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EDITORIAL

Mast cells and virus

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Mast cells (MCs) are hematopoietic cells that reside ubiquitously in all vascularized tissues. They are potential sources of a wide variety of biologically active secreted compounds, including diverse cytokines, chemokines and growth factors. In addition, they participate in innate and adaptive immune responses. MCs are the most important cells in immediate reactions and chronic IgE-associated allergic disorders and enhance the host resistance to certain biological agents, including viruses. Therefore, MCs influence many biological responses to viruses and other microbiological agents. Viruses activate MCs through TLR4 leading to the generation of several pro-inflammatory cytokines, including those of the IL-1 family. Here, we report how viruses can activate MCs producing severe inflammation and how these interesting cells can activate the immune system by carrying out a protective action for our organism.

Despite the unprecedented success of antiretroviral treatments, many question marks remain in the fight against viruses. Mast cells (MCs) derive from hematopoietic stem cells and are ubiquitously present in all vascularized tissues near blood vessels, epithelial, nerves, airways, etc. (1).

They are activated through the aggregation of the FcεRI receptor leading to the production of a spectrum of pro-inflammatory mediators, including many growth factors, cytokines, and chemokines. The binding of IgE antibody to the FcεRI receptor initiates events that lead to the secretion of preformed mediators stored in cytoplasmic granules, such as vasoactive amines, proteoglycan neutral proteases,

the cytokine TNF, and the *de novo* synthesis of pro-inflammatory lipid mediators, such as leukotrienes and prostaglandins, and the secretion of cytokines and chemokines (Table I).

IgE mediates hypersensitivity to allergens and stimulates pro-inflammatory cytokines, and the type 2 adaptive immune response in the anti-viral defense may aid in host protection. Therefore, it is possible that the type 2 response, characterized by the participation of T helper-2 cells and other immune cells, improves the host's systemic defense against viral infections and other noxious substances. In the adaptive response some important cytokines are secreted such as IL-4 and IL-13 involved in allergic sensitization and

Key words: mast cell; virus; allergy; inflammation; cytokines; SARS-CoV-2

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antibody production (2). In primary immunization or the type I response, the cytokines that are produced are of the type of the IL-1 family, along with IL-6, TNF and other inflammatory cytokines (3).

IL-1 and IL-6, and chemokines CXC and CC, mediate infectious processes without MC degranulation. MCs produce a wider range of cytokines/chemokines and pro-inflammatory and anti-inflammatory mediators. They influence many biological processes, including responses to bacteria and viruses with a broad spectrum of cytokines and growth factors. Mature MCs are important for host defense against various pathogens, including viruses, found in many areas of the human body, such as mucosal surfaces, although the true role of MCs in host anti-viral responses is not entirely clear. Viruses have evolved mechanisms to break epithelial barriers and enter the human body, where they find MCs and other immune cells that act as sentinels and contribute to anti-viral responses.

Tissue MCs exposed to the virus and various viral proteins during infection can activate cell receptors of MCs and other immune cells both in vitro and in vivo. MCs are critically involved not only in allergic disorders, but also in infectious diseases, with different roles due to their ability to mediate pro- and anti-inflammatory responses. They can capture viruses and infect other immune cells, suggesting that inhibiting MCs could be a new therapeutic strategy.

The ability of MCs to capture viruses, with very complex mechanisms, leads to infection of the whole body. MCs located on the surfaces of tissues exposed to the external environment, where they reside for a long time, induce a higher immune response due to the synergistic effect of IFN and IL-18 produced by macrophages when infected with viruses.

Cytokines and chemokines play an important role in allergic and infectious diseases by activating MCs and other immune cells, and they are considered potential therapeutic targets. Thus, allergic reactions can offer an important host defense mechanism against viruses and other harmful biological substances, although these reactions are not entirely clear. Mast cells participate in the allergic process through the activation of IgE on the FcεRI receptor and they can have a dual function, the inflammatory one and the protective one of the host (4). Infection with pathogenic viruses is associated with the development of specific IgEs that participate in the allergic phenomenon and constitute an important host defense mechanism. Therefore, MCs participate in innate immunity by expressing TLR and producing IL-1 family members, while acquired immunity produces IL-33, an alarming cytokine. Viruses and other biological organisms (and molecular compounds) are mainly associated with innate immunity, while they activate only 5% of the acquired immunity mediated by T and B lymphocytes. TLRs activate inflammation via viruses and bacteria

Table I. *Features of human mast cells.*

Origin	Hematopoietic stem cells
Maturation	Connective tissue
Granule	Numerous
Lipids.....	Present
Tryptase.....	Present
Chymase	Present
Histamine	Present
Carboxypeptidase A	Present
Phospholipase A2.....	Present
Growth Factors.....	Present
Lipid mediators	PGD2, LTC4, LTD4, LTE4, PAF
Cytokines.....	IL-3, IL-4, IL-5, IL-6, IL-13, IL-18, TNF, VEGF, etc.
Chemokines.....	CXCL8, CXCL1, CCL1, CCL2, CCL3

and also induce the production processes of IL-33, which mediates acquired immunity. MCs can be activated by IL-33 via the ST2 receptor and produce various cytokines. IL-33 is involved in the activation of several cells, including MCs and ILC2 lymphocytes in allergic disease and virus infectious processes, releasing IL-6, TNF, IL-4, IL-13 and other cytokines. In addition, IL-33 can also stimulate MCs to generate chemokines, including CCL1, CCL2, CCL17, CCL22 and CXCL8, molecules with chemotactic activity on various immune cells (5). Since CD34⁺ stem cells are important in the maturation of MCs, IL-33 has been shown to aid this clonal expansion process. On the other hand, the chymase produced by MCs can degrade the active IL-33 to the inactive form.

Several TLRs reveal molecular patterns associated with pathogens with the involvement of different biological elements, including viruses, resulting in a specific immune response. TLRs can activate adapter proteins such as MyD88, TRIP, TIRAP, etc., which share the Toll-Interleukin1 receptor (TIR) (6). The consequent activation of NFkB and of the mitogen-activated protein kinase (MAPKs) pathways activated by TLR3 leads to the generation of inflammatory mediators.

Virus-infected MCs can recruit innate immune response NK cells and improve their effector functions through the production of CXCL8 and type I interferons (IFNs). MCs can express the functional Toll-like 4 receptor after preincubation with IFN-gamma (IFNg). The production of IFNg, a cytokine capable of activating various immune cells including MCs, results in an antagonistic response to viral infection and can be induced by IL-12. IFNg is an anti-viral TH1 cytokine that MCs and other cells can produce after treatment with various simulators, including IL-12. However, activation of MCs with IgE does not cause IFNg secretion. IFNg can activate MCs with both positive and negative effects, a topic that is still unclear.

The IL-18 receptor is activated by IL-12 resulting in the production of IFNg and, as we have already said above, IL-18 and IL-12 have a synergistic action on increasing the production of IFNg by macrophages. The IL-18 receptor is activated by IL-12, resulting in the production of IFNg. Experimental animals treated

with an IL-18 neutralizer can become resistant to viral and bacterial infections. In viral infections there is an overproduction of IL-18 by the host, participating in the cytokine storm with consequent hyperreactivity and exaggerated inflammatory reaction. For example, the SARS-CoV-2 virus responsible for the coronavirus disease 2019 (COVID-19) infects target cells in the airways. It then infects the entire body, a process mediated by MCs that efficiently capture viral particles causing robust infection. Viral transfer between cells probably occurs by contact with the involvement of these cells. SARS-CoV-2 virus provokes the release of inflammatory cytokines of the IL-1 family, causing lesions in lung tissue and the so-called cytokine storm syndrome.

IL-10 is an important anti-inflammatory cytokine generated by MCs and other immune cells such as Th1, Th2, Th17, Treg, CD8⁺, and B cells, but also by macrophages, NK cells, etc. IL-10 regulates allergic responses and inflammation by limiting severe hypersensitivity reactions (7). MCs produce IL-10 cytokine that can be secreted by other immune cells such as Th1, Th2, Th17, Treg, CD8⁺ and B cells, but also by macrophages, NK cells, etc., it regulates allergic responses and inflammation by limiting severe hypersensitivity reactions.

IL-10 is a multifunctional cytokine involved in the differentiation and function of T cells and correlates with different virus genomes by regulating immune and inflammatory responses and it can both impede pathogen clearance and ameliorate immunopathology.

This anti-inflammatory cytokine is a master regulator of immunity and infections and in immunological responses to viruses, where there is an overproduction of IFN and TNF, IL-10 improves the excessive reactions of Th1 and CD8⁺ T cells.

Here, we can conclude that in pathogenic virus infections, MCs are very important both for the immune defense and for having a local and systemic inflammatory power in the body.

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Effect of ULF TENS on the tonic response to eye opening of stomatognathic muscles in healthy subjects: sEMG analysis and correlations with psychometric self-assessment tests (CSI and DASS42)

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Literature suggests that sEMG (surface Electromiography) of stomatognathic muscles is different in subjects with open eyes and closed eyes in various pathological conditions. Ultra-Low Frequency Transcutaneous Nervous Stimulation (ULF TENS) is used in dentistry to evaluate the adaptation capacity of the autonomic and muscular system and, at the same time, some self-assessment scales (CSI and DASS42) can help the dentist to evaluate the state of central hypo-excitability/ dysregulation. The aim of this paper is to evaluate ULF TENS effect in condition of opened eyes and closed eyes and to link the sEMG response to Central Sensitization condition or psychological condition of evaluation scales score. 27 healthy patients have been analyzed with 8 channels sEMG. Four homologue muscle couples (masseters, anterior digastrics, sternocleidomastoid, and anterior temporalis) in condition of open eyes and closed eyes before and after ULF TENS were checked, after the compilation of CSI and DASS42 scales. Left Temporal Anterior sEMG scores with open eyes are significantly higher than those obtained with closed eyes. The increasing answer of electric tone is higher on the left side. After ULF TENS the left side muscles activation is reduced and this response to stimulation is inversely proportional to CSI and DASS42 scores. ULF TENS is highly effective on those patients acting on the nucleus for coupling between sensory stimulus and modulation of the autonomous behavioral response.

The ULF TENS has been used for years together with sEMG analysis in dentistry to treat pain in temporomandibular disorders (TMD) patients and to make planning for prosthetic and orthodontic rehabilitation (1, 2). One of the reasons because this technique is used is the “relaxation” effect of the musculature which would allow to obtain more physiological parameters for the study of the reciprocal position between the upper and lower jaw (3). This mechanism is unclear and an alternative hypothesis has emerged: according to this hypothesis, there is effect of ULF TENS for dental use on brainstem structures responsible for stimulus coupling sensory and behavioral responses, including autonomous and

muscular responses. Further evidence of recent years show that sEMG values of healthy subjects suffering for TMD are susceptible to variation passing from the condition with closed eyes to that with opened eyes. In particular, the dioptric correction modulated on sEMG signals is able to significantly modify this variation especially in subjects affected by TMD (4). The ULF TENS, acting on some of the crucial cores for the coupling circuit between sensory input or central processing and response, may be able to modify this coupling and modulate the motor response in a different way. To evaluate this hypothesis, two self-assessment scales of “central sensitization” (CSI) and psychological status (DASS42) are used to evaluate

Key words: TENS, CSI, DASS42, stomatognathic muscles, TMD

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the general arousal state, with particular regard to depression (D), anxiety (A) and stress (S). The DASS42 has been used to quantitatively evaluate the values of Depression, Anxiety and Stress associated with particular population samples on the ways of dealing with adverse socio-cultural and health events (4, 5).

The aim of the work is to evaluate the presence of different sEMG behavior of the stomatognathic muscles in different conditions of visual sensory input (closed eyes and opened eyes), to evaluate the central effect of ULF TENS stimulation on the coupling circuit between sensory signal visual and myoelectric tonic response and, finally, to correlate this response to the general arousal and psychometric state.

MATERIALS AND METHODS

This study was carried out in accordance with the fundamental principles of the Declaration of Helsinki. It was approved before commencement by the Ethics Committee of the University of L'Aquila (16137/2016). 50 patients aged 20-50 years were clinically examined at the Dental Clinic of the University of L'Aquila, Italy. The same clinician performed all examinations. Examination included: dental visit and compilation of CSI and DASS 42 scales. The scales were compiled in a room in which the patient, who underwent the self-assessment test, was alone. The average compilation time for each scale was approximately 5 min. The assessment and assignment of the numerical scores for both scales lasted about 3-4 min per subject (1 min for the CSI and 2-3 min for the DASS42). The following exclusion criteria were applied: presence of epilepsy or peacemaker, TMD, presence of systemic diseases, presence of ophthalmological diseases or visual problems corrected by glasses. Inclusion criteria were: healthy subjects, age between 20 and 50 years, written informed consent signed. Ultimately, 27 patients (12 male and 15 female aged 37.26 ± 9.63) were enrolled in this pilot study and analyzed with EMG analysis (SCAN 9-10).

sEMG analysis protocol

According to the literature electromyographic activity was recorded using an eight-channel Myotronics K7 Evaluation System (Seattle, WA, USA) equipped with bipolar electrodes with an inter-electrode distance of 20 mm. Before positioning the electrodes, the patient's

skin was thoroughly cleaned with alcohol. Electrodes were positioned on the left and right masseter muscles (LMM, RMM) and the left and right anterior temporal muscles (LTA, RTA), as described by Castroflorio et al. In addition, electrodes were also placed on the left and right anterior digastric muscles (RDA, LDA) and the left and right sternocleidomastoid muscles (LSC, RSC) bilaterally parallel to the muscular fibres and over the lower portion of the muscle to avoid the innervation point, as described by Falla et al (6).

Electrical signals were amplified, recorded, and digitized with the K7 clinical software package. The root mean square (RMS) values (in μV) were used as indices of the signal amplitude. Three consecutive sEMG rest position tracks, with a duration of 15 seconds each, were acquired, and any trace with interposing phasic event, such as swallowing, voluntary movement, or clenching, was discarded. Procedure for sEMG registration was performed by one examiner in a blinded fashion. For each studied muscle, the average between the three recordings, which was generated by the software and expressed as microvolts (μV), was reported in a spreadsheet for differential statistics.

In this study, the authors evaluated the scan 9 (before ULF TENS) and the scan 10 (after ULF TENS) with muscle tone being evaluated with eyes both closed and open in randomized way. During the open eye test the subject is invited to relax.

