	20.93 $\pm$ 3.34	0.137
3 ms postoperative	18.13 $\pm$ 3.11	17.63 $\pm$ 3.15	0.392

EORTC: European Organization for Research and Treatment of Cancer.

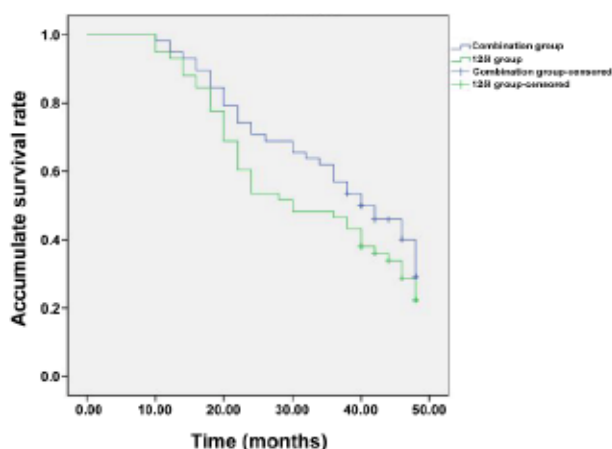


Fig. 2. Kaplan-Meier survival curves of patients in Combination group and ^{125}I group. The difference between overall survival rate of patients in Combination group and ^{125}I group had no statistical significance ($P=0.167$).

thereby raising the KPS score. It was found that the total effective rate and clinical benefit rate were significantly higher in Combination group than those in ^{125}I group ($P=0.013$, $P=0.010$). Stent implantation combined with TAI can increase the concentration of chemotherapy drugs in local tumor tissues (8-68 times that of intravenous chemotherapy), and greatly enhance the first-pass effect of chemotherapy drugs. As a result, tumor cells can be killed more effectively, and systemic adverse reactions caused by chemotherapy drugs can be alleviated, thus protecting important organs (10). In this study, nausea, vomiting, abdominal distension and fatigue occurred in some cases, and most of them were tolerable. A few cases had bone marrow suppression above degree III, and no severe liver and kidney dysfunction occurred. According to the follow-up results, the postoperative survival rate and quality of life were better in Combination group than those in ^{125}I group, but there were no statistically significant differences ($P>0.05$).

Multidisciplinary collaboration and comprehensive treatment are needed for the treatment of esophageal cancer (11). ^{125}I radioactive seed stent can effectively eliminate the local tumor, significantly improve the patient's eating status, and

increase the KPS score, which provides support for subsequent TAI (12). TAI can wash and kill cancer cells in the blood. There are certain limitations in this study. For example, the sample size was limited, the follow-up period was not long enough, the follow-up content was not comprehensive enough, and the tumor progression was not analyzed via follow-up. In the future, the conclusion in this study needs to be confirmed by more rigorous large-sample prospective multicenter randomized studies.

^{125}I seed stent implantation combined with TAI has definite efficacy in the treatment of advanced esophageal cancer, which can significantly ameliorate dysphagia and raise the quality of life of patients, with tolerable adverse reactions. Therefore, it is an effective minimally invasive treatment for advanced esophageal cancer.

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Circ_0006006 promotes the non-small cell lung cancer progression via modulating the miR-155-5p/ETS1 axis

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To the Editor,

Non-small cell lung cancer (NSCLC) accounts for approximately 75%-80% of all lung cancer cases (1). The 1-year survival rate of NSCLC patients with distant metastasis is only about 10%-15% (1). Therefore, it is important to clarify the molecular mechanism of the progression of NSCLC and find new therapeutic targets.

Circular RNAs (circRNAs) are endogenous RNA molecules with a stable closed-loop structure (2). As reported, some circRNAs, as competitive endogenous RNAs (ceRNAs), regulate mRNA translation by decoying miRNA via their microRNA (miRNA) response elements (3). In addition, some circRNAs are abnormally expressed in NSCLC and participate in its progression. For example, circPTK2 overexpression promotes TIF1 γ expression and inhibits TGF- β -induced epithelial-mesenchymal transition of NSCLC cells (4). However, the detailed role of circRNAs in NSCLC progression has not been fully clarified.

In the present study, we analyzed the microarray data of GSE112214, which was downloaded from the Gene Expression Omnibus (GEO) database, and found that circ_0006006 was up-regulated in NSCLC. Herein, we report that circ_0006006,

which is highly expressed in NSCLC tissues and cell lines, accelerates the growth, migration and invasion of NSCLC cells, and circ_0006006 up-regulates E26 ETS proto-oncogene 1 (ETS1) expression by adsorbing microRNA (miRNA, miR)-155-5p.

MATERIALS AND METHODS

Patients and clinical specimens

The collection and use of human tissues, with written informed consent, was endorsed by the Ethics Committee of Wuhan Hanyang Hospital, and the procedures were performed according to the Declaration of Helsinki. Forty-six cases of cancer tissues and adjacent non-cancer tissues available from NSCLC patients who underwent surgical resection were stored in liquid nitrogen at -196°C immediately after removal. All patients did not receive radiotherapy and chemotherapy before the surgery and did not take targeted drugs.

Cell culture and transfection

NSCLC cell lines (H1755, SK-MES-1, A549, H1975 cells) and human normal lung epithelial cells (BEAS-2B cells), available from American Type Culture Collection (ATCC; Rockville, MD, USA), were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS;

Key words: lung cancer, circ_0006006, miR-155-5p, ETS1

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Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO₂. Small interfering RNA against circ_0006006 (si-circ_0006006#1 and si-circ_0006006#2), siRNA negative control (si-NC), miR-155-5p mimics and its control (mimics NC), and miR-155-5p inhibitors were purchased from Genepharma (Shanghai, China). The above vectors and oligonucleotides were transfected into H1755 and A549 cells in the logarithmic growth phase by Lipofectamine®3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

Quantitative real-time PCR (qRT-PCR)

Total RNA in NSCLC tissues and cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reversely transcribed into cDNA with a One Step PrimeScript miRNA cDNA synthesis kit and PrimeScript RT kit (Takara Biotechnology Co., Ltd., Dalian, China), with the cDNA as the template, qRT-PCR was performed with an SYBR-Green Master Mix kit (Takara Bio, Inc., Otsu, Japan) on ABI7300 system (Thermo Fisher Scientific, Waltham, MA, USA). Circ_0006006, miR-155-5p, and ETS1 mRNA expression (U6 as the internal reference of miR-155-5p, GAPDH as that of circ_0006006 and ETS1 mRNA) were evaluated by 2^{-ΔΔCt} (Table I).

Cell-counting kit 8 assay (CCK-8)

The transfected NSCLC cells of each group were inoculated in a 96-well plate at a density of 2×10³ cells/well. After 24, 48, 72, 96 h of culture, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to each well. Then

the cells were incubated for 4 h, and subsequently, the absorbance of the cells in each well at 450nm wavelength was measured with a microplate reader.

BrdU assay

The transfected H1755 and A549 cells in the logarithmic growth phase were prepared into single-cell suspensions, then were inoculated into 24-well plates at the density of 1×10⁵ cells/well. After the cells were cultured with a serum-free medium for 12 h, they were incubated with BrdU labeling reagent for 8 h (AmyJet Scientific Inc., Wuhan, China). Then the cells were fixed with 4% paraformaldehyde for 10 min and rinsed with phosphate buffer saline (PBS). Subsequently, glycine at a 2 mg/mL concentration was supplemented to incubate the cells for 5 min, and then the cells were incubated with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). After PBS washed the cells, an anti-BrdU antibody (Abcam, ab8955, 1:1000) was added, and the cells were incubated for 2 h at room temperature in the darkness. Next, the cells were rewashed by PBS and incubated with the secondary antibody (Abcam, ab150116, 1:800) for 1 h at room temperature. Next, nuclei were stained with DAPI staining solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 min in the darkness. Cell immunofluorescence was observed under a microscope after PBS rewashed the cells. Cell proliferation rate = BrdU-positive cells/DAPI-positive cells.

Transwell assay

Transwell assay was adopted to evaluate the ability

Table I. Primer sequences

Gene	Primer sequences
circ_0006006	F-5'-GACACACTTGGGACATCGGA-3' R-5'-CCTCATTACCCAGCGTGACA-3'
miR-155-5p	F-5'- GAGGGTTAATGCTAATCGTGATAGG-3' R-5'- GCACAGAATCAACACGACTCACTAT-3'
ETS1	F-5'- GTGGTGAGGCAAGGACCTAG-3' R-5'-TGAGTTGCCATCTCATCCCA-3'
U6	F-5'-ATTGGAACGATACAGAGAAGATT-3' R-5'-GGAACGCTTCACGAATTTG-3'
GAPDH	F-5'-CTCTGCTCCTCCTGTTCGAC-3' R-5'-ACCAAATCCGTTGACTCCGA-3'

of NSCLC cell migration and invasion. The transfected H1755 and A549 cells, trypsinized with 0.25% trypsin, were centrifuged and then resuspended with a serum-free medium. Next, 5×10^4 cells were transferred into the upper chamber of the Transwell system (Corning, Beijing, China), and 400 μ L of RPMI-1640 medium containing 10% FBS was added into the lower compartment; the cells were cultured at 37°C for 24 h. The cells that failed to migrate were removed from the upper chamber. Then the cells on the below surface of the membrane were fixed with 4% paraformaldehyde for 10 min and then stained with 0.5% crystal violet solution. Then the cells were counted, after being washed in tap water, under an inverted microscope. Specifically, Matrigel was used to cover the membrane to mimic the extracellular matrix in the invasion experiment; hence, Matrigel is not in the migration experiment.

Nucleo-cytoplasmic separation

The separation and collection of RNA from the nucleus and cytoplasm of NSCLC cells was conducted with the NE-PER™ Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) and the RNeasy Midi Kit (Qiagen, Shanghai, China) according to the manufacturers' instructions. Then qRT-PCR was performed to detect the subcellular distribution of circ_0006006 in NSCLC cells. U6 and GAPDH were used to control the nucleus and cytoplasm, respectively.