During the open eye test, the subject is invited to read silently the first, second, and third lines, with the subsequent lines covered, of a standard Snellen optotype. The subject is positioned at 3 meters from the optotype, which corresponds to vision acuity of 1/10, 2/10, and 3/10, respectively.

The sEMG recording session lasted about 60 min plus 15 min of completing the self-assessment test (6-10).

ULF TENS procedure

The method for sensory ULF TENS was described previously (11, 12). Briefly, a J5 Myomonitor TENS Unit device (Myotronics-Noromed, Inc., Tukwila, WA, USA) with disposable electrodes (Myotrode SG Electrodes, Myotronics-Noromed, Inc., Tukwila, WA, USA) was used. This low-frequency neurostimulator generates a repetitive synchronous and bilateral stimulus delivered at 1.5-s intervals, with an adjustable amplitude of approximately 0-24 mA, a duration of 500 μs , and a frequency of 0.66 Hz. The two TENS electrodes were placed on both sides

on the cutaneous projection of the notch of the fifth pair of cranial nerves, which was located between the coronoid and condylar processes and was retrieved by manual palpation of the zone anterior to the tragus; a third grounding electrode was placed in the centre of the back of the neck. Based on the literature, central nervous system stimulation was obtained by sensory TENS stimulation.

Briefly, the amplitude of TENS stimulation started at 0 mA, with the stimulator turned on and the rheostat, which controls the amplitude, positioned at 0. The amplitude of stimulation was progressively increased at a rate of 0.6 mA/s until the patients reported the sensation of pricking. The amplitude was not further increased. Particular attention was paid to avoid reaching the threshold of motor (VII pair of cranial nerve and its mimic muscles) stimulation. The

same operator delivered all subjects the TENS according to the manufacturer's guidelines.

Statistics

Data normality was tested with Shapiro-Wilk test prior to apply parametric statistics. Paired t test was used for testing sEMG differences between open/closed eyes. Pearson analysis was used to determine correlations between either CSI/DAAS and muscles activity in terms of sEMG. Statistical significance was set at $p < 0.05$. SPSS (v.21.0) was used to perform all the statistical analysis.

RESULTS

Table I shows the average age values divided by

Table I. Average values of age and self-assessment test variables.

	Age	Males Age (12)	Females Age (15)	CSI	DASS D	DASS A	DASS S
27 patients	37.26 (9.63)	38.01 (9.08)	36.88 (10.16)	15,3 (10.05)	3,41 (3.73)	6,49 (5.22)	9,71 (5.47)

The values considered "normal" are the following: CSI <20; DASS D <9; DASS A <7; DASS S <14.

Table II. Mean RMS values and statistical comparisons in the conditions with closed eyes and open eyes before and after ULF TENS.

	CE	OE	ULF TENS CE	ULF TENS OE	T test (p)		
					Before ULF TENS CE vs OE	Before ULF TENS CE vs ULF TENS CE	Before ULF TENS OE vs ULF TENS OE
LTA	1.59 (0.76)	2.29 (1.25)	1.22 (0.48)	1.51 (0.77)	0.01	0.04	0.008
LMM	1.17 (0.41)	1.36 (0.73)	1.07 (0.42)	1.02 (0.40)	0.25	0.40	0.03
RMM	1.11 (0.41)	1.32 (0.79)	1.08 (0.62)	0.95 (0.44)	0.22	0.87	0.03
RTA	1.69 (0.76)	2.06 (0.96)	1.40 (0.60)	1.40 (0.46)	0.12	0.12	0.002
LSC	1.52 (0.96)	1.66 (1.28)	1.38 (0.81)	1.45 (0.94)	0.64	0.57	0.50
LDA	1.53 (0.58)	1.55 (0.60)	1.47 (0.45)	1.51 (0.56)	0.89	0.69	0.78
RDA	1.44 (0.63)	1.45 (0.65)	1.40 (0.61)	1.47 (0.78)	0.96	0.81	0.89
RSC	1.48 (0.61)	1.64 (0.80)	1.45 (0.76)	1.52 (0.83)	0.43	0.87	0.62

sex and the results obtained in the self-assessment scales (CSI and DASS42). The group mean values in the self-assessment scales are lower than those considered “clinical”. No correlation was observed between age, sex and psychometric variables.

Table II shows the mean sEMG values (RMS) of the various sEMG channels in different conditions with closed eyes and with opened eyes before and after sensory ULF TENS. The comparison between closed eyes and opened eyes values before ULF TENS differs significantly only for the LTA muscle (1.59 vs 2.29; $p = 0.01$). The ULF TENS in the condition with closed eyes reduces the LTA value significantly (1.29 vs 1.59; $p = 0.04$). No ULF TENS effect is observed in the closed-eye condition in the other sEMG channels. Statistical comparison of the condition with opened eyes before and after ULF TENS shows a significant reduction in TA values (LTA 2.29 vs 1.51; $p = 0.008$. LMM 1.36 vs 1.02; $p = 0.03$. RMM 1.32 vs 0.95; $p = 0.03$. RTA 2.06

vs 1.40; $p = 0.002$). No statistically significant effect was observed on the DA and SC muscles.

Table III shows relationship between opened eyes and closed eyes (OE / CE) in the conditions before and after ULF TENS. The statistical comparison shows a significant change between, before and after ULF TENS of the ratio in the LMM muscles (1.20 vs 0.95; $p = 0.03$) and RMM (1.26 vs 0.92; $p = 0.04$). At the level of TA, the tendency to reduce the ratio is evident although it does not reach statistical significance (LTA $p = 0.07$; RTA $p = 0.08$).

In the table IV is shown the correlation index (ρ) between the values of the OE / CE of the individual muscles with the scores of the self-assessment scales CSI and DASS42. As can be seen, no ρ values higher than 0.35 were reached in any muscle, a weak inverse relationship is observed above all in the case of RSC with CSI and DASS42 and RDA with DASS S (-0.420).

Table III. Relationship between sEMG values with open eyes and closed eyes (OE / CE) and statistical comparison in the conditions before and after ULF TENS.

	Pre OA/OC	Tens OA/OC	T test (p)
LTA	1.60 (0.96)	1.23 (0.45)	0.07
LMM	1.20 (0.56)	0.95 (0.17)	0.03
RMM	1.26 (0.83)	0.92 (0.18)	0.04
RTA	1.36 (0.74)	1.08 (0.38)	0.08
LSC	1.07 (0.26)	1.05 (0.24)	0.76
LDA	1.02 (0.22)	1.01 (0.21)	0.78
RDA	1.01 (0.24)	1.04 (0.30)	0.76
RSC	1.11 (0.41)	1.04 (0.24)	0.48

Table IV. Correlation (ρ) between the OE / CE ratio and the indices of the self-assessment scales (CSI, DASS D, DASS A, DASS S) before the ULF TENS.

	Lta oe/ce	Lmm oe/ce	Rmm oe/ce	Rta oe/ce	Lsc oe/ce	Lda oe/ce	Rda oe/ce	Rsc oe/ce
corr CSI	0.205	0.027	0.220	-0.302	-0.147	-0.005	-0.209	-0.314
corr DAS S D	0.163	0.044	0.168	-0.195	-0.302	-0.181	-0.350	-0.347
corr DAS S A	0.179	-0.137	0.098	-0.094	-0.132	0.106	-0.232	-0.337
corr DAS S S	0.060	-0.175	0.031	-0.087	-0.161	-0.078	-0.420	-0.357

DISCUSSION

The results are as follows:

- 1) There is a significant difference in basic myoelectric activity in some muscles of the stomatognathic system in comparison between closed and open eyes. This difference is mainly due to the canal that corresponds to the left anterior temporal muscle (LTA). The values with eyes open are significantly greater than those with eyes closed at the LTA level.
- 2) There is no correlation between absolute values (RMS) in the different conditions (closed eyes, open eyes, before and after ULF TENS) with age, sex and psychometric variables.
- 3) The sensitive ULF TENS seems to exert a greater effect (significant reduction of myoelectric tone) in the condition with eyes closed especially on the LTA muscle.
- 4) In the comparison before and after ULF TENS with open eyes, the effect of reduction of electromyographic values is more marked than in the same comparison with closed eyes. The reduction in values is distributed on all purely stomatognathic muscles (LTA, LMM, RMM, RTA).
- 5) The OE / CE ratio, which expresses a form of sensory "activation" or "provocation" of the myoelectric system, is significantly influenced by the effect of ULF TENS at the level of TA and MM. The direction of change is relative less muscle activation with open eyes compared with closed eyes after ULF TENS.
- 6) There is a significant inverse correlation at the level of the LTA and LMM muscles between the OE / CE ratio (visual sensory activation) after ULF TENS with some psychometric variables, in particular with CSI and DASS D and A. This correlation would show that the greatest effect ULF TENS (lower relative activation of the LTA and LMM muscles with open eyes) is obtained in subjects who have a higher score on CSI and DASS42 D and A and who may be subjects with higher arousal levels. This work confirms what has already been observed in the literature that with open eyes the sEMG values especially of the TA muscles are increased compared to closed eyes. These data were observed in spectacle wearers with standard eye correction and in subjects with TMD.

At the same time, the correction of the visual defect through the aid of the sEMG is able to improve the clinical and electromyographic picture in children suffering from headache and functional lateral deviation of the jaw. An interesting aspect of the problem posed by our study is the marked asymmetry of visual sensory activation on the left muscles (LTA and LMM) (4).

Infact, the left mimic musculature responds with an increase in tone, greater than the right, to sensory events of various kinds, regardless of the value of the stimulus itself. An explanation for the phenomenon may be that the right side of the brain has a prevalence in controlling the affective emotional valence of sensory inputs and that it mainly activates the peripheral motor circuits to the left of the face to communicate the emotional / affective condition characteristic of sensory processing (13).

To highlight a sEMG effect linked to psychophysical characteristics, two psychometric self-assessment scales (CSI and DASS42) were used in our study. The CSI and DASS42 scales have been widely used in the literature to characterize some aspects of central sensitization and psychometric characteristics and have shown a good degree of reliability and specificity in characterizing subjects with CS and with alterations in the psychological sphere attributable to depression, anxiety or stress in the course of various life conditions and / or illness (13-22). The DASS42 was also used to evaluate the coping modalities between the psychological characteristics and the daily actions in young subjects during the lock-down from COVID19 in various countries, showing its reliability to highlight different states of depression and / or anxiety, in various cultural and social spheres (23-26).

This type of work and its results have a particular clinical interest for dentistry and, more generally, for those involved in rehabilitation and prosthodontics. In particular, the techniques involving the use of dental ULF TENS in the rehabilitation and orthodontic field should consider the fact that any intervention which modifies the neuromuscular structure and its sensory afferents is necessarily framed within a central processing scheme input, which does not necessarily perceives a hypothetically neutral sensory stimulus

as a real neutral stimulus. Each variation, therefore, should not be evaluated in itself, but in a way that “tests” the response to a system “provocation”. Knowing how to respond to the “provocation” and the use of techniques that facilitate the least “expensive” response possible, this could be a way to explore and better study to obtain clinical results that can be more easily integrated into the specific and subjective system of modulation.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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Long non-coding RNA 01088 restrains breast cancer progression via regulating miR-498/PTEN axis

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Breast cancer (BC) is one of the most common tumors and the second leading cause of cancer-related death among women. Long non-coding RNA (lncRNA) is vital in regulating the progression of BC, however, its biological function in BC is inconclusive. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to quantify the expressions of LINC01088, microRNA (miRNA) miR-498, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mRNA in BC tissues and cells. Cell counting kit-8 (CCK-8) and 5-Ethynyl-2'-deoxyuridine (EdU) assays were used to detect cell proliferation. The apoptosis was detected by flow cytometry. Moreover, PTEN protein expression was determined by Western blot. Dual-luciferase reporter gene assay and RNA immunoprecipitation (RIP) assay were used to verify the targeting relationships among LINC01088, miR-498, and PTEN. LINC01088 expression level was declined in BC tissues and cells, which was related to higher TNM stage. LINC01088 overexpression significantly inhibited the growth of BC cells, induced apoptosis, promoted Bax and cleaved-caspase-3 expressions, and restrained Bcl-2 expression; LINC01088 knockdown worked oppositely. MiR-498 was confirmed as the target of LINC01088. PTEN expression was negatively modulated by miR-498 and positively regulated by LINC01088 in BC cells. LINC01088 up-regulates the expression of PTEN by targeting miR-498 and has the potential to repress the progression of BC.

Breast cancer (BC) is the most prevalent malignancy posing a serious threat to women's health, and its morbidity and mortality have been increasing yearly worldwide (1, 2). Although many advances have been made in the treatment of BC, the metastasis and drug-resistance is still the main cause of death among women (3, 4). Therefore, it is urgent to further study the mechanism of the pathogenesis

of BC and explore new targets for the effective treatment.

Long non-coding RNA (lncRNA) is a kind of RNA exceeding 200 nt without protein-coding capability (5). Previously, it was believed lncRNA did not have biological functions (6), however, in recent years, lncRNAs have been proved to have many functions as molecular sponge, molecular guider and molecular

Key words: breast cancer, LINC01088, miR-498, PTEN

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scaffold (7). LncRNAs are pivotal in the progression of various tumors, including BC (8). For example, in BC, LINC00899 plays an inhibitory role in cancer progression through the competitive combination with miR-425 (9). LINC01116 is found to enhance ESR1 expression and expedite the progression of BC as a competing endogenous RNA (ceRNA) of miR-145 (10). In addition, it is reported that LINC01088 inhibits the tumorigenesis of ovarian epithelial cells by regulating miR-24-1-5p/PAK4 axis (11). However, the biological function of LINC01088 in BC has not been completely elucidated.

MicroRNAs (miRNA) can regulate gene expression post-transcriptionally via restraining the translation of mRNA or inducing its degradation (12). MiRNAs are also crucial regulators in cancer development (13). For instance, miR-448 expression level is raised in BC tissues and cells, and miR-448 can promote the progression of BC by targeting ZEB1 (14). As reported by Chai et al., miR-498 expression is elevated in BC cell lines, which promotes BC proliferation and migration by inhibiting PTEN (15). In the present study, we found that LINC01088 could inhibit the progression of BC via modulating the miR-498/PTEN axis. The research deepened the understanding of the pathogenesis of BC and may provide a new molecular target for targeted therapy of BC.

MATERIALS AND METHODS

BC sample collection

Thirty-three BC patients who underwent mastectomy in The Third Affiliated Hospital of Kunming Medical University were enrolled in this study. The patients had did not received radiotherapy or chemotherapy prior to surgery. Subsequent to surgical resection, BC tumor tissues and adjacent normal tissues were frozen at -196°C in liquid nitrogen. Written informed consent was obtained from all the enrolled patients, and the Ethics Committee of The Third Affiliated Hospital of Kunming Medical University, and the Ethics Review Board of Xi'an No. 3 Hospital approved the study,

Cell culture and transfection

Human embryonic kidney cells (HEK293T), BC cell lines (MDA-MB-468, MDA-MB-453, SKBR3, MDA-

MB-231, T47D, and MCF7), and human mammary epithelial cell line MCF-10A were all purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were accordingly cultured in Dulbecco's modified Eagle's medium (DMEM, Shanghai Biyuntian Biotechnology Co., Ltd, Shanghai, China) with 10% fetal bovine serum (FBS, Shanghai Biyuntian Biotechnology Co., Ltd, Shanghai, China) and 1% penicillin/streptomycin (Shanghai Biyuntian Biotechnology Co., Ltd, Shanghai, China) at 37°C in 5% CO_2 . The medium was changed at intervals of every 2–3 days, and cells growing to 70–80% confluence were routinely subcultured with 0.25% trypsin (Roche, Basel, Switzerland). LINC01088 overexpression plasmids (pcDNA-LINC01088), empty vector plasmids (pcDNA-NC), small interfering RNA (siRNA) oligonucleotides targeting LINC01088 (si-LINC01088-1 LINC01088-2), siRNA negative control (si-NC), miR-498 mimics and controls (miR-NC) and miR-498 inhibitors and controls (Inh NC) were all from GenePharma (Shanghai, China). Lipofectamine[®]3000 (Invitrogen, Carlsbad, CA, USA) was used to transfect the above vectors/oligonucleotides into SKBR3 and MDA-MB-231 cells. After 24 h, quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the efficiency.

qRT-PCR

Total RNA was extracted from BC tissues and cells by TRIzol Regent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed by the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). With cDNA as the template, SYBR[®]Premix-Ex-Taq[™] (Takara, Tokyo, Japan) was used for qRT-PCR on ABI7300 system (Thermo Fisher Scientific Inc., Rockford, IL, USA), with GAPDH as the internal control of mRNA and lncRNA, and U6 as that of miRNA. To identify the subcellular localization of LINC01088, a PARIS[™] kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used for nucleo-cytoplasmic separation. Then qRT-PCR was performed to detect the expression level of LINC01088 in cytoplasm and nuclei, respectively. Specific primer sequences were as follows: LINC01088 Forward: 5'-CTCCCTTCCTCCATGATT-3', LINC01088 Reverse: 5'-ATGTGGGGACATTGATGCTT-3'; miR-498 Forward: 5'-TTTCAAGCCAGGGGGCGTTTTTC-3', miR-498 Reverse: 5'-GCTTCAAGCTCTGGAGGTGCTTTTC-3'; PTEN Forward: 5'-TCCCAGACATGACAGCCATC-3',

PTEN Reverse: 5'-TGCTTTGAATCCAAAAACCTTACT-3';
 U6 Forward: 5'-CTCGCTTCGGCAGCACA-3', U6 Reverse:
 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH Forward:
 5'-GCACCGTCAAGGCTGAGAAC-3', GAPDH Reverse:
 5'-TGGTGAAGACGCCAGTGGA-3'.

Cell counting kit-8 (CCK-8) assay

SKBR3 or MDA-MB-231 cells at logarithmic phases of growth were trypsinized, with the cell density adjusted to 2×10^4 cells/mL. After that, these cells were transferred into a 96-well plate with 100 μ L of cell suspension per well and cultured. After 1, 2, 3, and 4 days, these cells were incubated with 10 μ L of CCK-8 solution (MedChemExpress, Monmouth Junction, NJ, USA) for 1 h. Subsequently, the 96-well plate was placed in a microplate reader, and the absorbance of each well at 450 nm was measured.

5-Ethynyl-2'-deoxyuridine (EdU) assay

An EdU kit (Beyotime Biotechnology, Shanghai, China) was used to detect the proliferation of BC cells. Briefly, SKBR3 or MDA-MB-231 cells were transferred into 24-well plates to normal growth stage, and then 200 μ L 5 μ mol/L EdU medium (Beyotime Biotechnology, Shanghai, China) was added into each well, and the cells were incubated for 2 h, and then washed with phosphate buffer saline (PBS). Subsequently, these cells were fixed with paraformaldehyde for 10 min at ambient temperature. Then, the cells were incubated with 200 μ L of 2mg/mL glycine for 5 min, and then incubated with 100 μ L of 0.5% Triton X-100 penetrant. After being decolorized for 10 min on a shaking table, the cells were washed with PBS twice for 5 min each time and subsequently stained with anti-BrdU antibody at room temperature for 30 min at ambient temperature and with DAPI staining solution for 20 min at room temperature. Then, the number of cells was counted under a fluorescence microscope.

Western blot

RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) was used to lyse BC cells, and the lysate was collected by high-speed centrifugation to extract the total protein of cells, with total protein concentration determined by the BCA method. The supernatant was heated in a water bath at 100°C for 10 min to denature the proteins, and the proteins were subjected to 12% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Afterwards, the membranes were blocked with 5% skimmed milk for 1 h at room temperature, rinsed with Tris Buffered Saline Tween (TBST), and then incubated with the primary antibody anti-PTEN (ab267787, 1:500, Abcam, Cambridge, UK), anti-Bax antibody (ab182733, 1:500, Abcam, Cambridge, UK), anti-Bcl-2 antibody (ab32124, 1:500, Abcam, Cambridge, UK), cleaved caspase-3 (c-caspase-3, ab32042, 1:500, Abcam, Cambridge, UK) and anti-GAPDH antibody (ab9485, 1: 500, Abcam, Cambridge, UK) at 4°C overnight. After being immersed in TBST, the membranes were incubated with Goat Anti-Rabbit IgG H&L (ab150077, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature, and then washed with TBST four times. GAPDH was used as an internal reference, and the protein bands were developed by an ECL chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

Flow cytometry

The apoptosis of SKBR3 or MDA-MB-231 cells was detected by flow cytometry with Annexin V-FITC and propidium iodide (PI) double staining. Briefly, the BC cells in different groups were suspended in annexin V-binding buffer (about 1×10^5 cells in 1 mL buffer, per sample) and stained with 5 μ L of Annexin V-FITC staining solution and 5 μ L of PI solution (Invitrogen, Carlsbad, CA, USA) for 15 min at ambient temperature. Stained cells diluted in annexin V-binding buffer were detected by a flow cytometer (BD Biosciences, Bedford, MA, USA). The percentage of apoptotic cells was analyzed by Flowjo software (TreeStar, San Carlos, CA, USA).

RNA immunoprecipitation

RNA immunoprecipitation (RIP) assay was conducted with a Magna RIP RNA binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) according to the manufacturer's instruction. RIP lysis buffer was adopted to lyse SKBR3 and MDA-MB-231 cells, and 100 μ L of the whole-cell extract was accordingly incubated with RIPA buffer (Beyotime, Shanghai, China) containing magnetic beads coupled with human anti-Argonaute2 (Ago2) antibody (Millipore, Billerica, MA, USA) at 4°C for 8 h, with a normal mouse

IgG (Millipore, Billerica, MA, USA) as a negative control. Subsequently, the samples were washed with washing buffer and incubated with proteinase K at 55°C for 30 min to separate RNA-protein complexes from magnetic beads. Ultimately, the immunoprecipitated RNA was extracted and analyzed by qRT-PCR.

Dual-luciferase reporter assay

The wild type or mutant type binding sequence between miR-498 and LINC01088 or miR-498 and PTEN mRNA 3'-UTR was amplified and cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to construct wild type luciferase reporter gene vector (LINC01088 WT, PTEN WT) or mutant type luciferase reporter gene vector (LINC01088

MUT, PTEN MUT). The reporter vector, miR-498 mimics, miR-498 inhibitors, or their negative control were subsequently co-transfected into 293T cells, respectively. After 48 h, the luciferase activity was determined by a dual luciferase assay system (Promega, Madison, WI, USA).

Statistical analysis

The experiments were repeated three times. The data were statistically analyzed by SPSS 23.0 software (SPSS Inc., Chicago, IL, USA), with the quantitative data expressed as mean $\pm$ standard deviation. Student's *t*-test was employed for the comparison between the two groups, and a one-way analysis of variance (ANOVA) was used for the comparison among multiple groups. Statistically, $P < 0.05$ was meaningful.

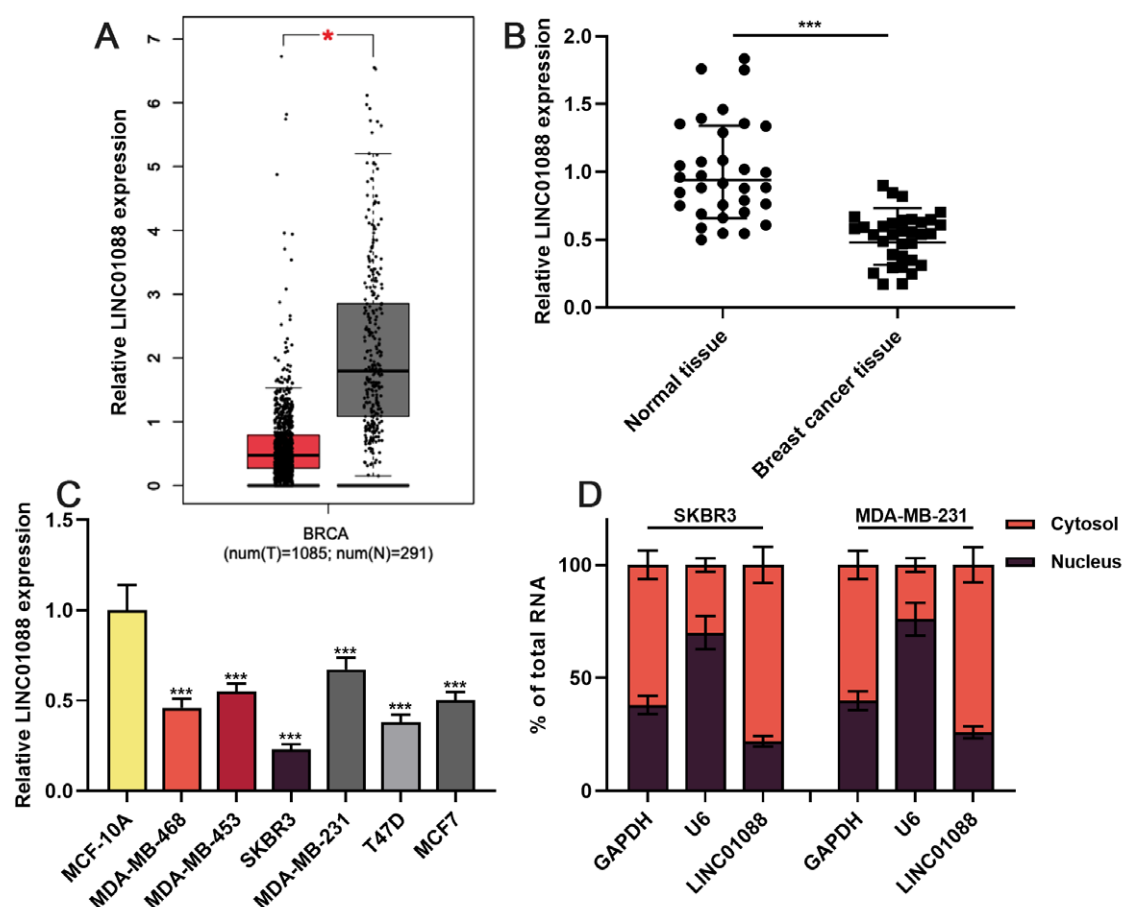


Fig. 1. LINC01088 expression was down-regulated in BC. **A)** GEPIA database was used to analyze LINC01088 expression in BC tissues (red columns, $n=1085$) and normal tissues (grey columns, $n=291$). **B)** LINC01088 expression in BC tissues ($n=33$) and normal breast tissues ($n=33$) was detected by qRT-PCR. **C)** LINC01088 expression in normal human mammary epithelial cell MCF-10A and BC cell lines (MDA-MB-468, MDA-MB-453, SKBR3, MDA-MB-231, T47D, and MCF7) was detected by qRT-PCR. **D)** Nuclear-cytoplasmic separation was used to detect the subcellular localization of LINC01088 in SKBR3 and MDA-MB-231 cells. All of the experiments were performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$.

RESULTS

LINC01088 was lowly expressed in BC

First of all, to investigate the expression pattern of LINC01088 in BC, the data of GEPIA were analyzed, with the results displaying a remarkable down-regulation of LINC01088 expression level in BC tissue samples, compared with normal breast tissues (Fig. 1A). Then, qRT-PCR was used to detect LINC01088 expression in 33 cases of BC tissue samples and corresponding normal breast tissue samples, and the results showed that LINC01088

expression was down-regulated in BC tissue samples (Fig. 1B). Furthermore, the expression of LINC01088 in BC cell lines (MDA-MB-468, MDA-MB-453, SKBR3, MDA-MB-231, T47D, and MCF7), compared with that in normal human mammary epithelial cell lines MCF-10A, was significantly down-regulated (Fig. 1C). In addition, the expression was also detected of LINC01088 in the nucleus and cytoplasm, respectively, and it was found that LINC01088 was mainly distributed in the cytoplasm through nucleo-cytoplasmic separation (Fig. 1D).