Dual-luciferase reporter gene assay

The wild type (WT) binding sequence or mutated (MUT) binding sequence of circ_0006006 or ETS1 mRNA 3'-UTR was amplified and cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to construct the luciferase reporter gene vectors (circ_0006006 WT, ETS1 WT, circ_0006006 MUT, ETS1 MUT). The vectors were co-transfected with miR-155-5p mimics or mimics NC into H1755 and A549 cells. 24 h later, the luciferase activity of the cells was detected by a dual-luciferase detection kit (Promega, Madison, WI, USA).

Western blot

The medium was discarded, and the NSCLC cells were lysed in RIPA lysis buffer (Beyotime, Shanghai, China) to extract the total protein. First, 20 μ g of protein

sample in each group was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was then blocked with 5% skimmed milk at ambient temperature for 1 h. The PVDF membrane was then washed in tris buffered saline tween (TBST) 3 times and subsequently incubated with primary antibodies anti-ETS1 (ab220361, 1:1000, Abcam, Shanghai, China) and Anti- β -actin (ab6276, 1:1000, Abcam, Shanghai, China) at 4°C for 12 h. After the membrane was rewashed with TBST, the membrane was incubated with horseradish peroxidase (HRP) labeled goat anti-rabbit secondary antibody (ab150077, 1:2000, Abcam, Shanghai, China) or goat anti-mouse secondary antibody (ab205719, 1:2000, Abcam, Shanghai, China) for 1 h at room temperature. Then, the PVDF membranes were washed again in TBST 3 times for 10 min. Ultimately, the ECL Western blot substrate solution (Promega, Madison, WI, USA) was added to the PVDF membrane to develop the protein bands.

Statistical analysis

SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) was applied for statistical analysis. The experiments were performed in triplicate, with the data presented as "mean $\pm$ standard deviation." The student's t-test compared the difference between the two groups, and one-way ANOVA compared the data among multiple groups. Pearson correlation analysis was conducted for analyzing the correlation among the expression levels of circ_0006006, miR-155-5p, and ETS1 mRNA. The counting data were expressed in a contingency table, and differences between the two groups were analyzed by χ^2 test. Statistically, $P < 0.05$ is meaningful.

RESULTS

Circ_0006006 expression was elevated in NSCLC tissues and cells

First, we downloaded the GSE112214 dataset from the GEO database and analyzed the differentially expressed circRNAs in NSCLC tissues compared with adjacent normal tissues by GEO2R. The results showed that circ_0006006 expression was significantly up-regulated in NSCLC tissues

(Fig. 1A, B). Consistently, qRT-PCR showed that circ_0006006 expression in NSCLC tissues was significantly up-regulated compared with adjacent tissues (Fig. 1C). Besides, compared with BEAS-2B cells, circ_0006006 expression in four NSCLC cell lines (H1755, SK-MES-1, A549, and H1975) was significantly enhanced (Fig. 1D). Furthermore, the Chi-square test suggested that circ_0006006 expression was markedly interrelated with higher TNM stage and larger tumor size of the patients (Table II, $P < 0.05$). These data suggested that circ_0006006 may be a tumor-promoter in NSCLC.

Knocking down circ_0006006 inhibited the malignant biological behavior of NSCLC cells

From four kinds of NSCLC cells, we selected H1755 and A549 cells with the higher expression of circ_0006006 to construct circ_0006006 expression knockdown cell models (Fig. 2A). CCK-8 and BrdU assays suggested that knocking down circ_0006006 demonstrably impaired the proliferation of H1755 cells and A549 cells relative to the control group (Fig. 2B-D). Furthermore, the Transwell assay showed that knocking down circ_0006006 impeded H1755 and A549 cells

(Fig 2E&F). These data proved that circ_0006006 was implicated in repressing the multiplication, migration, and invasion of NSCLC cells.

Circ_0006006 directly targeted miR-155-5p

The Circular RNA Interactome database predicted a potential binding site between circ_0006006 and miR-155-5p (Fig. 3A). Subsequently, dual-luciferase reporter gene assay was confirmed, and it showed that overexpression of miR-155-5p could markedly inhibit the luciferase activity of circ_0006006 WT, but that of circ_0006006 MUT was not significantly affected (Fig. 3B). We then examined the subcellular localization of circ_0006006 by cell fractionation followed by qRT-PCR and found that circ_0006006 was mainly located in the cytoplasm of NSCLC cells (Fig. 3C). qRT-PCR showed that knocking down circ_0006006 promoted miR-155-5p expression in H1755 and A549 cells (Fig. 3D). It was further revealed that miR-155-5p expression was reduced in NSCLC cells and tissues (Fig. 3E&F). We also observed that, in the tumor tissues of the 46 patients, circ_0006006 expression and miR-155-5p expression were negatively correlated (Fig.

Table II. Correlation between circ_0006006 expression and multiple clinicopathological characteristics of NSCLC patients ($n=46$).

Characteristics	Number	Circ0006006 expression		χ^2	P value
		High (23)	Low (23)		
Age(years)					
<60	20	13	7	3.1846	0.0743
≥60	26	10	16		
Gender					
Male	27	16	11	2.2417	0.1343
Female	19	7	12		
Smoking					
Yes	29	13	16	0.8398	0.3594
No	17	10	7		
Tumor size (cm)					
≤3	15	4	11	4.8473	0.0276*
>3	31	19	12		
TNM stage					
I~II	16	4	12	6.1333	0.0132*
III~IV	30	19	11		
Differentiation					
Well/moderate	28	17	11	3.2857	0.0698
Poor	18	6	12		

3G). These data suggested that circ_0006006 was a negative regulator of miR-155-5p in NSCLC cells.

Circ_0006006 up-regulated ETS1 expression by adsorbing miR-155-5p

We found that miR-155-5p had a binding site with ETS1 mRNA 3'-UTR through the TargetScan database (Fig. 4A). Dual-luciferase reporter gene assay showed that miR-155-5p overexpression could remarkably restrain the luciferase activity of ETS1 WT, but that of ETS1 MUT was not markedly affected (Fig. 4B). qRT-PCR and Western blot showed that transfection of si-circ_0006006#1 could significantly inhibit ETS1 mRNA and protein expression in NSCLC cells, while

transfection of miR-155-5p inhibitors could partially reverse this effect (Fig. 4C-D). qRT-PCR showed that ETS1 mRNA expression was significantly up-regulated in NSCLC (Fig. 4E). Pearson correlation analysis implied that ETS1 mRNA expression was negatively correlated with miR-155-5p expression and positively correlated with circ_0006006 expression (Fig. 4F&G). These findings suggested that circ_0006006 up-regulated ETS1 expression by adsorbing miR-155-5p in NSCLC cells.

DISCUSSION

Recently, circRNA has been a hot spot in cancer

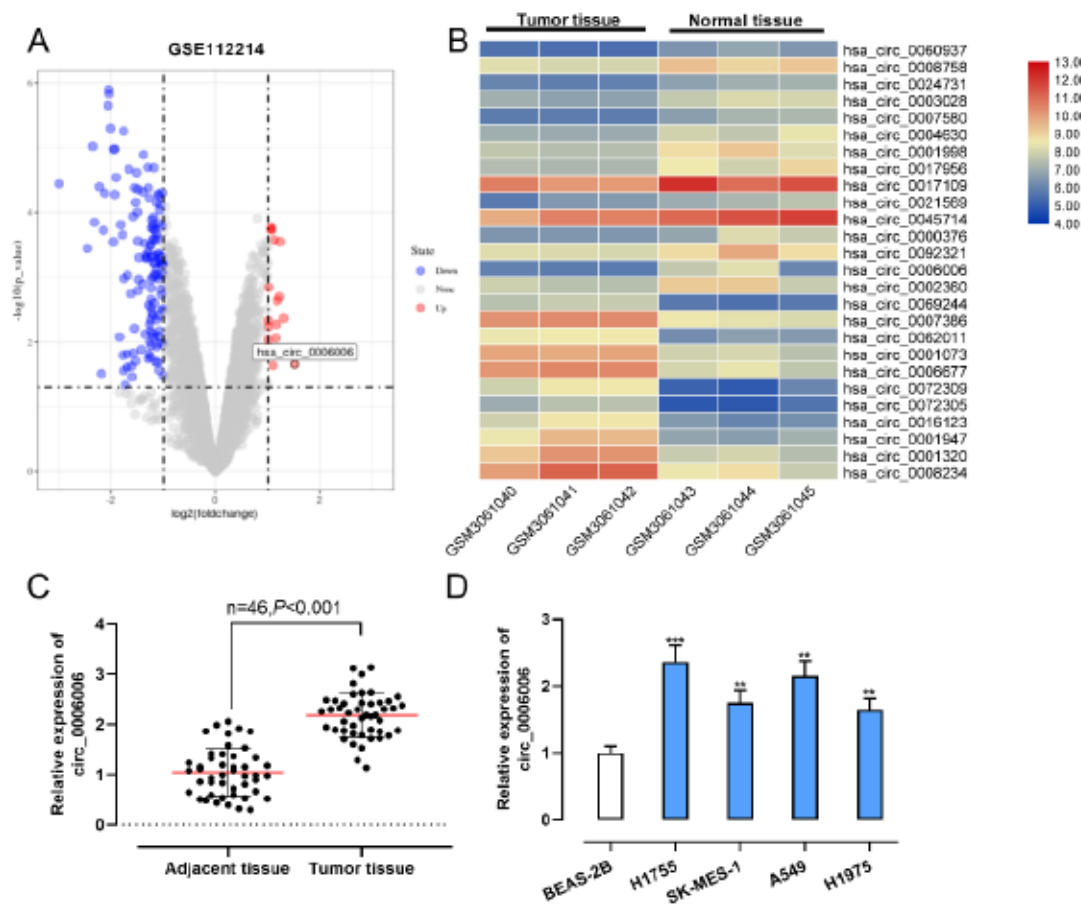


Fig. 1. Circ_0006006 was highly expressed in NSCLC tissues and cells. **A)** Volcano plot was used to show the differently expressed circRNAs in NSCLC tissues (v.s normal tissues adjacent to cancer). CircRNAs which were significantly increased were marked in red, and the circRNAs which were significantly decreased were marked in blue. **B)** Heatmap was adopted to show the expression of circRNAs in NSCLC tissues and adjacent normal tissues. **C)** Circ_0006006 expression in tumor tissues and adjacent tissues of 46 NSCLC patients was detected by qRT-PCR. **D)** The expression of circ_0006006 in NSCLC cell lines (H1755, SK-MES-1, A549 and H1975 cells) and human normal lung epithelial cells (BEAS-2B cells) was detected by qRT-PCR. ** $P < 0.01$, and *** $P < 0.001$.

research. Reportedly, circFGFR1 expression is raised in NSCLC tissues, which is associated with the poor prognosis of patients (5). Another study reports that circFARSA expression is elevated in NSCLC tissues, and its overexpression significantly expedites migration and invasion of NSCLC cells (6). We found that circ_0006006 was highly

expressed in NSCLC, and the abnormal expression of circ_0006006 is associated with the malignant phenotypes of NSCLC cells. To our best knowledge, this is the first report about circ_0006006 in NSCLC. MiR-155-5p has been identified as a tumor suppressor in multiple malignancies, including breast cancer, ovarian cancer, and NSCLC (7, 8, 9).