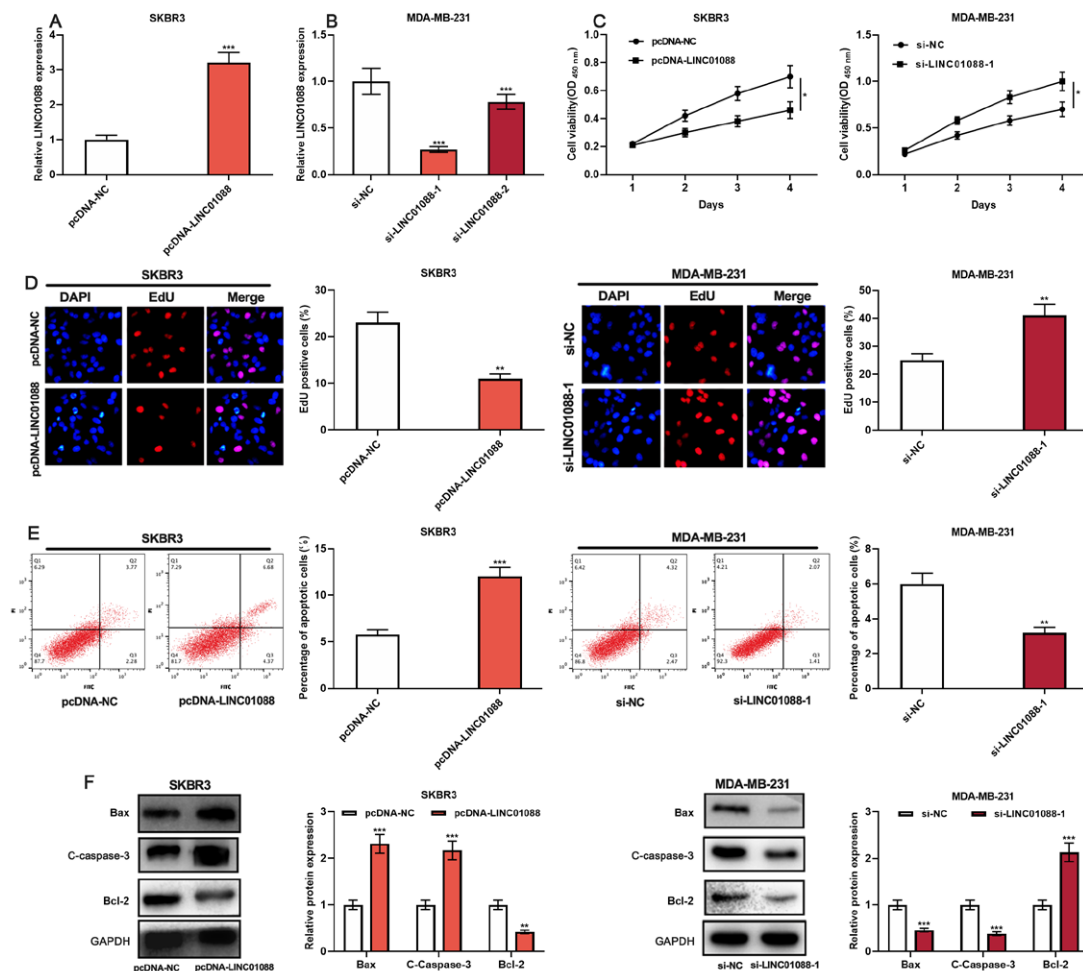


Fig. 2. LINC01088 inhibited BC cell proliferation and promoted apoptosis. **A, B)** LINC01088 overexpression plasmid was transfected into SKBR3 cells, and si-LINC01088-1 and si-LINC01088-2 were transfected into MDA-MB-231 cells, and then LINC01088 expression was detected by qRT-PCR. **C, D)** The effects of LINC01088 overexpression or knockdown on the proliferation of SKBR3 and MDA-MB-231 cells were detected by CCK-8 (**C**) and EdU assay (**D**). **E)** The effects of LINC01088 overexpression or knockdown on the apoptosis of SKBR3 and MDA-MB-231 cells were detected by flow cytometry. **F)** The effects of LINC01088 overexpression or knockdown on the expressions of Bax, cleaved-caspase-3, and Bcl-2 proteins in SKBR3 and MDA-MB-231 cells were examined by Western blot. All of the experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

LINC01088 inhibited BC cell proliferation and promoted apoptosis

As shown, the expression of LINC01088 was the lowest in SKBR3 cells and the highest in MDA-MB-231 cells (Fig. 1C), so we transfected LINC01088 overexpression plasmid into SKBR3 cells and transfected si-LINC01088-1 and si-LINC01088-2 into MDA-MB-231 cells. si-LINC01088-1 was selected for subsequent experiments because of its better knockdown efficiency, and qRT-PCR results indicated that LINC01088 overexpression and low expression cell models were constructed successfully (Fig. 2 A, B). Subsequently, the biological functions of LINC01088 *in vitro* were determined by CCK-8 assay, EdU assay,

flow cytometry assay, and Western blot analysis. It was shown that LINC01088 overexpression significantly inhibited cell proliferation, promoted apoptosis, enhanced the expressions of Bax and cleaved-caspase-3 protein, and impeded Bcl-2 protein expression; while LINC01088 knockdown promoted cell proliferation and expressions of Bax and C-caspase-3 protein, and inhibited apoptosis and Bcl-2 protein expression (Fig. 2 C, F).

LINC01088 directly targeted miR-498

To further explore the mechanism of LINC01088, LncBase Predicted database was utilized to predict the targets of LINC01088, and miR-498 was found to be a potential target of LINC01088 (Fig. 3A).

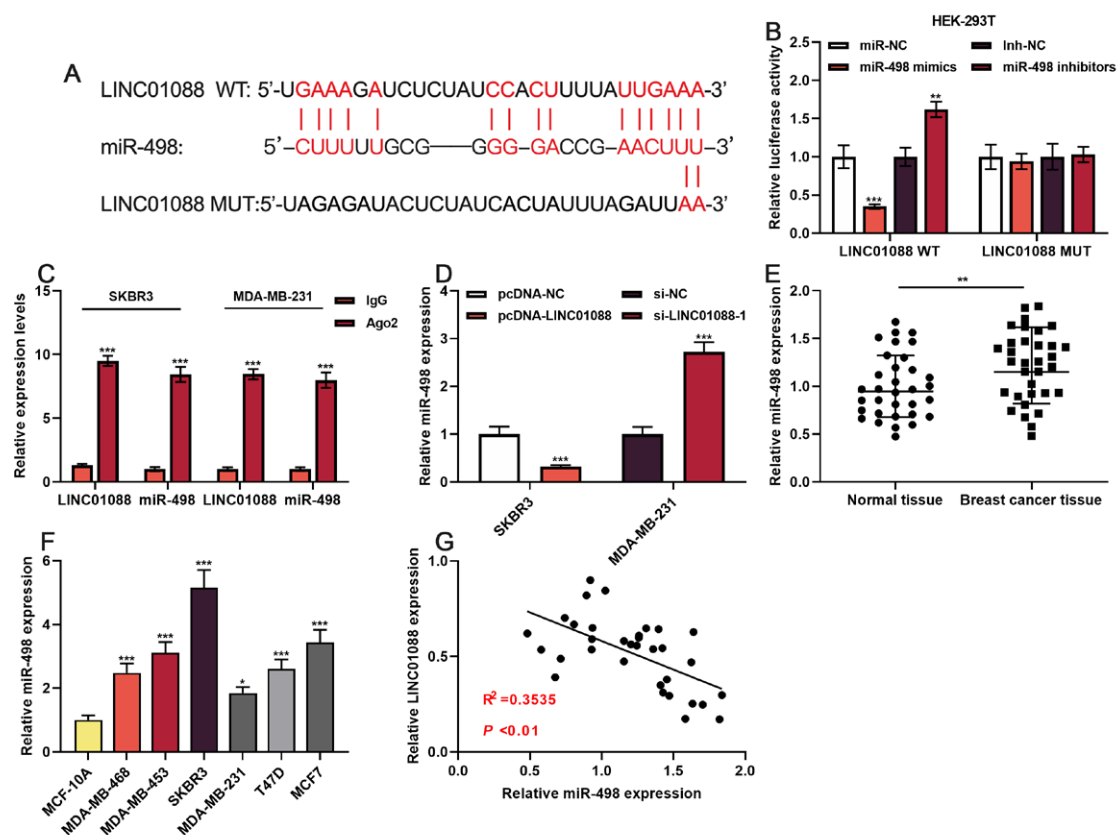


Fig. 3. LINC01088 specifically regulated miR-498 expression. A) The LncBase Predicted database was used to predict the potential binding sites between LINC01088 and miR-498. B) Dual-luciferase reporter gene assay was used to identify the binding site between LINC01088 and miR-498. C) RIP assay was used to confirm the direct interaction between LINC01088 and miR-498. D) The expression of miR-498 was detected by qRT-PCR after LINC01088 overexpression or knockdown in BC cells. E) MiR-498 expression in BC tissues ($n=33$) and normal breast tissues ($n=33$) was detected by qRT-PCR. F) MiR-498 expression in normal human mammary epithelial cell MCF-10A and BC cell lines was detected by qRT-PCR. G) Pearson correlation analysis was adopted to analyze the correlation between LINC01088 and miR-498 expressions in BC tissues. All of the experiments were performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$.

To further verify the targeting relationship between the two molecules, we performed a dual-luciferase reporter assay. The results showed that the up-regulation of miR-498 expression significantly decreased the luciferase activity of LINC01088 WT, and the inhibition of miR-498 expression exerted opposite effects, but neither of them had a significant effect on that of LINC01088 MUT (Fig. 3B). Then, an RIP assay was further conducted to verify the interaction between LINC01088 and miR-498. It was shown that LINC01088 and miR-

498 were significantly enriched in Ago2 group as against the IgG group (Fig. 3C). To further pinpoint the role of LINC01088 in the regulation of miR-498 expression, the expression of miR-498 in LINC01088 overexpression and knockdown cell models was detected. qRT-PCR showed that the up-regulation of LINC01088 expression significantly inhibited miR-498 expression in SKBR3 while the down-regulation of LINC01088 expression promoted miR-498 expression in MDA-MB-231 (Fig. 3D). Furthermore, qRT-PCR results showed

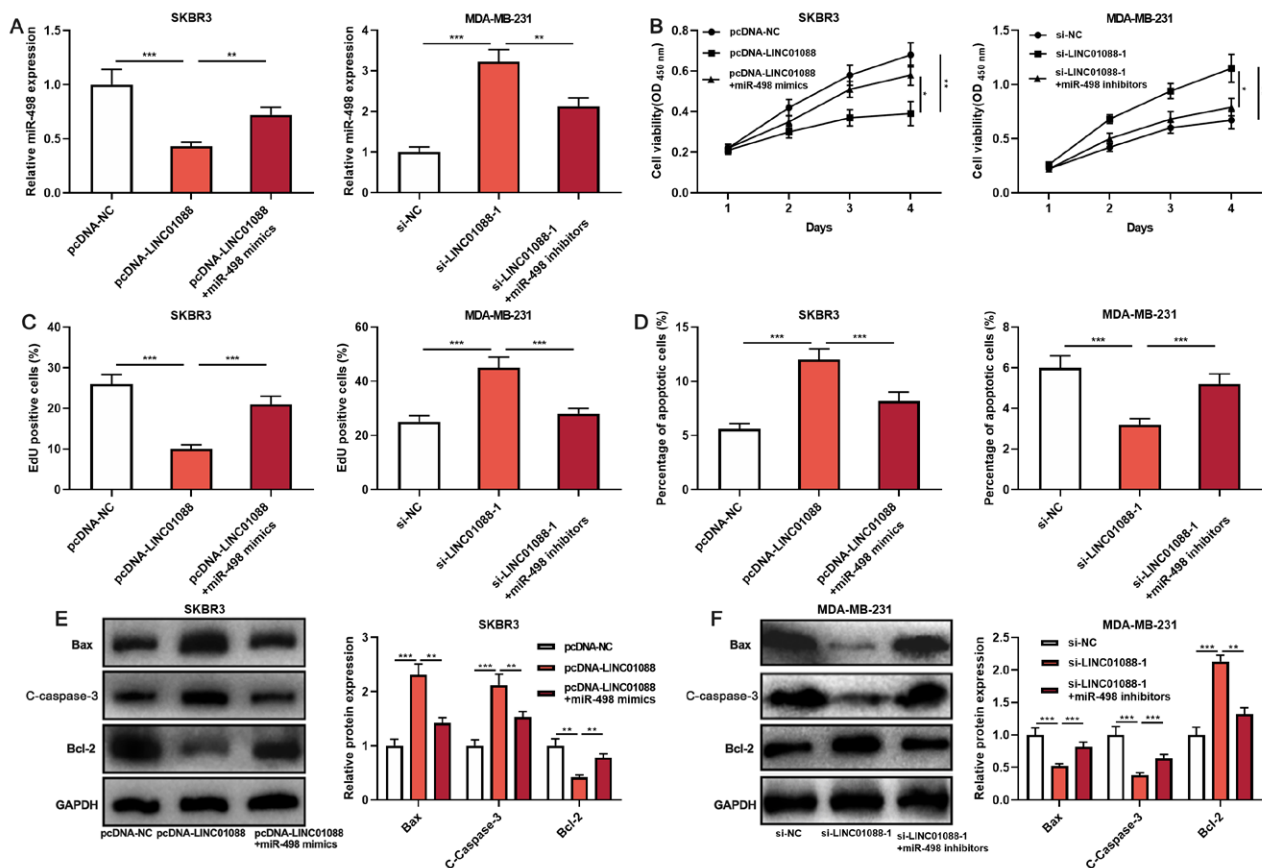


Fig. 4. LINC01088 inhibited the malignant phenotypes of BC cells by targeting miR-498. **A)** pcDNA-NC, pcDNA-LINC01088, or pcDNA-LINC01088+miR-498 mimics were transfected into SKBR3 cells, and si-NC, si-LINC01088-1, si-LINC01088-1+miR-498 inhibitors were transfected into MDA-MB-231 cells, respectively. Then the expression of miR-498 was detected by qRT-PCR. **B)** The effects of the co-transfection of pcDNA-LINC01088 and miR-498 mimics and the co-transfection of si-LINC01088-1 and miR-498 inhibitors on the proliferation of SKBR3 and MDA-MB-231 cells were detected by CCK-8 assay. **C)** The effects of the co-transfection of pcDNA-LINC01088 and miR-498 mimics and the co-transfection of si-LINC01088-1 and miR-498 inhibitors on the proliferation of SKBR3 and MDA-MB-231 cells were detected by EdU assay. **D)** The effects of the co-transfection of pcDNA-LINC01088 and miR-498 mimics and the co-transfection of si-LINC01088-1 and miR-498 inhibitors on the apoptosis of SKBR3 and MDA-MB-231 cells were detected by flow cytometry. **E, F)** The effects of the co-transfection of pcDNA-LINC01088 and miR-498 mimics and the co-transfection of si-LINC01088-1 and miR-498 inhibitors on the expressions of apoptosis-related proteins in SKBR3 and MDA-MB-231 cells were detected by Western blot. All of the experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

that miR-498 expression was up-regulated in BC tissue samples compared with normal breast tissues (Fig. 3E). Also, the expression of miR-498 was significantly up-regulated in BC cell lines compared with that in MCF-10A cells (Fig. 3F). Subsequently, the correlation between the expressions of miR-498 and LINC01088 was analyzed with Pearson's correlation analysis, and it was shown that there was a negative correlation between them in BC tissues (Fig. 3G).