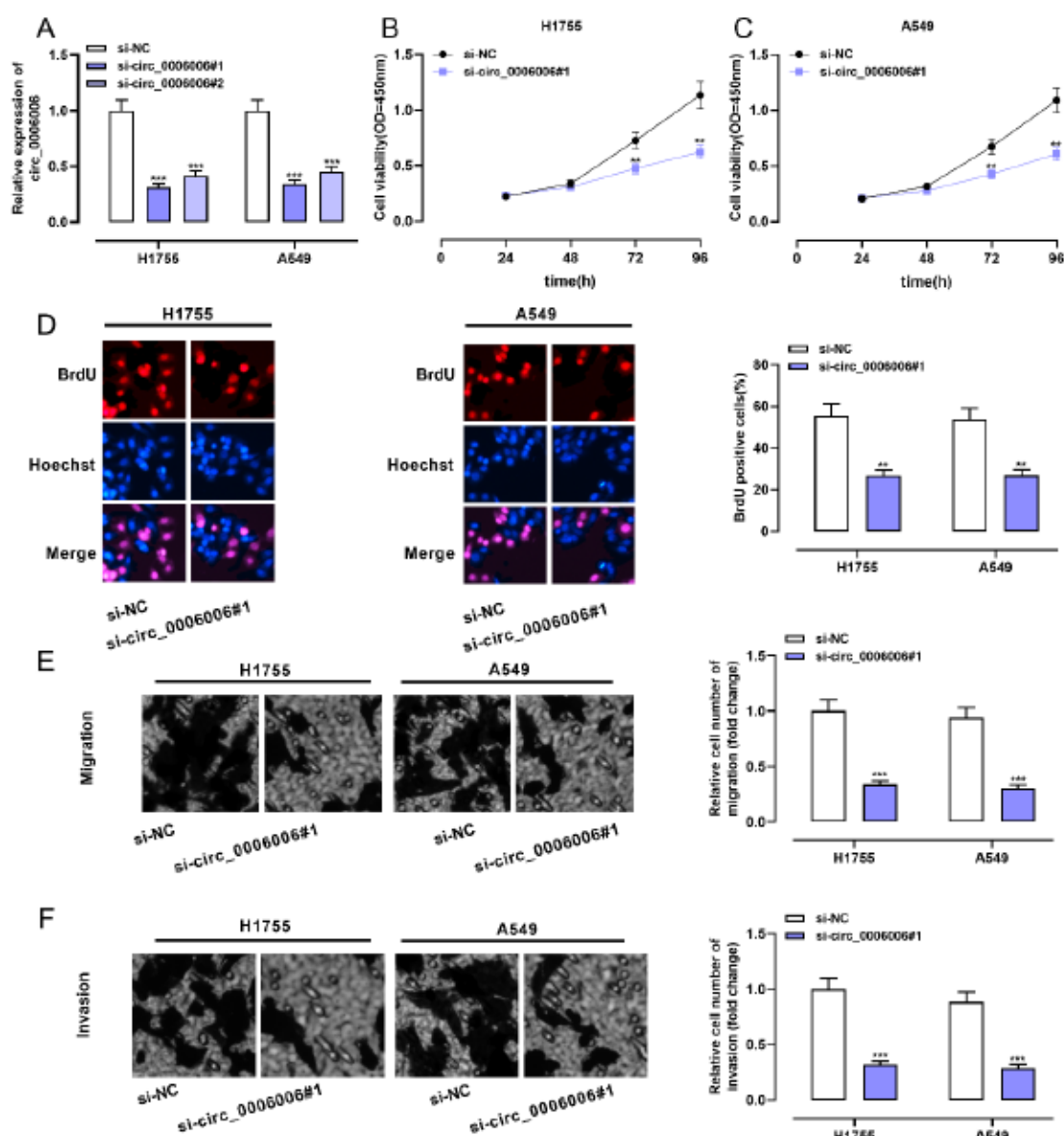


Fig. 2. Effects of circ_0006006 on proliferation, migration and invasion of NSCLC cells. **A)** qRT-PCR was used to detect the expression of circ_0006006 in H1755 cells and A549 cells transfected with si-circ_0006006#1/2. **B-D)** CCK-8 and BrdU assays were used to detect the proliferation of NSCLC cells with circ_0006006 knockdown. **E-F)** Transwell assay was used to detect the migration and invasion of NSCLC cells with circ_0006006 knockdown. ** $P < 0.01$, and *** $P < 0.001$.

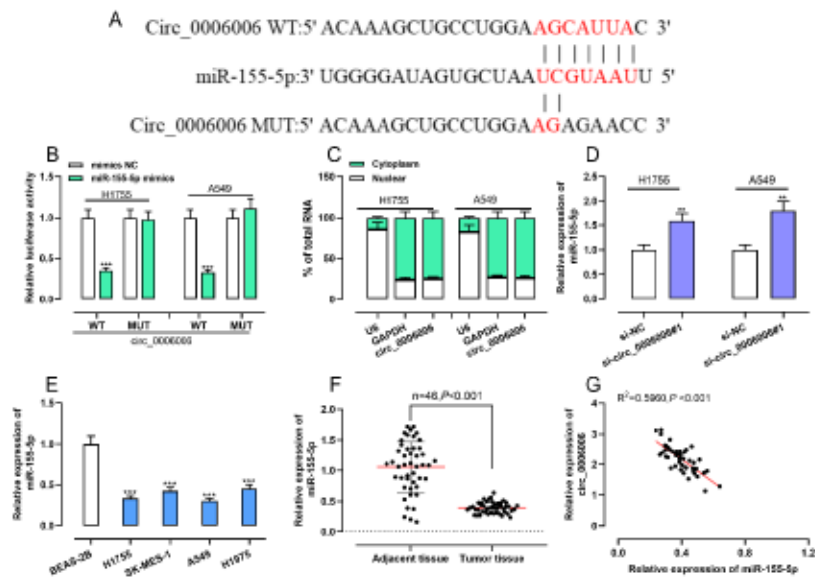


Fig. 3. MiR-155-5p was the target of circ_0006006 in NSCLC cells. **A)** Bioinformatics was adopted to predict the binding sites between circ_0006006 and miR-155-5p. **B)** Dual-luciferase reporter gene experiment was used to detect the targeting relationship between miR-155-5p and circ_0006006 in NSCLC cells. **C)** Subcellular localization of circ_0006006 was detected after nucleocytoplasmic separation. **D)** qRT-PCR was adopted to detect miR-155-5p expression in NSCLC cells with circ_0006006 knockdown. **E)** qRT-PCR was used to detect the expression of miR-155-5p in NSCLC cells. **F)** The expression of miR-155-5p in 46 cases of NSCLC tissues and adjacent tissues was detected by qRT-PCR. **G)** Pearson correlation analysis was employed to examine the correlation between circ_0006006 expression and miR-155-5p expression in NSCLC tissues. ** $P < 0.01$, and *** $P < 0.001$.

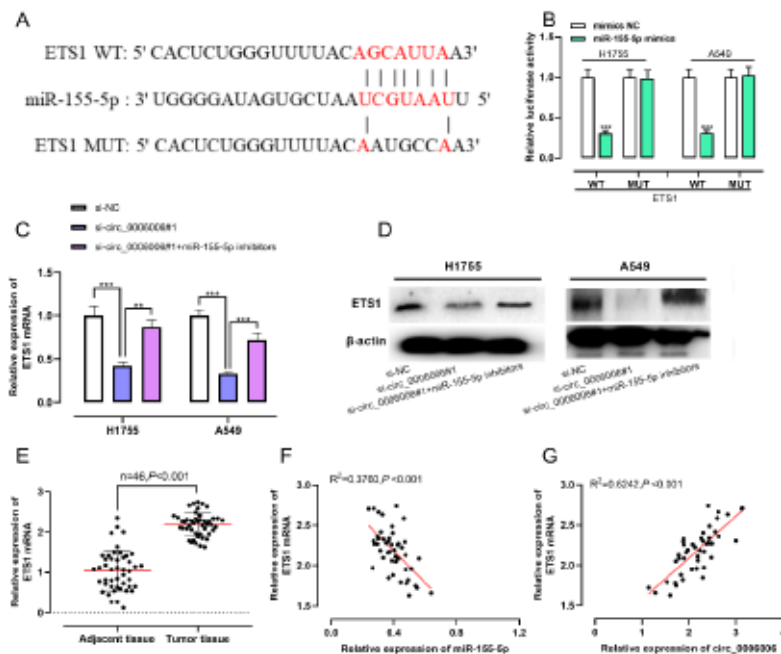


Fig. 4. Circ_0006006 upregulated the expression of ETS1 by adsorbing miR-155-5p. **A)** Bioinformatics was executed to predict the binding site between miR-155-5p and ETS1 mRNA 3'UTR. **B)** Dual-luciferase reporter gene assay was used to detect the binding relationship between miR-155-5p and ETS1 mRNA 3'UTR in NSCLC cells. **C-D)** qRT-PCR and Western blot were used to detect the regulatory effects of circ_0006006 and miR-155-5p on the expression of ETS1 in NSCLC cells. **E)** The expression of ETS1 mRNA in 46 cases of NSCLC tissues and adjacent tissues was detected by qRT-PCR. **F-G)** Pearson correlation analysis was used to detect the correlation between ETS1 mRNA expression and miR-155-5p expression or circ_0006006 expression in NSCLC tissues. ** $P < 0.01$ and *** $P < 0.001$.