LINC01088 exerted its function by targeting miR-498

To elaborate on whether LINC01088 could regulate the proliferation and apoptosis of BC cells by modulating miR-498 expression, pcDNA-NC, pcDNA-LINC01088, pcDNA-LINC01088+miR-498 mimics were transfected into SKBR3 cells, and si-NC, si-LINC01088-1, si-LINC01088-1+miR-498 inhibitors into MDA-MB-231 cells, respectively, with qRT-PCR showing the models were constructed successfully (Fig. 4A). CCK-8 assay, EdU assay, flow cytometry assay, and Western blot revealed that the up-regulation of LINC01088 expression could restrain the multiplication and expedite the apoptosis of SKBR3, while the transfection of miR-498 mimics could weaken these effects; knocking down LINC01088 could accelerate the growth and inhibit the apoptosis of MDA-MB-231 cells, while the transfection of miR-498 inhibitors could reverse these effects (Fig. 4 B-F).

LINC01088 participated in the progression of BC by regulating miR-498/PTEN axis

To expound the mechanism of LINC01088 in BC, we analyzed the downstream genes of miR-498 by Targetscan database and found that there was a possible binding sequence between PTEN mRNA 3'UTR and miR-498 (Fig. 5A). To further verify the targeting relationship between miR-498 and PTEN, we performed a dual-luciferase reporter assay. It was proved that the up-regulation of miR-498 expression observably inhibited the luciferase activity of PTEN WT while the down-regulation of miR-498 expression worked oppositely; the up-regulation or down-regulation of miR-498 had no significant effect on that of PTEN MUT (Fig. 5B).

To further investigate the roles of LINC01088 and miR-498 in the regulation of PTEN expression, the cell models mentioned above were constructed (Fig. 4A). We found that the up-regulation of LINC01088 expression promoted PTEN protein and mRNA expressions while the up-regulation of miR-498 expression weakened this effect; knocking down LINC01088 inhibited PTEN protein and mRNA expressions, and the inhibition of miR-498 expression abolished this effect (Fig. 5 C, D). qRT-PCR revealed that PTEN mRNA expression in BC tissue was demonstrably down-regulated compared to that in normal breast tissues (Fig. 5E). Pearson's correlation analysis highlighted that PTEN mRNA expression was negatively correlated with miR-498 expression and positively correlated with LINC01088 expression in BC tissues (Fig. 5 F, G).

DISCUSSION

LncRNA features prominently in regulating many biological processes, including cell vitality, apoptosis, and differentiation, as well as tumorigenesis and cancer progression, which may predict the prognosis of some types of cancers (16). As reported, the abnormal expression of lncRNA is related to the occurrence and development of BC. For example, lncRNA NKILA binds to NF- κ B/I κ B, and inhibits I κ B phosphorylation and NF- κ B activation induced by I κ B kinases (IKK), to inhibit BC metastasis (17). Another study shows that LINC00899 expression is decreased in BC tissues and cell lines, and LINC00899 competitively combines with miR-425 and can work as a tumor suppressor by up-regulating DICER1 (9). LINC01088 is a newly discovered RNA that affects cancer progression. For example, LINC01088 expression is significantly down-regulated in ovarian cancer, and the overall survival rate of patients with low expression of LINC01088 is poor (18). Another study also confirms that LINC01088 expression is decreased in benign epithelial ovarian tumors, and LINC01088 promotes the expression of PAK4 through competitively binding to miR-24-2, which inhibits the progression of ovarian cancer (11). In this work, it was revealed that LINC01088 was significantly lowly expressed

in BC tissues and cells. Further studies proved that LINC01088 overexpression dramatically restrained the proliferation and promoted the apoptosis of BC cells. These data substantiated that LINC01088 was a tumor suppressor in BC.

MiRNA, a short non-coding RNA molecule, modulates gene expression in eukaryotes and can be used as biomarker for many diseases, including cancer (19). MiRNAs, reportedly, are pivotal in regulating the proliferation, survival, chemosensitivity and other malignant biological behaviors of cancer cells (20). It is reported that miR-498 expression in liver cancer tissues is significantly lower than that

in adjacent tissues, and miR-498 overexpression markedly inhibits the proliferation, metastasis, and epithelial-mesenchymal transition (EMT) of liver cancer cells (20). MiR-498 expression in ovarian cancer tissue is also significantly down-regulated as against that in adjacent tissues, and miR-498 overexpression significantly inhibits the viability of ovarian cancer cells by targeting FOXO3 (21). The role of miR-498 in BC has also been reported. Vitamin D upregulates miR-498 expression, thereby downregulating hTERT expression and promoting BC cell growth (22). In this work, we authenticated that miR-498 expression was raised in BC, and

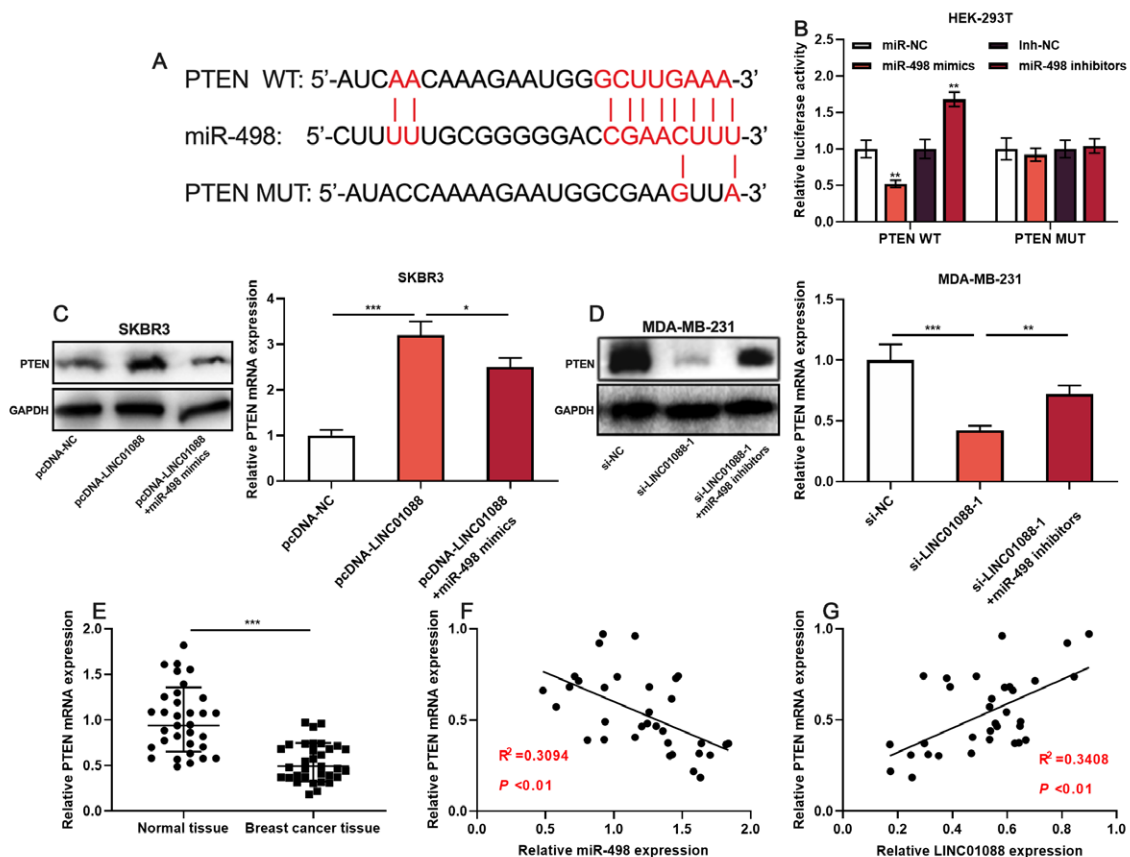


Fig. 5. LINC01088 was a tumor-suppressor by regulating miR-498/PTEN axis in BC cells. **A)** The potential binding sites between PTEN mRNA 3'UTR and miR-498 were predicted by TargetScan database. **B)** The relative luciferase activity of the cells in each group was detected by dual-luciferase reporter gene assay to confirm the targeting relationship between miR-498 and PTEN. **C, D)** pcDNA-NC, pcDNA-LINC01088, or pcDNA-LINC01088+miR-498 mimics were transfected into SKBR3 cells, and si-NC, si-LINC01088-1, si-LINC01088-1+miR-498 inhibitors were transfected into MDA-MB-231 cells, respectively. PTEN protein expression and PTEN mRNA expression were then detected by Western blot and qRT-PCR. **E)** The expression of PTEN mRNA in BC tissues ($n=33$) and adjacent tissues ($n=33$) was detected by qRT-PCR. **F, G)** Pearson correlation was used to analyze the correlation between PTEN mRNA and miR-498, or LINC01088 expression, respectively. All of the experiments were performed in triplicate. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

LINC01088 inhibited the progression of BC by negatively regulating miR-498 expression.

PTEN protein contains 403 amino acid residues, with a protein tyrosine phosphatase (PTP) domain at the N-terminal and PDZ domain at the C-terminal segment (23, 24). PTEN, as a protein phosphatase, can degrade inositol triphosphate, thus being a very effective negative regulator of the PI3K-AKT pathway and regulating signal transduction in tumor cells (25). As reported, the abnormal expression of PTEN is related to the occurrence and development of BC (26). For example, PTEN-mediated AKT phosphorylation is associated with cancer cell migration and apoptosis and induces BC chemotherapy resistance (27). Reportedly, PTEN is poorly expressed in triple-negative breast cancer cells and can be used as a potential predictor of patient prognosis (28). In the current study, we demonstrated that miR-498 could bind to PTEN mRNA 3'UTR and negatively modulate the expression of PTEN, and LINC01088 could positively regulate PTEN. In addition, LINC01088 could impede the multiplication and accelerate the apoptosis of BC cells by adsorbing miR-498 to up-regulate PTEN expression. Our study provides a novel explanation of the mechanism of PTEN dysregulation in BC.

To recapitulate briefly, LINC01088 can inhibit the proliferation and promote the apoptosis of BC cells by regulating miR-498/PTEN axis, which is expected to provide new clues for BC treatment. In the following work, more clinical samples should be collected to further analyze the prognostic evaluation value of LINC01088 expression, and *in vivo* experiments are required to further validate the tumor-suppressive role of LINC01088.

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EIF4E3 is an immune infiltration correlated prognosticator in colon cancer

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Colon cancer is a highly malignant gastrointestinal cancer, and genes in the tumor infiltrating cells and microenvironment are emerging as pivotal players in its development and prognosis. In this study, TIMER and StarBase tools were used to find whether EIF4E3 gene was downregulated in colon tissues, and whether lower expression of EIF4E3 was associated with poor prognosis in colon cancer patients, as well as to ascertain that EIF4E3 could regulate immune-related pathways by *gene set enrichment analysis* (GSEA). Using TIMER (Tumor Immune Estimation Resource) database and ImmuneCellAI tools, it was found that EIF4E3 expression was correlated with tumor immune infiltrates in colon cancer. The results identified EIF4E3 as an immune infiltrate-correlated prognosticator in colon cancer.

Colon adenocarcinoma (COAD) is one of the most prevalent malignancies in the world (1), and it is the second leading cause of cancer-related deaths in the United States (2). Despite the advances in chemoradiotherapy and surgery, the prognosis of patients remains unsatisfactory (3-5). Studies have illustrated that dysregulation of the immune system can promote the occurrence and development of cancer (6). Immunotherapy is rapidly attracting attention in cancer-associated treatment as it has shown efficacy in some advanced and aggressive cancers (5, 7, 8). However, not all patients respond well to immunotherapy. It is essential to understand the mechanism of immunotherapy and further identify novel biomarkers for better predicting this treatment.

Increasing evidence has indicated that changes in tumor microenvironment (TME) play an important role in the occurrence, progression, treatment and prognosis of COAD (9, 10). For example, Wang

et al. found that TGFβ1 could be an indicator for tumor immune microenvironment of colon cancer (11). Also, PD-L1⁺ dendritic cells in the tumor microenvironment correlate with good prognosis and CD8⁺ T cell infiltration in colon cancer (12). TME is a highly complex milieu composed of diverse factors that are associated with cancer progression and metastasis, as well as with the development of cancer resistance to therapeutics (13). Immune cells and stromal cells are two major types of non-tumor components in TME. Immune cells within the tumor TME play important roles in tumorigenesis (14).

EIF4E3 is a significant member of the EIF4E family which maps to chromosome 3p13. It acts as a transcription initiation factor and recruits mRNAs to the ribosome (15). Several studies have reported the function of EIF4E3 in cancers. For example, Osborne et al. (16) found that eIF4E3 relies on the methyl-7-guanosine (m(7)G) cap-binding activity to act as a tumor suppressor. Moreover, EIF4E3

Key words: EIF4E3; immune infiltration; prognosticator; colon cancer; Th17 cells

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overexpression could marginally inhibit eIF4E1-driven translation, which was regulated by MNKs in diffuse large B-cell lymphoma (17). However, these are still rarely reported in colon cancer.

In this study, we first integrated TIMER, StarBase database and bioinformatics to indicate that EIF4E3 was downregulated in COAD and associated with poor prognosis. Next, comprehensive analysis showed that EIF4E3 could regulate immune-related pathways and affect immune infiltration levels in COAD.

MATERIALS AND METHODS

Data acquisition

The RNA-seq data of colon and normal tissue were obtained from the The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). Some samples with incomplete clinical information were deleted when COAD samples were analyzed by comprehensive bioinformatics. Also, the related phenotype information of the patients (e.g., age, sex, and TNM stage) and corresponding survival information (e.g., survival status and time to latest follow-up) were downloaded. Subsequently, by *chi*-squared test, the statistical difference of these characteristics with EIF4E3 expression was analyzed.

TIMER database analysis

TIMER (<http://cistrome.dfci.harvard.edu/TIMER/>) is a comprehensive database for systematic analysis of immune infiltrates with different kinds of cancer (18). It used a previously published statistical method to analyze RNA-seq expression profile data to detect the infiltration of immune cells in tumor tissues (19). The current release of TIMER incorporated 10,009 samples across 23 cancer types from TCGA dataset to estimate the abundance of immune infiltrates.