Interestingly, some studies report that miR-155-5p is regulated by circRNAs. For example, circ_0008532 promotes the growth and invasion of bladder cancer cells by sponging miR-155-5p (10). Here, we found that miR-155-5p was repressed by circ_0006006 in NSCLC cells, partly explaining the mechanism of miR-155-5p dysregulation in cancer cells.

ETS1 is involved in extracellular matrix remodeling, cell migration, and angiogenesis (11). It is reported that ETS1 enhances the expression of WIPF1 by binding to its promoter and promotes the proliferative and migratory capacity of NSCLC cells (12). Here, we proved that miR-155-5p directly targeted ETS1, and circ_0006006 could up-regulate ETS1 expression by adsorbing miR-155-5p.

Collectively, these findings suggest that the ceRNA network consisting of circ_0006006, miR-155-5p, and ETS1 is involved in NSCLC progression.

Furthermore, our data imply that circ_0006006 is a promising biomarker and therapy target for NSCLC.

Authors contribution

Hui Chen conceived and designed the experiments. Liwei Teng, Guifang Zhang, and Hui Chen performed the experiments, conducted the statistical analysis, and wrote the paper. All the authors have read and approved the final manuscript.

Competing interests

None.

Ethics statement

The Ethics Committee approved the Ethics Review Board of Wuhan Hanyang Hospital study.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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CircBCL11B regulates colorectal carcinoma progression via the miR-579/LASP1 axis

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To the Editor,

Colorectal cancer (CRC) is the third most common cancer and the second most frequent cause of cancer-resulted deaths globally. The prognosis remains poorly known for the CRC patients who suffer from the tumors but are not diagnosed until developing into advanced ones (1); therefore, it is necessary to develop novel therapeutic strategies for the treatment of patients with advanced CRC.

CircRNAs (circRNAs) are regarded a novel class of non-coding RNA molecules. Previous studies have shown that various circRNAs play important roles in a variety of physiological activities and disease developments *in vivo*. CircRNAs usually act as miRNA sponges, regulating splicing and transcription of RNA, and modifying the expression of their parent genes as well (2, 3). Previous research pointed out that hsa_circ_0033144 (circBCL11B) was upregulated in oral squamous cell carcinoma (4).

As we know, microRNAs can target specific mRNAs for the regulation of their transcriptional levels, and these regulatory roles have been studied in various diseases. MiR-579 was regarded as a novel tumor suppressor and a novel target by regulating the PI3K/AKT pathway in spongioblastoma (5). It was reported that miR-579 influenced the development of osteosarcoma by regulating MMP13 expression

(6). In this study, we predicted that miR-579 acted as a target of circBCL11B.

It has been reported that a variety of tumors and diseases are induced by the abnormal expression level of miR-579, which however, has never been reported in CRC. Therefore, our study determined to conduct an inquiry into the expression and function of circBCL11B and miR-579 in CRC tissues and cell lines.

MATERIALS AND METHODS

Cell culture

CRC cell lines including SW-480, HCT-116, HT-29, LOVO, CACO2 and RKO, as well as colon cell HCoEpiC were obtained from American Type Culture Collection. All of them were cultivated in DMEM (Gibco) medium containing 10% FBS (Gibco), according to the manufacturer's instructions. Cells were incubated at 37°C with 5% CO₂, and the medium was refreshed every 2 days to ensure the sufficient nutrients for cell growth.

Clinical data

Data of about 50 patients with CRC were collected from the Qinghai Provincial People's Hospital. All the patients who participated in this study had not received chemotherapy or radiotherapy before their surgeries.

Key words: *circBCL11B, miR-579, LASP1, CRC*

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Samples were gathered from primary tumor tissues of CRC patients, and normal colon tissues near the tumor resection margins were used as controls. Then obtained tissues were quickly kept in liquid nitrogen for further experiments. This study was approved by the Committee of Ethics, Qinghai Provincial People's Hospital. The informed consent documents were obtained from the participants.

Cell transfection

SiRNA, miR-579 mimics, and miR-579 inhibitor were transfected into SW-480 and HCT-116 cells, respectively, according to instructions of LipofectamineTM3000 transfection reagents. The medium was refreshed after 6 h, and cells were harvested for subsequent experiments after 48 h of culture. SiRNA sequences were as follows:

si-circBCL11B#1: 5'-ATTGCAGCAGAGGCTGACCAT-3';
 si-circBCL11B#2: 5'-TGCAGCAGAGGCTGACCATGT-3';
 si-circBCL11B#3: 5'-CATTGCAGCAGAGGCTGACCA-3';
 negative control: 5'-TTCTCCGAACGTGTCACGT-3'

CCK-8 assay

CRC cells were grown in 96-well plates with 2×10^4 cells per well. LipofectamineTM 3000 was used for transfection when the convergence of cell growth reached 60%. Cell activity was assayed at 24 h, 48 h, 72 h and 96 h, by the CCK-8 kit, according to the instructions of the CCK-8 kit.

Real-time quantitative PCR

RNA in tissues or cells was achieved by TRIzol reagent and then reversely transcribed into cDNA by Prime ScriptTMRT Master Mix. SYBR Premix Ex TaqTM kit was applied for RNA quantification. FTC-3000p qPCR system was served to complete the experiments, and the relative expression levels of the target genes were calculated by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

CRC cells were harvested at 48 h after transfection and then lysis buffer protease inhibitor (MedChemExpress, American) was employed for the extraction of the total proteins. Proteins were quantified by BCA, denatured, and went through electrophoresis. PVDF membrane was incubated with the primary antibody overnight at 4°C and then incubated with the secondary antibody at room

temperature for 2 h. Finally, protein levels were analyzed using the Image-Pro Plus Image analysis system.

Clonogenic assay

Cells were grown in 6-well plates for 10 days after transfection for 48 h. Then cells were fixed with formaldehyde and stained with crystal violet for 15 min at room temperature. The number of clones was calculated under a low-power microscope (more than 50 cells were regarded as one clone) after drying.

Transwell migration and invasion assays

Cell invasion ability was monitored by Transwell assay with Matrigel. Transwell chambers (Sigma, American) were pre-coated with Matrigel (Sigma, American). After transfection for 48 h, cells (1×10^5) were prepared and aliquoted into single-cell suspension and were then added into the upper chamber. Meanwhile, in the lower chamber, 500 μ l medium with 10% serum was placed. Transwell chambers were placed in cell incubators for 8 h. Finally, the cells on the lower surface of the compartment were fixed with formaldehyde and subsequently stained with hematoxylin. Cell invasion ability was evaluated according to the number of cells penetrating the membrane. Cell migration ability was determined by Transwell assay without Matrigel. The rest of the operation was similar to the cell invasion assay.

Luciferase reporter assay

The plasmids harboring fluorescence labelled wild-type circBCL11B, mutated circBCL11B, LASP1 3'UTR WT, and LASP1 3'UTR MUT, were synthesized at the beginning. Subsequently, these plasmids were transfected into CRC cells, respectively. After 48 h culture, the luciferase activity of SW-480 and HCT-116 cells was measured by a double luciferase activity detection kit (Promega, American).

Flow cytometer assay

Annexin V-FITC/PI apoptosis kit (Thermo Fisher, American) was used to measure cell apoptosis. CRC cells were harvested at 48 h after transfection, followed by PBS washing. Cells were mixed with 100 μ l 1X binding buffer and 5 μ l Annexin V-FITC/PI staining solution and then were incubated in dark for 10 min. Cell apoptosis was determined by flow cytometry (BD Biosciences).

EDU assay

Cells were grown in 96-well plates and transfected with siRNA for 48 h. EDU solution (Abbkine, American) was diluted to 50 μ M in cell medium, and then cells were incubated with EDU mixture for 2 h in a cell incubator. CRC cells were fixed with formaldehyde, stained with Apollo, and were examined under an inverted fluorescence microscope.

RNA pull-down assay

The RNA plasmid was provided by TAKARA (Japan). RNA was marked with biotin probe and then co-incubated with cell lysate to form RNA-protein complex. Afterwards, This RNA-protein complex was co-purified, and the separated proteins were characterized by immunoblot.

Statistical analysis

SPSS 19.0 software was used for statistical analysis, and the results of at least 3 independent biological

replicates, were collected. Data were reported as mean $\pm$ SEM. $P < 0.05$ was regarded as statistically significance.

RESULTS

CircBCL11B was substantially upregulated in CRC tissues and cell lines

The expression of circBCL11B in 50 paired CRC and adjacent tissues was quantified by qRT-PCR. As shown in Fig. 1, the expression level of circBCL11B was remarkably higher in CRC tissues (Fig. 1A), and enhanced expression of circBCL11B was associated with lower survival (Fig. 1B). Furthermore, the expression of circBCL11B was upregulated in two CRC cell lines including SW-480 and HCT-116, compared with normal colon cells (HCoEpiC) (Fig. 1C). We next confirmed that circBCL11B was circular since it failed to be digested by RNase R SW480 and HCT116,

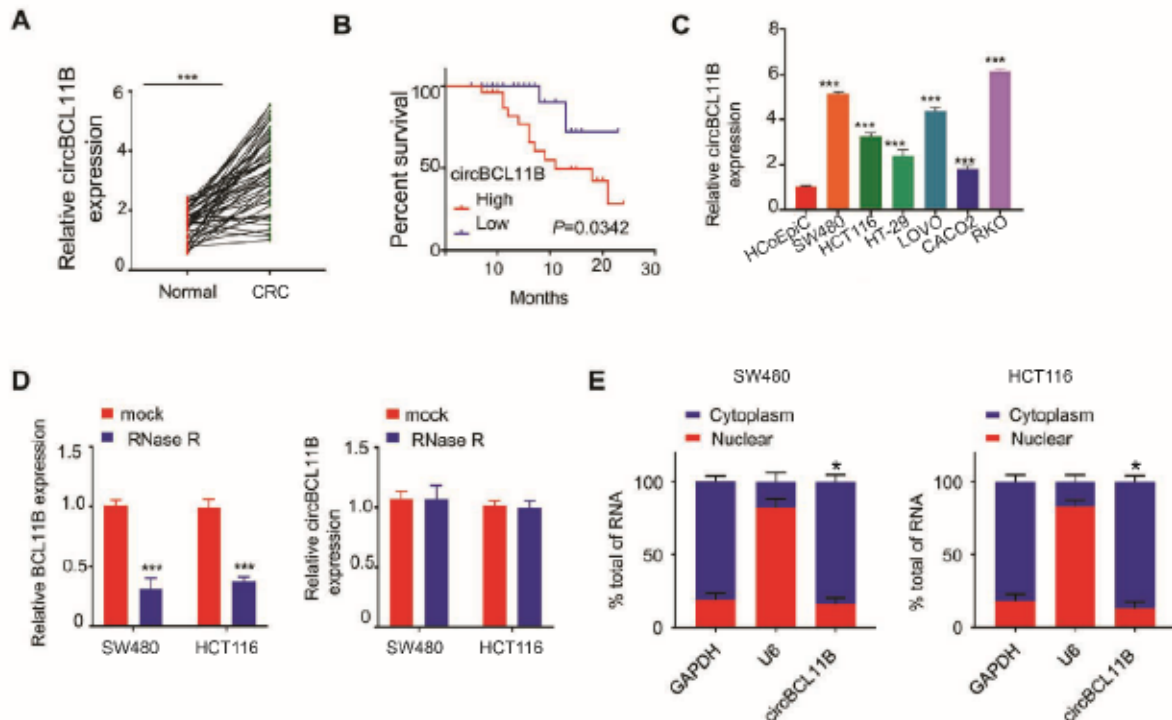


Fig. 1. *circBCL11B* expression was remarkably increased in CRC carcinoma tissues and cell lines. **A)** *circBCL11B* expression in 50 couples of CRC tissues and cell lines was detected via qRT-PCR assay, $p < 0.001$; **B)** Kaplan-Meier survival curves were used to evaluate overall survival time in CRC patients with low *circBCL11B* expression ($n=25$) and high *circBCL11B* expression ($n = 25$), $p < 0.001$; **C)** The mRNA levels of *circBCL11B* in a series of CRC cell lines (SW-480 and HCT-116) and normal colon cells (HCoEpiC) were measured by qRT-PCR, $p < 0.01$; **D)** The expression levels of *BCL11 B* and *circBCL11 B* in SW-480 and HCT-116 cells treated with RNase R were measured by qRT-PCR; **E)** Nucleoplasmic separation assay showed the cell sublocalization of *circBCL11B* via qRT-PCR, $p < 0.01$.

while BCL11B did (Fig. 1D). Subsequently, we performed nuclear/cytoplasmic separation assay in SW-480 and HCT-116 cells to identify the cellular sublocalization of circBCL11B (Fig. 1E). The results of qRT-PCR indicated that circBCL11B was mainly localized in the cytoplasm. To further figure out the relationship between circBCL11B and clinical features, the median expression value of circBCL11B in CRC tissues was set as the cut-

off value, and 50 CRC patients were divided into two groups: the high expression group ($n = 25$) and the low expression group ($n = 25$). The chi-square test was used to determine the relationship between the expression of circBCL11B and the clinicopathological data of CRC. The results showed that the expression of circBCL11B was closely correlated with tumor sizes, TNM stages, and distal metastasis, and the differences

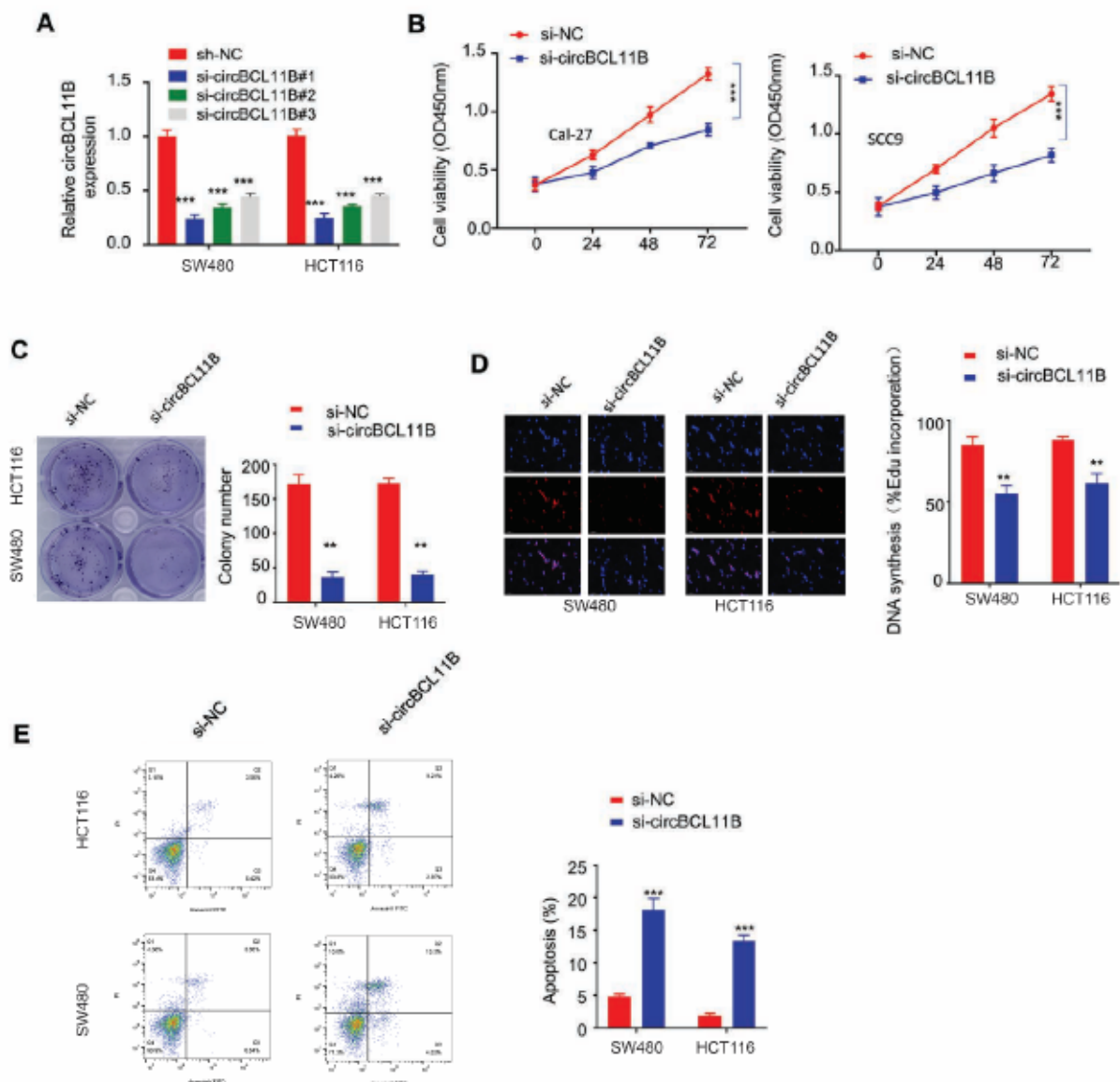


Fig. 2. circBCL11B silencing inhibited cell progression and induced cell apoptosis. **A**) Knockdown effect of two siRNA oligos targeting circBCL11B in SW-480 and HCT-116 cells. $p < 0.01$; **B-D**) CCK-8 (**B**), clonogenic (**C**), and EDU (**D**) assays showed that knockdown of circBCL11B inhibited cell progression in CRC cells, $p < 0.01$; **E**) The apoptosis level of different groups of SW-480 and HCT-116 cells (si-NC, si-circBCL11B) was detected by flow cytometry, $p < 0.01$.

were statistically significant ($p < 0.05$), but not related to the patient's ages, genders, or tumor differentiations.

Downregulated circBCL11B arrested the malignant activity of CRC and promoted cell apoptosis

To further explore the biochemical roles of circBCL11B in CRC progression, we constructed three siRNA oligos to silence the transcriptional level of circBCL11B and the knockdown efficiency was determined by qRT-PCR in SW-480 and HCT-116 cells (Fig. 2A). As shown in Fig. 2, the oligo of si-circBCL11B#1 was selected for the silence of circBCL11B due to its better knockdown efficiency than others. We found that the silence of circBCL11B suppressed CRC cell viability as shown in Fig. 2 based on CCK-8 and EDU assays. Similarly, downregulated circBCL11B expression also

affected cell cloning efficiency (Fig. 2C). As a result, we also examined whether circBCL11B affected apoptosis in CRC cells. Flow cytometry results showed that cell apoptosis was induced by the silence of circBCL11B (Fig. 2F).