StarBase database

StarBase has powerful data capabilities. This database provides a comprehensive analysis of the expression profiles of different types of RNA. It also decodes the interaction networks of lncRNAs, miRNAs, competing endogenous RNAs, RNA-binding proteins and mRNAs from large-scale CLIP-Seq data (20, 21). In this study, StarBase was used to evaluate the expression level and prognostic value of EIF4E3

in COAD. P-value < 0.05 was considered to have statistically significant value.

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) is a computational method used to determine whether a set of priori-defined genes show statistically significant, concordant differences between two biological states (22). GSEA preliminarily classified the genes according to their correlation with the EIF4E3 expression from TCGA. This computational method illustrates the significant difference in survival observed between the high and low EIF4E3 samples. For each analysis, we ran 1000 repetitions of gene set permutations. In addition, nominal P values and standardized enrichment scores (NES) were used to classify enrichment pathways in each phenotype (23).

Correlation analysis between EIF4E3 and immune infiltration in COAD

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 analyze the correlation between EIF4E3 expression and TIICs, the cutoff was p-value < 0.01. The Immune Cell Abundance Identifier (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/>) (24). The ImmuCellAI detected the abundance of immune cells, and the differences in the Th17 cells were analyzed in this study.

Statistical analysis

All statistical analyses were enforced with corresponding packages of R (Version 3.6.3). For all analyses, the threshold p-value < 0.05 was considered as statistically significant.

RESULTS

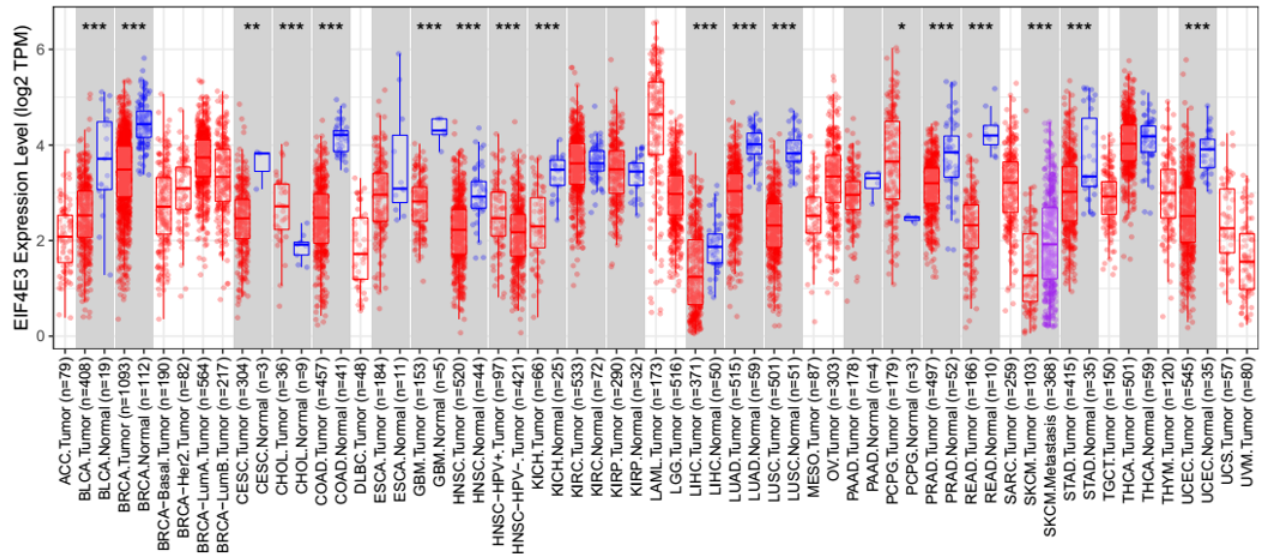
EIF4E3 was highly expressed in colon cancer

The mRNA-seq data of colon cancer from TCGA datasets were analyzed by the TIMER web tools, which showed the mRNA expression levels of EIF4E3 in

different kinds of tumors. In Fig. 1A, EIF4E3 showed a lower expression level in most cancers, including bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), cervical squamous

cell carcinoma and endocervical adenocarcinoma (CESC), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), liver hepatocellular

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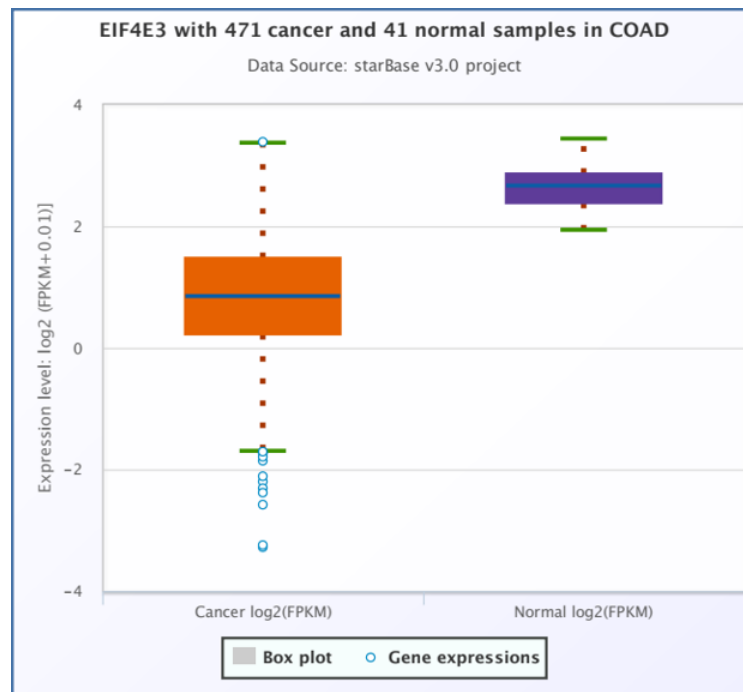


Fig. 1. EIF4E3 mRNA expression levels in normal and cancer tissues. A) EIF4E3 mRNA expression levels in different tumor types from TCGA database were determined by TIMER database (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). B) EIF4E3 in COAD compared with normal tissues in the Starbase database (Fold change=0.33, $p=1.9e-27$).

carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), prostate adenocarcinoma (PRAD), rectum adenocarcinoma (READ), skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), uterine corpus endometrial carcinoma (UCEC) as well as COAD. Next, the expression level of EIF4E3 in colon cancer and normal tissues were analyzed by StarBase database. As shown in Fig. 1B, EIF4E3 was significantly upregulated in 471 cancer tissues compared to 41 normal tissues.

Clinicopathology and prognostic value of EIF4E3

In order to investigate the clinical value of dysregulated EIF4E3. The colorectal cancer data from TCGA was used to conduct our analysis. The patients' clinical classification information is shown in Table I. From the table, EIF4E3 expression was closely correlated with history of colon polyps ($p=0.044$) and lymphatic invasion ($p=0$). These suggested that EIF4E3 could be an indicator of colorectal cancer occurrence and development. Subsequently, Kaplan-Meier survival analysis of the colon dataset showed that EIF4E3 expression was significantly correlated with overall survival ($p=0.043$) (Fig. 2). Higher EIF4E3

expression was associated with better prognosis, and lower expression of EIF4E3 was associated with poor prognosis in colon cancer patients.

Biological function analysis of EIF4E3 in colon cancer

Next, GSEA was used to reveal the significantly enriched KEGG pathways associated with EIF4E3 expression. As shown in Fig. 3A, some pathways were found to be associated with immunity and inflammation, such as inflammatory bowel disease (Fig. 3B), intestinal immune network for IgA production (Fig. 3C), Toll-like receptor signaling pathway (Fig. 3D), T-cell receptor signaling pathway (Fig. 3E), Th17 cell differentiation (Fig. 3F) and chemokine signaling pathway (Fig. 3G). As known, immunity and inflammation constitute the basic features of the tumor microenvironment (25). These pathways may indicate that EIF4E3 was involved in the tumor microenvironment and thus affect the progression of colon cancer.

Correlation of EIF4E3 with tumor infiltrating immune cells

Subsequently, TIMER database was used to investigate the correlation between EIF4E3

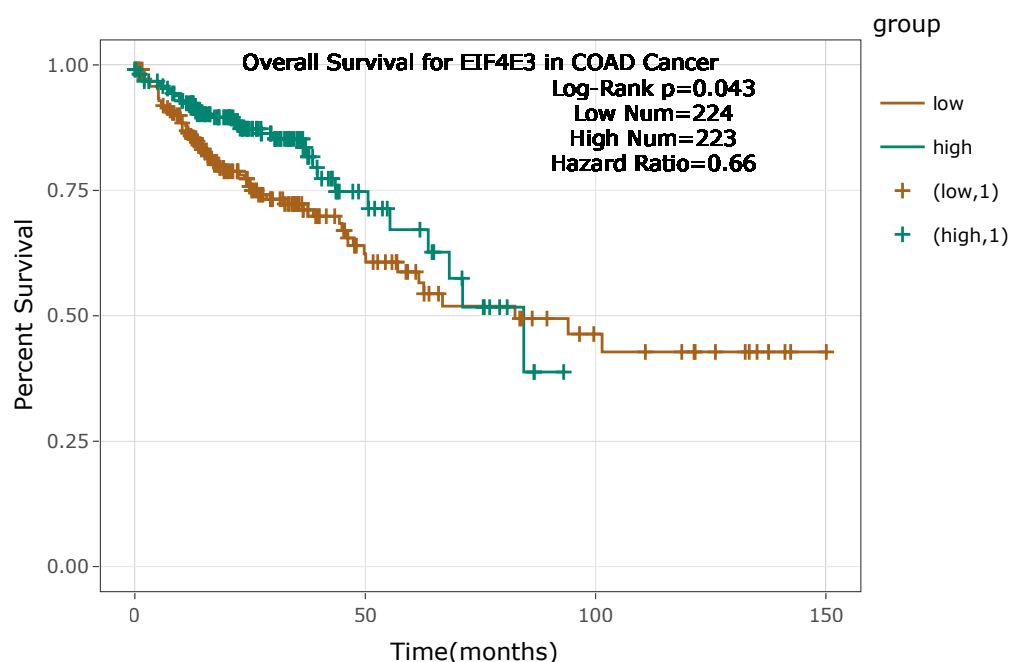


Fig. 2. The overall survival curve of differential EIF4E3 expression in COAD ($p=0.043$).

expression and tumor immune infiltrates in COAD. As shown in Fig. 4. The results showed that the expression of EIF4E3 in COAD was significantly related to CD4⁺ T cells, CD8⁺ T cells, myeloid dendritic cells and neutrophils. These results were consistent with the KEGG pathway analysis. CD4⁺ T cells play critical roles in mediating adaptive

immunity to cancers. During T cell receptor activation in a particular cytokine milieu, naive CD4⁺ T cells may differentiate into one of several kinds of T helper (Th) cells, including Th1, Th2, Th17, and iTreg (26). Th17 cells and regulatory T cells (Tregs) have emerged as a prominent factor in regulating cancer (27). In our analysis, we found that

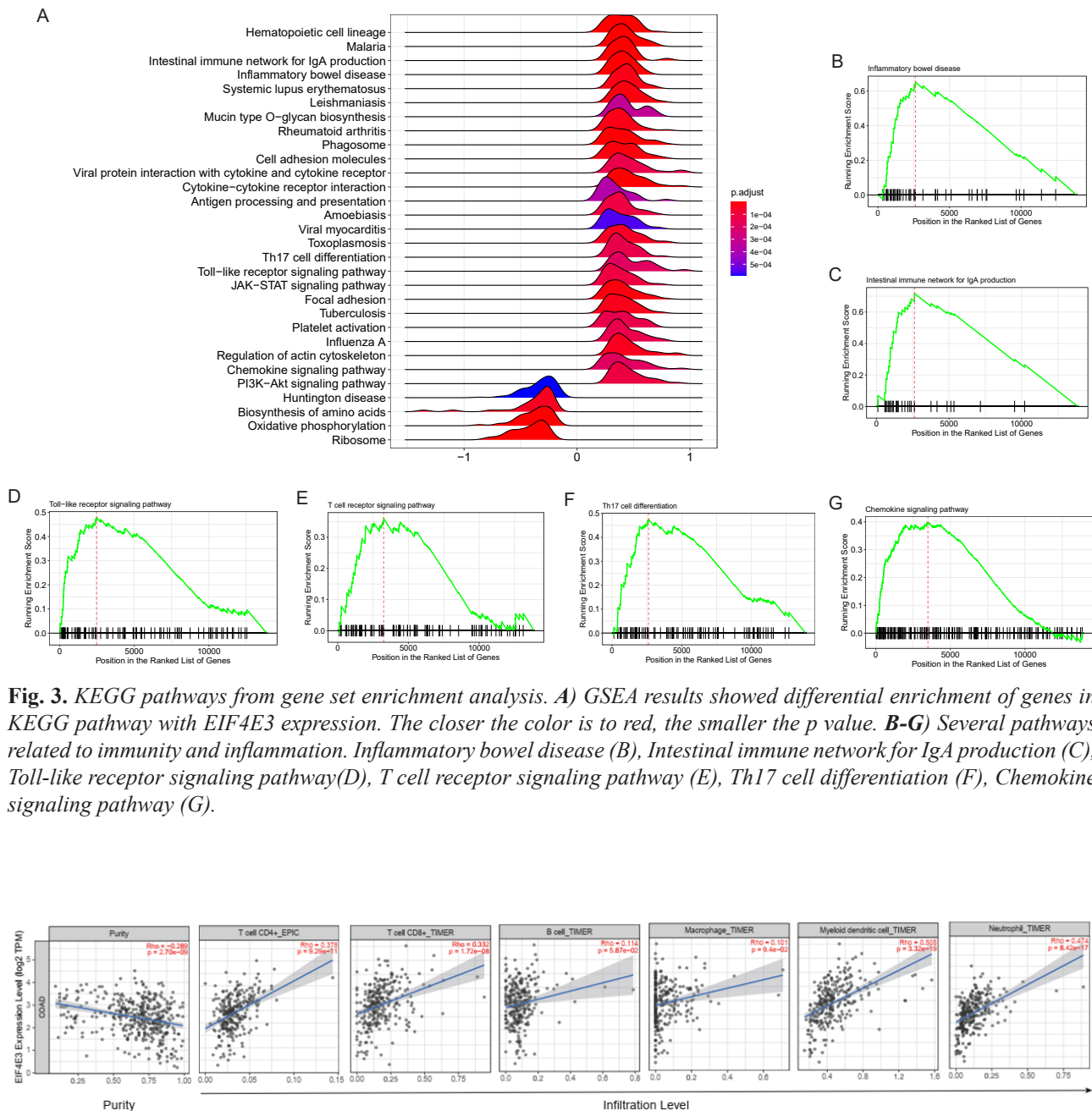


Fig. 4. Correlations between EIF4E3 expression and immune infiltration levels in COAD by TIMER web tool.