Silencing circBCL11B inhibited migration and invasion of CRC cells

Transwell assay without Matrigel was performed in SW-480 and HCT-116, suggesting that cell migration was affected by downregulated circBCL11B (Fig. 3A). Besides, knockdown of circBCL11B also hindered cell invasion (Fig. 3B). Generally, the migration and invasion of tumor cells are mainly caused by epithelial mesenchymal transformation (EMT), which results in the loss of epithelial phenotypes and the decreased level of E-cadherin, reducing cell adhesion, and subsequently enabling cells to acquire characteristics that are

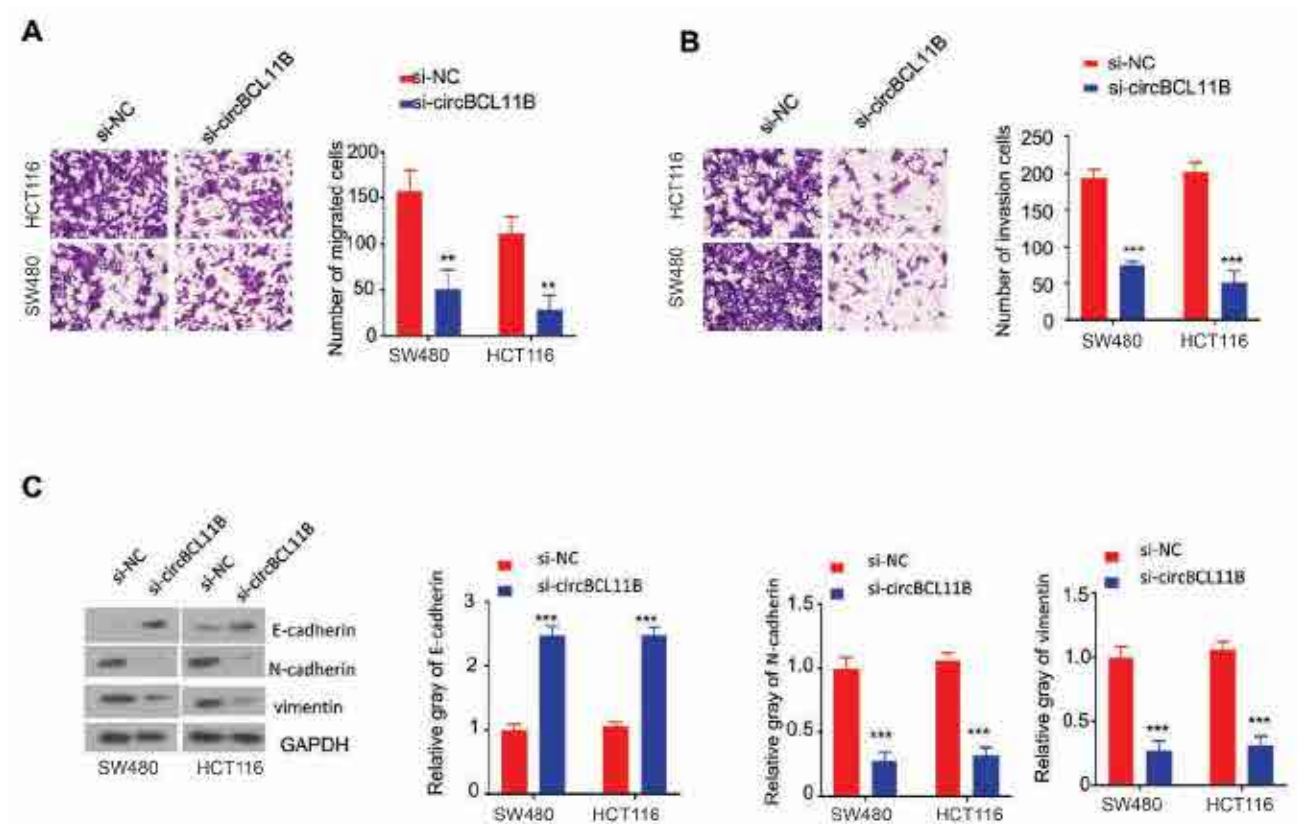


Fig. 3. Knockdown of circBCL11B inhibited cell migration and invasion in vitro. **A-B)** Transwell assays detected cell migration (**A**) and invasion (**B**) in SW-480 and HCT-116 cells, $p < 0.01$; **C)** The protein levels of E-cadherin, N-cadherin, and vimentin in SW-480 and HCT-116 cells (si-NC, si-circBCL11B), as revealed by western blot.

easily invading and transferring. Meanwhile, cells can acquire mesenchymal phenotypes, such as the increased expression of vimentin and N-cadherin. Western blot analysis indicated that the knockdown of circBCL11B upregulated the expression of E-cadherin protein, whereas the expression levels of N-cadherin and vimentin protein were downregulated (Fig. 3C). Taken together, circBCL11B promoted cell migration and invasion by regulating EMT in CRC cells.

CircBCL11B interacted with miR-579 and regulated its expression

We predicted that miR-579 probably acted as a target of circBCL11B since miR-579 may interact with circBCL11B based on Circinteractome analysis (Fig. 4A). Moreover, the expression level of miR-579 was closely related to CRC progression. qRT-PCR results showed that the expression of miR-579 was drastically reduced in cancer tissues (Fig. 4C). Similarly, downregulation of miR-579

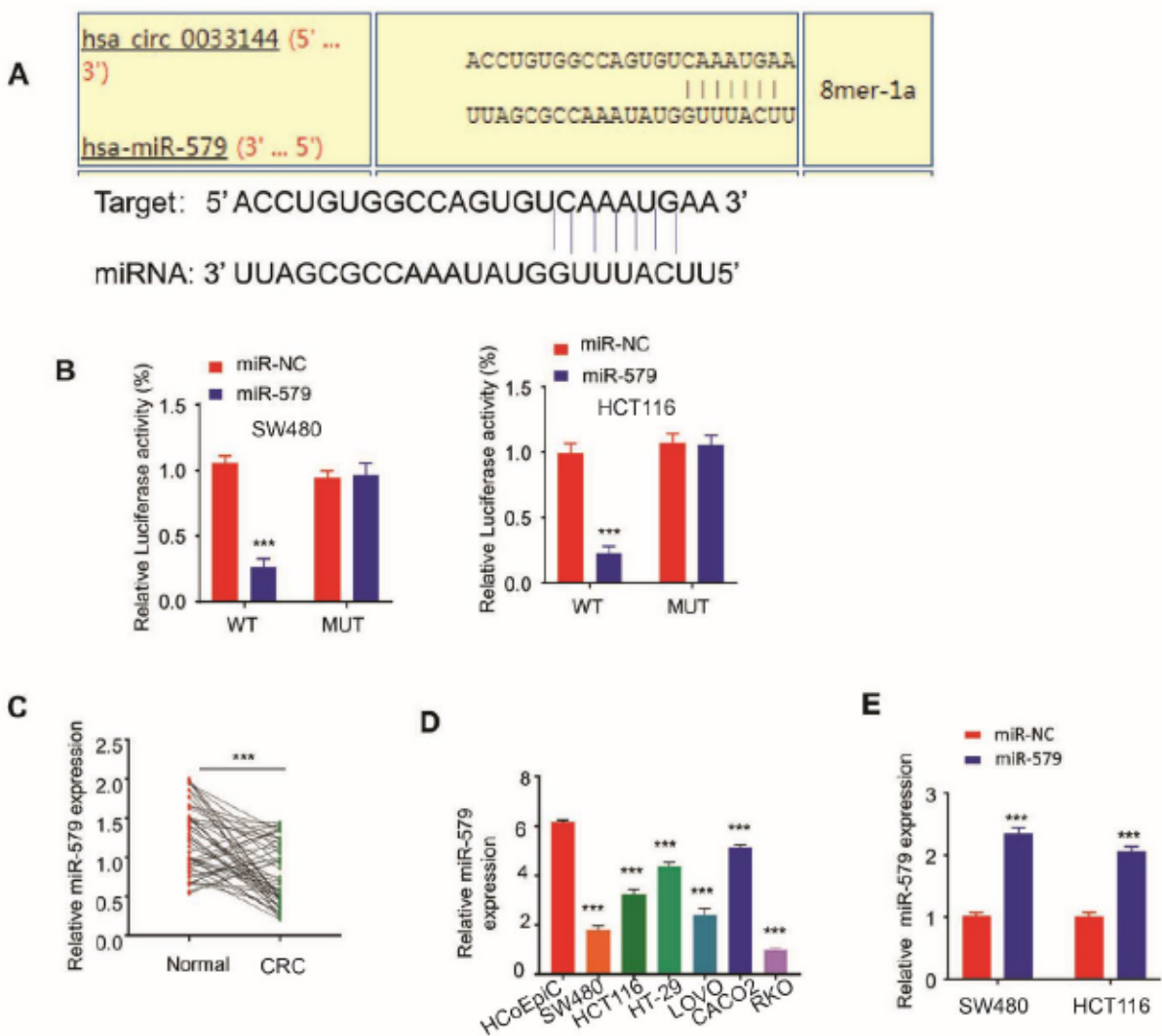
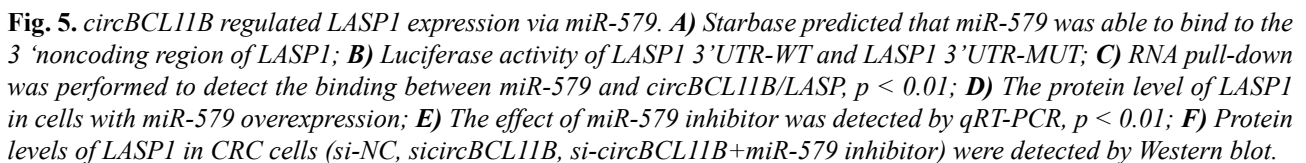


Fig. 4. *circBCL11B interacted with miR-579 and regulated its expression. A) Circinteractome analysis showed that circBCL11B contained the binding site for miR-579; B) The miR-579 expression in 50 pairs of CRC and normal tissues was detected by qRT-PCR, $p < 0.001$; C) The qRT-PCR showed the downregulation level of miR-579 in CRC cells, compared with normal cells, $p < 0.01$; D) The transfection effect of miR-579 mimics was demonstrated by qRT-PCR, $p < 0.01$; E) Dual-luciferase reporter assay measured the luciferase activity of wild-type circBCL11B vector and mutated circBCL11B vector in SW-480 and HCT-116 cells.*

binding site (Fig. 4E). Thus, we considered that miR-579 probably acted as the potential target of circBCL11B.