Th17 was significantly high expressed in adjacent tumor tissues (Fig. 5A). Meanwhile, higher Th17 cell expression was associated with better prognosis in COAD (Fig. 5B). Furthermore, the marker genes CD3D4, CD4 and STAT3 of Th17 cells were verified to highly correlate with the expression of EIF4E3, as shown in Fig. 5 C-E.

DISCUSSION

Colon cancer is the most common pathological type of colorectal cancer. Tumor metastasis is the main cause of poor outcome of colon cancer patients (28). Approximately 25% of colon cancer patients present with hepatic metastases on initial diagnosis, and about 50% will develop hepatic metastases within 3 years of the primary surgery (29). Thus,

it is necessary to find novel specific diagnostic biomarkers in early COAD (30, 31). Although many studies have reported possible diagnostic or therapeutic targets for colon cancer, they remain mostly theoretical. As a well-known heterogeneous disease, COAD still lacks specific diagnostic biomarkers for individualized therapy, especially in immunotherapy.

The eIF4E protein family is comprised of three components: eIF4E1, eIF4E2 and eIF4E3 (32), which are essential for the initiation of translation. Previous studies showed EIF4E family plays an important role in proliferation, survival (33), mRNA export and oncogenic transformation, and these functions are dependent on its cap-binding activity (34). The family is reported to be an oncogene elevated in an estimated 30% of human cancers (35). Among

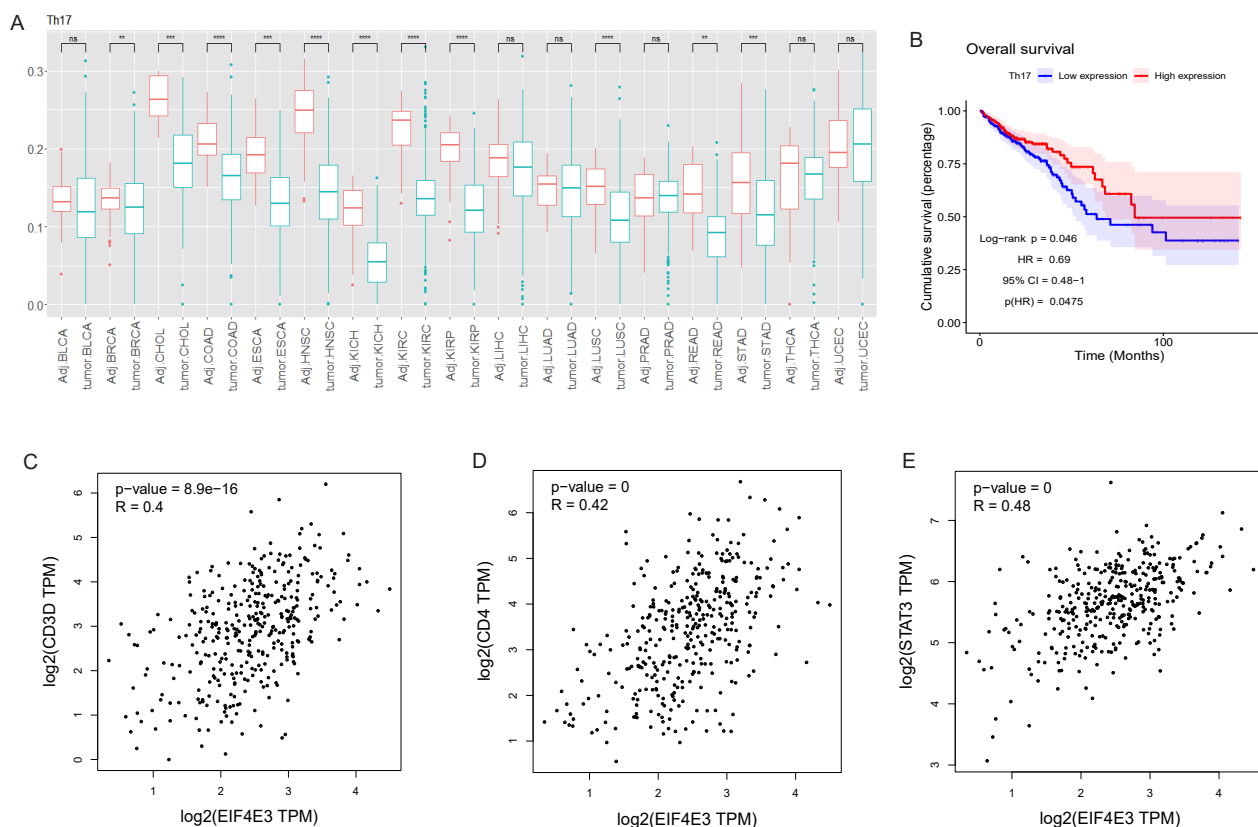


Fig. 5. EIF4E3 expression correlated with Th17 cells in COAD. **A)** The Th17 expression levels in different tumor types from TCGA database were determined by IMMUECELLAI database (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). **B)** Th17 cell content could be used as a prognostic factor. Higher Th17 cells expression was associated with better prognosis in COAD. **C-E)** The correlation between marker genes CD3D4, CD4 and STAT3 of Th17 cells, respectively with EIF4E3 expression.

the family members, the most commonly studied is EIF4E1 (36). However, several studies reported that EIF4E3 is a tumor suppressing factor, rather than a promoter, of both target transcript expression and oncogenic transformation (16, 37). The high expression of EIF4E3 marginally suppresses EIF4E1-driven translation, which exhibits a new role for EIF4E3 in translation initiation (17). These results match our findings in the COAD investigation. In this study, we found that EIF4E3 was downregulated

and high expression of EIF4E3 showed a better prognosis in COAD.

GSEA was then used to access the potential regulation pathways. We found that the role of EIF4E3 is diversified. Oxidative phosphorylation, cell focal adhesion, cytokine-cytokine receptor interaction and many other important biological processes were significantly changed. Among these, many inflammation and immune system-related pathway were dysregulated, such as inflammatory

Table I. *EIF4E3 expression level and clinical pathological factors in colorectal patients*

Characteristics	Case (No.)	No. of Patients	expression level		p value
			low (No. cases)	high(No. cases)	
Status	593				0.891
Alive		467(78.8)	234	233	
Deceased		126(21.2)	64	62	
Gender	593				0.832
Female		280(47.2)	142	138	
Male		313(52.8)	156	157	
Age	593				0.845
< 60		169(28.5)	86	83	
≥ 60		424(71.5)	212	212	
History of colon polyps	509				0.044
Yes		159(31.2)	93	66	
No		350(68.8)	171	179	
Lymph node examined count	565				0.068
Low		299(52.9)	140	159	
High		266(47.1)	145	121	
Lymphatic invasion	536				0
Yes		217(40.5)	128	89	
No		319(59.5)	134	185	
Recurrence	489				0.887
Yes		104(21.3)	47	57	
No		385(78.7)	177	208	
Non nodal tumor deposits	290				0.925

Yes		47(16.2)	16	31	
No		243(83.8)	81	162	
Pathologic M	520				0.538
M0		436(83.8)	228	208	
M1		84(16.2)	47	37	
Pathologic N	590				0.372
N0		333(56.4)	173	160	
N1-2		257(43.6)	124	133	
Pathologic T	591				0.065
T1-2		119(20.1)	69	50	
T3-4		472(79.9)	229	243	
Pathologic stage	574				0.706
I-II		316(55.1)	163	153	
III-IV		258(44.9)	129	129	
Preoperative pretreatment CEA level	382				0.617
Low		194(50.8)	91	103	
High		188(49.2)	93	95	
Shortest dimension	164				0.542
Low		83(50.6)	24	59	
High		81(49.4)	20	61	

P < 0.05 (red highlighted font) represents significant difference

bowel disease, intestinal immune network for IgA production, Th17 cell differentiation, Toll-like receptor signaling pathway and chemokine signaling pathway. The immune system recognizes and responds to tumor cells.

Tumor immunotherapy brings new hope for cancer treatment. As reported, immunotherapy improved the treatment and outcome in a variety of malignant diseases such as melanoma, and renal and lung cancer, but rarely responds to colon cancer cases (38). In order to study the immunotherapy potential of EIF4E3, we found that EIF4E3 expression was significantly related to CD4⁺ T cells, CD8⁺ T cells, myeloid dendritic cells and neutrophils. Also, Th17 differentiates from CD4⁺ T cells, and Th17 was

highly expression in COAD. Higher Th17 cells expression was associated with better prognosis in COAD. Moreover, the marker genes CD3D4, CD4 and STAT3 of Th17 cells were verified to have a high correlation with the expression of EIF4E3. These may indicate that EIF4E3 could regulate Th17 cells to be involved in immune infiltrates in COAD.

In conclusion, this study integrated bioinformatics to identify EIF4E3 whether could be a prognostic factor. EIF4E3 was correlated with tumor infiltrating immune cells. These findings can help researchers to find targets for immunotherapy and personalize treatment for patients with COAD. However, there are some limits to the study. The function of EIF4E3 in TME still needs to be verified by future experiments.

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BUB1B, negatively regulated by miR-486-5p, enhances the proliferation, migration and invasion of ovarian cancer cells

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BUB1 mitotic checkpoint serine/threonine kinase B (BUB1B) belongs to the spindle assembly checkpoint protein family and is linked to the pathogenesis of diverse human cancers. This work intends to probe its expression, function and underlying molecular mechanism in ovarian cancer (OC). In the present work, gene expression profiles of GSE14407, GSE18520, and GSE54388 were obtained from the GEO database and screened for differentially expressed genes (DEGs) in ovarian cancer tissues. KM-plotter database was employed to analyze the relationship between BUB1B expression and the prognosis of OC patients. Immunohistochemistry was utilized to examine BUB1B expression in OC tissues and adjacent ovarian tissues. BUB1B expression level in OC cells was also examined using qRT-PCR. The relationship between BUB1B expression and clinicopathological parameters in OC patients was analyzed using the *Chi*-squared test. CCK-8 and BrdU experiments were employed to examine cell proliferation. Cell migration and invasion were detected by Transwell experiment. Western blot, qRT-PCR and dual-luciferase reporter gene analyses were employed to validate the regulatory relationship between miR-486-5p and BUB1B. In this work, we demonstrated that BUB1B was remarkably overexpressed in OC specimens compared with adjacent non-tumor tissue, which was associated with higher FIGO stage and differentiation status of the patients. OC cell proliferation, migration and invasion were substantially enhanced by BUB1B overexpression; conversely, knocking down BUB1B worked the opposite effect. Mechanistically, BUB1B was one of the downstream targets of miR-486-5p and was negatively modulated by the latter. It was concluded that, BUB1B, negatively regulated by miR-486-5p, facilitates OC progression, which is a promising target for OC therapy.

Ovarian cancer (OC) is one of the most lethal malignancies in the female reproductive system (1). In 2018, there were 295,414 new cases of OC and 184,799 deaths worldwide (2). The current standard treatment for OC is surgery combined with chemotherapy, but recurrence occurs in around 70% of patients after treatment (3). Therefore, further study of the mechanisms of OC progression will help to find novel therapeutic targets.

BUB1 mitotic checkpoint serine/threonine kinase

B (BUB1B), one of the essential constituent proteins of the mitotic checkpoint, is a multi-domain protein kinase. BUB1B participates in the spindle assembly checkpoint (SAC) mechanism, which impedes anaphase-promoting complex/cyclosome (APC/C) activity and ensures proper chromosome segregation (4-6). BUB1B under-expression results in the failure to adequately form kinetochore-microtubule attachments (kinetochore-microtubules) and increases the rate of missegregation (7-9). This implies BUB1B

Key words: BUB1B; miR-486-5p; OC

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dysfunction is associated with genomic stability (10, 11). It is now well documented that BUB1B is overexpressed in diverse cancers, including brain tumors, bladder cancer, breast cancer, and gastric cancer (12–16). In the present study, bioinformatics indicates that BUB1B is significantly upregulated in OC, suggesting that BUB1B plays a cancer-promoting role in OC.

MicroRNA (miRNA) is a short-stranded non-coding RNA, usually 20–24nt in length (17). MiRNAs are implicated in multiple cellular activities by influencing the stability and translation processes of mRNAs (18). Many miRNAs are involved in regulating tumorigenesis and cancer progression (19–21), for instance, miR-125a represses the proliferation, migration, and invasion of cervical cancer cells by targeting STAT3 (20). MiR-345-5p inhibits pancreatic cancer progression by directly targeting CCL 8 to block the NF- κ B signaling pathway (21). MiR-486-5p is reported to suppress colorectal cancer growth and migration by blocking pleomorphic adenoma gene-like 2/insulin-like growth factor-2/ β -catenin signaling pathway (22). Nevertheless, the role of miR-486-5p in OC and its mechanism remain undefined.

In this work, BUB1B expression is unveiled to be up-modulated in OC, and its overexpression is remarkably linked to unfavorable clinicopathological parameters in patients. OC cell proliferation, migration and invasion are enhanced by BUB1B overexpression. Mechanistically, BUB1B is one of the downstream targets of miR-486-5p and is negatively modulated by the latter. This work is expected to provide novel therapeutic targets for OC patients.

MATERIALS AND METHODS

Microarray data information

Three OC datasets (GSE14407, GSE18520, and GSE54388) were selected from the Gene Expression Omnibus (GEO) database and screened using the criteria $|\log FC| > 2$, $P < 0.05$ to obtain differentially expressed genes (DEGs) in OC (23).

Clinical specimens

Thirty-one pairs of cancerous and paracancerous tissues from subjects with newly diagnosed OC were selected. None

of the patients had undergone radiotherapy or chemotherapy before sampling. All collected specimens were placed in liquid nitrogen for conservation. The subjects all signed an informed consent before tissue specimens were obtained, and this research was endorsed by the Ethics Committee of Renmin Hospital of Wuhan University.