As we know, mRNA regulates tumorigenesis by regulating the expression of tumor-associated proteins. To this end, we further confirmed the target gene of miR-579. Starbase predicted the presence of the miR-579 binding site in the 3' non-coding region



of LASP1, suggesting that LASP1 was the potential target gene of miR-579 (Fig. 5A). Interestingly, the luciferase activity of vector carrying the LASP1 3'UTR-WT was reduced by miR-579 overexpression, and upregulated miR-579 failed to inhibit the activity of dual-luciferase reporter plasmid carrying the LASP1 3'UTR-MT (Fig. 5B). Furthermore, RNA pull-down assay results also proved that Biomir-579c was able to interact with circBCL11b and LASP1, as both of them were identified as interacting partners (Fig. 5C). Functionally, miR-579 overexpression reduced the protein level of LASP1 in CRC cells (Fig. 5D). To further confirm that, we used a miR-579 inhibitor, which could markedly inhibit miR-579 expression by transfecting it into CRC cells (Fig. 5E). Western blot results showed that the silence of circBCL11b reduced the protein expression level of LASP1, which was rescued by co-transfected with miR-579 inhibitor.

DISCUSSION

Our study supported that circBCL118 may serve as a new class of potential biomarkers or therapeutic targets for cancers based on the following evidence: (1) circBCL11B was upregulated in both CRC tissues and cell lines; (2) circBCL11B expression was involved in CRC progression; (3) circBCL11B silencing arrested CRC cell proliferation, invasion, and migration *in vitro*; (4) knockdown of circBCL11B also promoted cell apoptosis. MiRNAs, a class of small single-stranded non-coding RNAs, regulate the expression of mRNAs after transcription. Diverse miRNAs are involved in a range of physiological activities including metabolisms, cell differentiations, and tumorigenesis (7).

Functionally, our study determined that circBCL11B regulated tumor progression via miR-579/LASP1 axis. Using bioinformatics tools, we predicted that circBCL11B was able to bind to miR-579 and LASP1. LASP1 is an actin-binding protein, and multiple reports have shown that it promoted invasion and metastasis in a variety of tumors including pancreatic cancer, colorectal cancer, and medulloblastoma (8-10). Moreover, the predicted binding site was confirmed based on dual-luciferase reporter assay. Similarly, RNA pull-down assay also

proved that bio-miR-579c was able to enrich and pull down circBCL11B and LASP1 *in vitro*. Taken together, circBCL11B was confirmed to be able to interact with miR-579 and LASP1. Besides, miR-579 was downregulated in CRC tissues and cell lines, and both the miR-579 overexpression and circBCL11B silencing reduced the protein level of LASP1 in SW-480 and HCT-116 cells. Meaningfully, co-transfection of miR-579 inhibitor rescued the decreased level of LASP1 protein, which was caused by circBCL11B knockdown.

Our findings suggest that CircBCL11B was upregulated in CRC patients that regulated tumor progression. Targeting the regulatory axis of miR-579/LASP1 implicates that circBCL11B is a promising therapeutic possibility for CRC patients.

Disclosure of Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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The clinical implications of peripheral serum levels of IL-35 in patients with allergic rhinitis

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To the Editor,

Allergic rhinitis (AR) is a quite common disease globally. The number of AR patients in China has been increased significantly since the 1960s, ranging from 4%-38% (1). AR is an inflammatory disorder of the nasal mucosa induced by an immunoglobulin E (IgE) mediated reaction to inhaled allergens (2). It is characterized by symptoms of nasal itch, sneeze, watery rhinorrhea, nasal congestion, nasal and ocular pruritus, redness, and lacrimation.

It is crucial to understand the pathophysiology of AR. T helper 2 (Th2) cells are involved in developing allergic sensitization and the pathology of allergic inflammation (3). The cytokines secreted by Th2 cells play an essential role in mediating allergic inflammation and polarizing the sensitization process into a Th2 cell response.

IL-35 is a new member of the IL-12 family. It is a heterodimeric protein, composed of the subunit Epstein-Barr virus-induced gene 3 (EBI3) of IL-27 and the p35 subunit of IL-12, signaling through the receptors GP130 and IL-12R β 2, or IL-27R α and IL-12R β 2 (4). Moreover, IL-35 promotes tumor progression and inhibits anti-tumor immunity (5) but improves wound healing after myocardial infarction (6), and low levels of IL-35 signal the initiation of autoimmune diseases. In contrast, high levels of IL-

35 advance the progression of infectious diseases (7). Therefore, the present study aimed to investigate the clinical relationship and prognostic value of IL-35 in AR patients.

MATERIALS AND METHODS

Subjects

AR patients (n=47) were recruited from the Department of otolaryngology, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, from June 2018 to June 2019. Age and gender-matched volunteers without allergic and systemic disease history and drug use within the last one month (n=12) were selected as healthy controls. This study complies with the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. Therefore, written informed consents were obtained from all participants.

Diagnosis of Allergic rhinitis

Diagnosis of AR was based on medical history, physical examination, and laboratory findings. In addition, the Score for Allergic Rhinitis (SFAR) was applied to help the diagnosis. Briefly, SFAR involves eight components, including the following:

Nasal symptoms in the past year, including sneezing,

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runny nose, and blocked nose.

Nasal symptoms accompanied with itchy-watery eyes (rhinoconjunctivitis).

Months of the year in which the nasal symptoms occur.

Triggers of nasal symptoms, including pollens and house dust.

Perceived allergic status.

A previous medical diagnosis of allergy.

Previous positive tests of allergy.

Familial history of allergy.

The SFAR cut-off value of 7 was found to optimally discriminate between individuals with AR and those without (8).

Allergic sensitization was demonstrated by the patients' medical histories. All AR patients were divided into four groups according to allergen sensitivities as (1) cold-dry air (CDA), (2) smell, (3) dust, and (4) CDA & dust. AR clinic severity after treatment was evaluated by the mean total rhinoconjunctivitis symptom score (RCSS). The mean total RCSS is based on the evaluation of six to eight individual rhinitis and conjunctivitis symptoms, usually on a four-point scale. The 0-3 symptom score is:

Grade 0 = no symptoms (or signs);

Grade 1 = mild symptoms (sign/symptom present, but minimal awareness, easily tolerated);

Grade 2 = moderate symptoms (definite awareness of sign/symptom that is bothersome but tolerable);

Grade 3 = severe symptoms (sign/symptom that is hard to tolerate; causes interference with activities of daily living and sleeping) (9).

Visual Analogue Scale (VAS) score was used for the assessment of patients' subjective symptoms. Three groups were considered based on their feelings:

Patients with a VAS score below 50 mm (categorized as having mild rhinitis).

Those with a score of 60 mm or more (classified as having severe rhinitis).

Those with a score of 50 mm or more (10).

Patients with immunosuppressive treatment within the last month, or antihistamine treatment within the previous ten days, were excluded from the study (11). In addition, the study also excluded patients who smoked, had an active infection, or had a chronic disease such as rhinosinusitis, nasal polyposis, or cancer.

Sample collection and detection

Peripheral blood samples from all the subjects were

taken and rapidly centrifuged at 4°C. Sera were frozen at -80°C until analysis. The levels of serum immunoglobulin E (IgE) and IL-35 were measured by enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter DXI-800, USA) according to the manufacturer's protocols (human IgE ELISA kit, eBioscience; human IL-35, ELISA kit, Biolegend). The minimal detectable concentration for IgE was 0.08 to 7.8 ng/mL for IL-35. All samples were detected in duplicate.

Statistical analysis

All tests were performed by GraphPad Prism 5.0 (San Diego, CA, USA). T-test was used in comparing two independent groups. Pearson's correlation test examined the correlation of variables with each other. In the comparison of categorical data, Fisher's exact test was used. Quantitative data were presented in the tables as Mean $\pm$ SEM (standard error of the mean) and median range (maximum-minimum) values. Categorical data were expressed as n (number) and percentages (%). The study examined the data in the 95% reliability range, and the P-value was accepted as significant if below 0.05.

RESULTS

The primary characteristics of the study subjects

The present study enrolled 47 AR patients (patient group) and 12 healthy controls (HCs) from June 2018 to June 2019. Table 1 shows the demographic characteristics data of the study population. The mean age of AR patients was similar to HCs (34.11 $\pm$ 12.29 vs 32.17 $\pm$ 11.69 years, $P=0.391$). The AR group had a slightly higher female/male ratio than HCs (54.5% vs 61.7%, $P=0.612$). In contrast, the IgE levels in the AR group were significantly higher than in the HCs (205.70 $\pm$ 326.30 vs 12.72 $\pm$ 3.12) ($P=0.047$). Furthermore, the duration of the AR patients was (8.82 $\pm$ 5.29) years (Table I).

IL-35 was up-regulated in AR patients and positively correlated with IgE

Serum levels of IL-35 in AR patients were about two folds ($P=0.037$) higher than HCs (Fig. 1A), as well as the levels of IgE ($P=0.047$) (Table I). Also, the levels of IL-35 were positively correlated with those of IgE ($R^2=0.2023$, $P=0.0025$) (Fig. 1B).

Table 1. *Characteristic of the AR patients and healthy individuals*

Variables	Control (n=11)	Patients (n=47)	P-value
Sex, (female/male)	6(54.5%)/5(45.5%)	29(61.7%)/18(38.3%)	0.612
Age (mean±SEM)	32.17±11.69	34.11±12.29	0.391
Duration of AR		8.82±5.29	
IgE (IU/ml)	12.72±3.12	205.70±326.30	0.0471

The P value were compared using t test.

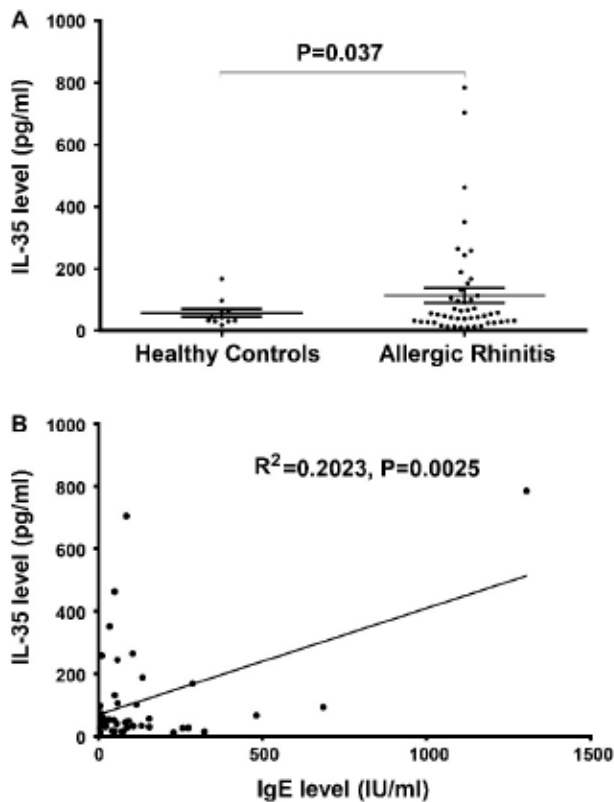


Fig. 1. Serum levels of IL-35 in allergic rhinitis patients. (A): Serum levels of IL-35 between healthy controls (HCs) and allergic rhinitis (AR) patients; (B): The correlation between the serum levels of IL-35 and immunoglobulin E (IgE) in AR patients. The bars represent as means $\pm$ SEM. The p-values were compared using t test. Pearson's correlation test was used to examine the correlation between serum levels of IL-35 and IgE.

IL-35 was higher in AR patients triggered by cold-dry air

There are lots of allergies or non-allergies that would trigger AR. The AR patients were divided into four subgroups according to different allergens, including dust, smell, cold-dry air, and cold-dry air with dust groups. IL-35 levels were higher in cold-dry air patients than in non-cold-dry air ($P=0.0418$) (Fig. 2C). However, there were no significant differences among dust, smell, and cold-dry air with dust groups.

Severe AR patients tended to have higher peripheral IL-35 levels

According to the VAS, the AR patients were grouped into moderate (VAS score below 50 mm) and severe symptoms groups (a VAS score of 60 mm or more). Serum levels of IL-35 in the severe groups were three folds higher than that in the moderate group at the time of AR diagnosis ($P=0.042$) (Fig. 3). According to visual analogue scale (VAS) scores which were used to evaluate the severity of symptoms, the levels of IL-35 in moderate and severe symptoms patients were much higher than those with no symptoms and mild symptoms patients ($P=0.042$) (Fig. 3).

Down-regulation of peripheral IL-35 levels indicates sound therapeutic effects

Based on our data after six months of follow-up, prognostic analysis was adopted to illuminate the relationship between peripheral IL-35 and therapeutic effects. The severity of clinical symptoms after

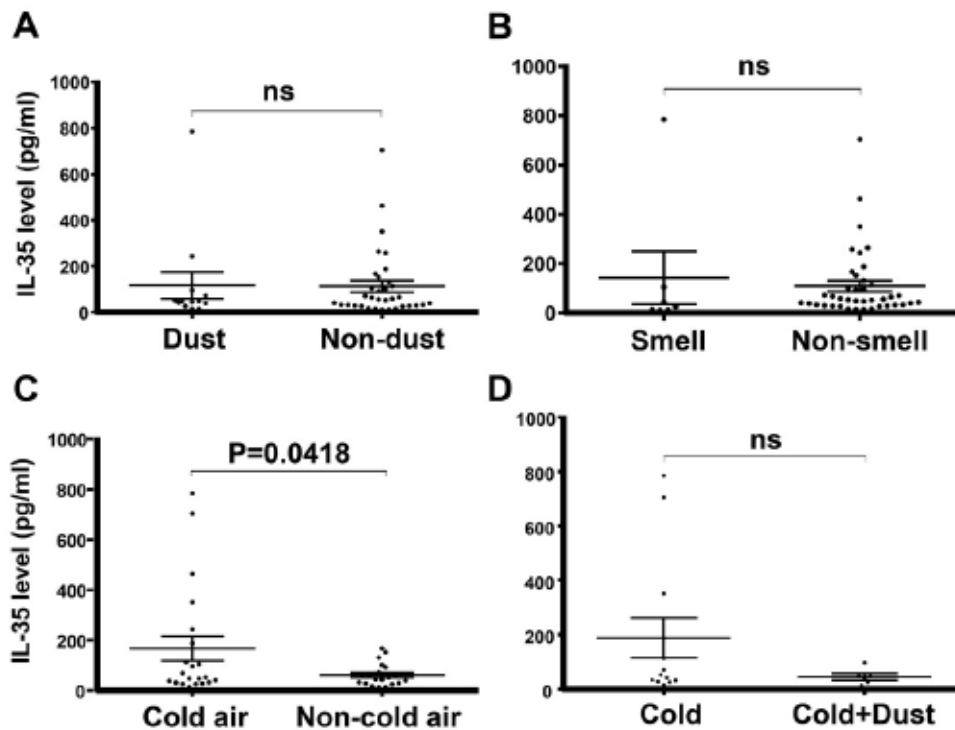


Fig. 2. Serum levels of IL-35 between different allergens that trigger AR. (A): Dust allergen group vs non-dust allergen group; (B): Smell allergen group vs Non-smell allergen group; (C): Cold-air allergen group vs Non-cold-air allergen group; (D): Cold-dry-air allergen group vs Cold-dry-air & Dust allergen group; The bars represent as means $\pm$ SEM. The P values were compared using a t-test.

treatment was rated by using RCSS. As illustrated in Fig. 4, the levels of IL-35 were much higher in patients with severe clinical signs than those with moderate symptoms ($P=0.041$) (Fig. 4).

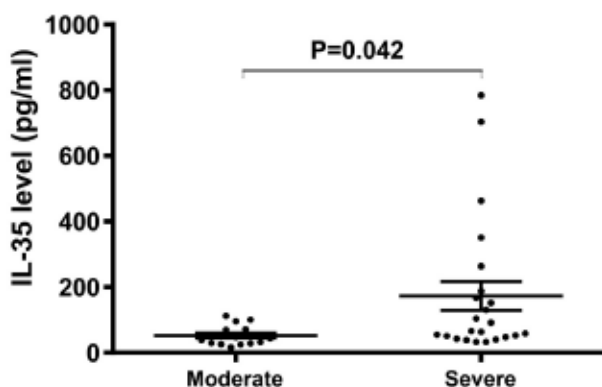


Fig. 3. Serum levels of IL-35 between AR patients with moderate and severe symptoms. The AR patients enrolled were divided into moderate group and severe groups by VAS according to their subjective feelings. The bars represent as means $\pm$ SEM. The P values were compared using t test.

DISCUSSION

The present study investigated the clinical relationship and prognosis between serum IL-35 levels and the severity of clinical symptoms in adults with AR. IL-35, being the attractive-suppressive cytokine and mainly secreted by regulatory T (Treg) cells, is an essential mediator of Treg cells (12). Data suggested that serum levels of IL-35 were significantly up-regulated in AR patients compared to the healthy control group, and serum IL-35 was elevated significantly among cold-dry air patients. More importantly, peripheral levels of IL-35 were positively correlated with IgE levels in AR patients, and severe AR patients usually had high serum levels of IL-35. Moreover, we found that lower levels of peripheral IL-35 were associated with better outcomes after treatments, indicating serum levels of IL-35 might serve as a new biomarker for evaluating the severity and prognosis of therapeutic effects in AR patients.

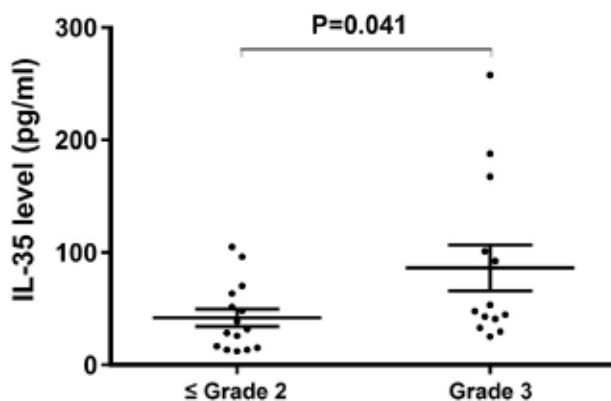


Fig. 4. Serum levels of IL-35 between AR patients with different clinical therapeutic outcomes. Serum levels of IL-35 were detected after following-up for at least 6 months. The levels of IL-35 were compared between different therapeutic effects. According to a four-point scale of the rhinoconjunctivitis symptom score (RCSS) for the severity of symptoms after treatments, the levels of IL-35 in AR patients with grade 3 who still had serious symptoms were much higher than those with grade 0 to 2 who had obvious attenuated symptoms. The bars represent as means $\pm$ SEM. The P values were compared using t test.

There were also some limitations in the present study. Firstly, studies should be conducted to reveal the mechanism of the role of IL-35 in AR. Secondly, more subjects with diseases should be enrolled in the control group. Additionally, further comprehensive and long-term follow-up studies need to be performed to obtain more integrated results.

In conclusion, our study showed that peripheral levels of IL-35 were positively correlated with AR patients and had a potential protective role in modulating pathological immunoreactions. These results might shed some light on the pathophysiology of AR and indicate a novel prognostic biomarker and therapeutic strategy with IL-35 for AR.

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GZ, JM conducted this study and wrote the paper; JZ, SH, LZ helped design the study, performed the data analysis and edited the manuscript; XJ, JT

helped collect the clinical specimens; SZ supervised the research and edited the paper.

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