Cell culture

The OC cell lines OVCAR-3, ES-2, OV-90 and the normal ovarian epithelial cell line IOSE80 were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The above cells were cultured in RPMI-1640 medium (Invitrogen, Shanghai, China) containing 10% fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences, Little Chalfont, UK), 100 IU/mL Penicillin G and 0.1 mg/mL streptomycin (Invitrogen, Shanghai, China) at 37°C in 5% CO₂.

Cell transfection

OC cells were trypsinized using trypsin (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and cell concentration was modulated to 1×10^5 cells / mL with serum-free medium, and planted in 6-well plates; 24 h later, BUB1B overexpression plasmid, small interfering RNA (siRNA) targeting BUB1B (si-BUB1B), miR-486-5p mimics and inhibitors were transfected into OC cell lines with Lipofectamine™ 2000 transfection reagent (Invitrogen, Shanghai, China) according to the manufacturer's instruction.

Immunohistochemical (IHC) staining

The tissue specimens in paraffin were sectioned, dewaxed and rehydrated. Next, the blocking solution was supplemented, and the tissues were blocked for 1 h at room temperature. Subsequently, in a wet box, the tissues were incubated with anti-BUB1B antibody (Abcam, ab54894, 1:100) at 4°C overnight. Subsequently, after the tissues were washed by phosphate buffer saline (PBS), the secondary antibody (Beyotime, Shanghai, 1:1000, China) was supplemented and the tissues were incubated at 37°C for 1 h in the wet box. After the tissues were washed by PBS, DAB reaction (Biossci Biotechnology Co., Ltd., Wuhan, China) was added onto the sections to develop the color, and the staining of the samples was evaluated by two independent pathologists.

Western blot

The cells were lysed by RIPA lysis buffer (Beyotime, Shanghai, China) for 30 min and centrifuged (12,000 r/min, 30 min). The supernatant was used as the protein sample. The protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China), and the protein was denatured by boiling for 5 min. After electrophoresis, the protein was transferred to NC membranes and then blocked with 5% skim milk for 2 h. Next, primary anti-BUB1B antibody (Abcam, ab254326, 1:500) was supplemented and the membrane was incubated at 4°C overnight. Subsequently, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG H&L (Abcam, ab150077, 1:2000) was supplemented and the membrane was incubated for 2 h. After the membrane was washed by tris buffered saline tween (TBST), the enhanced chemiluminescent reagent (Biozym, Hessisch Oldendorf, Germany) was used for the development of protein bands. GAPDH was considered as the internal reference.

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

Total RNA was extracted using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China). Reverse transcription was conducted with MMLV reverse transcriptase (Takara, Dalian, China) to produce cDNA. With the cDNA as template, according to the manufacturer's instruction, the SYBR-Green Master Mix kit (Takara, Dalian, China) was used to amplify the target genes on ABI 7900 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA). BUB1B and miR-486-5p expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method. GAPDH and U6 served as the internal references. The primer sequences are exhibited in Table I.

CCK-8 analysis

OC cells were transferred into 96-well plates (1×10^3 cells/well, 100 μ L cell suspension/well) and cultured for 12 h, 24 h, 48 h, 72 h, and 96 h, respectively. At each time point, 10 μ L of CCK-8 reagent (Dojindo Molecular Technologies, Japan) was supplemented to each well, and incubation was continued at 37°C for 2 h before termination. After that, the optical density (D value) at 450 nm was determined by a microplate reader.

BrdU analysis

OC cells were inoculated into 96-well plates. After

the cells were cultured with serum-free medium for 24 h, fresh completed medium was added, and the cells were incubated with 10 μ M BrdU solution (Beyotime, Shanghai, China) at 37°C for 30 min and then rinsed with PBS. Next, the cells were fixed with paraformaldehyde. Then 200 μ L of glycine at a concentration of 2 mg/mL was supplemented, and the cells were incubated for 5 min, and rinsed with PBS for 5 min on a shaker. Subsequently, 100 μ L of PBS containing 0.5% Triton X-100 was added into each well, and the cells were washed twice with PBS for 5 min each time. The cells were then treated with 2M HCL for 30 min, and washed with PBS 3 times. Next, the cells were incubated in the dark for 20 min at room temperature with anti-BrdU antibody (Beyotime, Shanghai, China, 1:1000), after which the cells were rinsed with PBS, and the nuclei were counterstained with DAPI staining solution in the dark for 30 min. Finally, the cells were observed, photographed and counted under a fluorescence microscope.

Transwell analysis

For cell migration, OC cells were suspended in serum-free medium, and transferred into the upper compartment (1×10^5 cells/well) of Transwell system (8 μ m pore size, Costar, Cambridge, MA, USA) and 500 μ L of RPMI-1640 containing 10% FBS was added into the lower

Table I. Primer sequence of qRT PCR

Name		Primer sequences
BUB1B	Forward	5'-AAATGACCTCTGGATGTTTG-3'
	Reverse	5'-GCATAAACGCCCTAATTTAAGCC-3'
GAPDH	Forward	5'-AGCCACATCGCTCAGACAC-3'
	Reverse	5'-GCCCAATACGACCAATCC-3'
miR-486-5p	Reverse transcription primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCGGG-3'
	Forward	5'-TCCTGTACTGAGCTGCCC-3'
	universal R	5'-GTGCAGGGTCCGAGGT-3'
U6	Reverse transcription primer	5'-GCTTCACGAATTTGCGTGTTCAT-3'
	Forward	5'-GCTTCGGCAGCACATATACTAAA-3'
	Reverse	5'-GCTTCACGAATTTGCGTGTTCAT-3'

Table II. Relationship between BUB1B expression level and clinical indicators

Tumor characteristics		BUB1B expression		P-value
		High (n=23)	Low (n=8)	
Age (years)	≥ 55	14	4	0.6894
	<55	9	4	
FIGO stage	I-II	5	6	0.0120
	III-IV	18	2	
Differentiation	Well	4	5	0.0272
	Poorly	19	3	
T	T0-T2	6	5	0.0947
	T3	17	3	
TNM stage	N	9	6	0.1134
	Present	14	2	
M	Absent	15	7	0.3791
	Present	8	1	

compartment. After 24 h of incubation, the cells on the upper surface of the membrane were removed with a cotton swab, and the cells on the surface below the membrane were fixed with methanol and stained with crystal violet solution. Five fields of view were randomly selected under an inverted microscope to count the number of cells. For cell invasion analysis, OC cells were transferred into the upper compartment of the Transwell system after the membrane was coated with a layer of Matrigel.

Dual-luciferase reporter

The dual-luciferase reporter assay system (Promega, Madison, WI, USA) was employed to perform the dual-luciferase reporter assay. The binding site of miR-486-5p on BUB1B was predicted by bioinformatics, and the binding fragment was amplified by PCR. The amplified fragment was then inserted into the pmiR-GLO report vector to construct the BUB1B wild-type plasmid (BUB1B-WT). Mutagenesis was adopted to mutate some of the nucleotides to construct a BUB1B mutant-type plasmid (BUB1B-MT). The OC cell lines were transfected with miR-486-5p mimics + BUB1B-WT, miR-486-5p mimics + BUB1B-MT, control miRNA mimics + BUB1B-WT, control miRNA mimics + BUB1B-MT, and the luciferase activity was determined 48 h post-transfection.

Statistical analysis

SPSS 20.0 software was applied for statistical analysis, and GraphPad Prism 8.0.1 software was employed to plot the figures. Measurement data were expressed as ($\bar{x} \pm s$), and comparisons between two groups were conducted using *t*-tests, and among multiple groups were conducted using one-way ANOVA. $P < 0.05$ signified statistical significance.

RESULTS

Remarkable up-modulation of BUB1B was observed in OC

Microarray data from GSE14407, GSE18520, and GSE54388, were downloaded from the GEO database, and the up-modulated DEGs were obtained with $\log FC > 2$, $P < 0.05$. Venn diagram showed that 119 gene expressions in OC were remarkably augmented, including BUB1B (Fig. 1A). Thirty-one cases of OC tissue and their corresponding paracancerous tissues were collected. Representative images of IHC are displayed in Fig. 1B, and statistical analysis revealed that BUB1B expression was markedly higher in OC tissues than in paracancerous tissues (Fig. 1C). The data of qRT-PCR revealed that there was a remarkable elevation of BUB1B in OC tissue expression relative to paracancerous normal tissues (Fig. 1D). Additionally, Western blot and qRT-PCR data showed that the expression of BUB1B was remarkably higher in the 3 OC cell lines relative to IOSE80 cells (Fig. 1 E, F).

BUB1B overexpression was strongly linked to unfavorable clinical parameters and prognosis in OC patients

Analysis of the relationship between BUB1B expression status and clinical parameters in 31 OC patients, revealed that BUB1B overexpression was strongly linked to a higher FIGO stage and poor differentiation (Table II). Moreover, KM-plotter database showed that OC patients with high levels of BUB1B had remarkably shorter overall survival (Fig. 2A), progression-free survival (Fig. 2B), and post-progression survival (Fig. 2C). The findings indicated BUB1B participated in facilitating OC progression.

BUB1B enhanced the proliferation, migration and invasion of OC cells

To probe the role of BUB1B in OC cells,

OVCAR-3 and ES-2 cells were transfected with BUB1B overexpression plasmid and si-BUB1B, respectively. Western blot analysis validated the successful transfection (Fig. 3A). CCK-8 and BrdU experiments suggested that BUB1B overexpression remarkably enhanced OC cell viability relative to the control group (Fig. 3 B-D). The data of Transwell experiments revealed that the up-modulation of BUB1B markedly facilitated the migration and invasion of OC cells (Fig. 3 E, F). Conversely, knocking down BUB1B remarkably suppressed these malignant features of OC cells relative to the control group (Fig. 3 B-F).

BUB1B was a downstream target of miR-486-5p

Four databases, miRDB, TargetScan, miRWalk, and ENCORI, were employed to predict the upstream modulators of BUB1B, and miR-486-

5p was predicted to be one of the upstream targets of BUB1B (Fig. 4A). The potential binding sites between them are shown in Fig. 4B. The data of qRT-PCR and Western blot suggest that miR-486-5p negatively modulated BUB1B expression at the mRNA and protein levels (Fig. 4 C, D). Dual-luciferase reporter gene experiment suggested that miR-486-5p remarkably impeded the reporter gene activity of BUB1B-WT, but the activity of BUB1B-MT was unchanged (Fig. 4E). Pearson analysis implied a remarkable negative correlation between miR-486-5p expression and BUB1B expression in OC tissues (Fig. 4F). The findings indicated that BUB1B was a downstream target of miR-486-5p.

MiR-486-5p impeded the proliferation, migration and invasion of OC cells

qRT-PCR showed that miR-486-5p expression

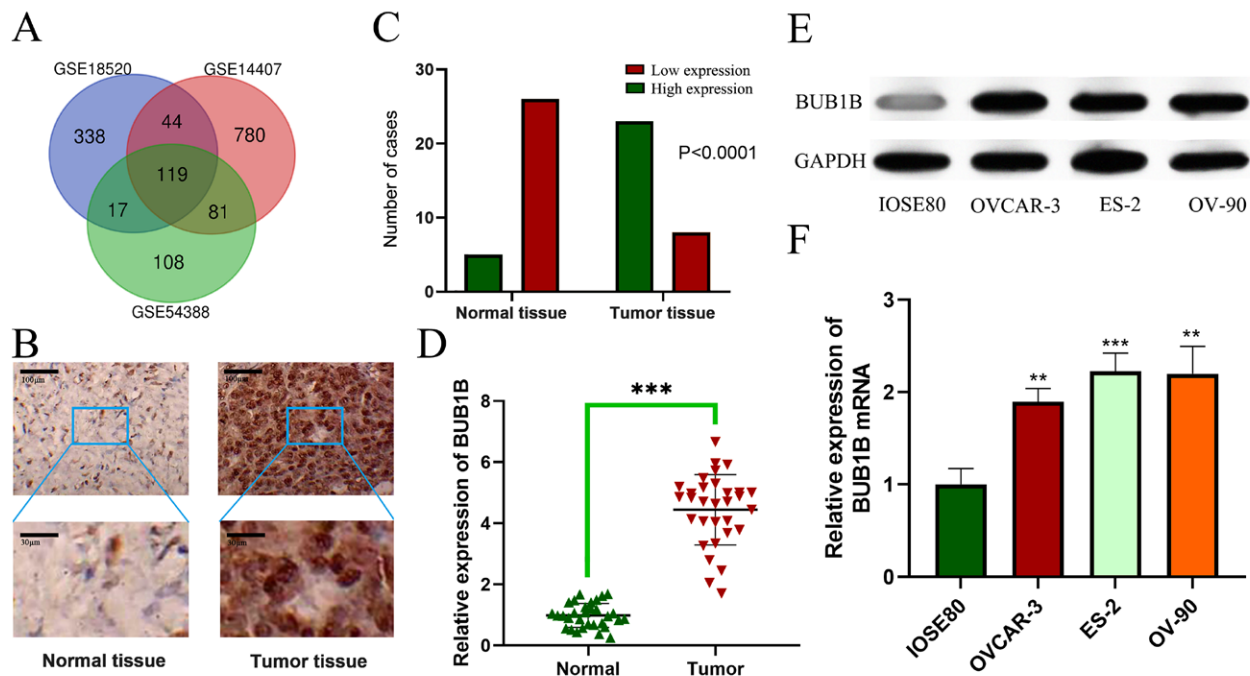


Fig 1. *BUB1B was overexpressed in ovarian cancer. A)* The DEGs in ovarian cancer tissues were obtained from gene expression profiles of GSE14407, GSE18520, and GSE54388. *B)* Immunohistochemistry was employed to examine BUB1B expression in ovarian cancer tissues and paracancerous tissues. *C)* BUB1B protein expression level in ovarian cancer tissues and adjacent ovarian tissues were compared. *D)* BUB1B mRNA expression in cancerous and paracancerous tissues of 31 ovarian cancer patients was analyzed by qRT-PCR. *E)* Western blot was applied to detect BUB1B expression in ovarian cancer cell lines (OVCAR-3, ES-2, and OV-90) and normal ovarian epithelial cell line (IOSE-80). *F)* BUB1B mRNA expression in the ovarian cancer cell lines OVCAR-3, ES-2, OV-90 and normal ovarian epithelial cell line IOSE-80 was determined by qRT-PCR. ** $P < 0.01$; *** $P < 0.001$.

in OC tissues was markedly lower than that in paracancerous tissues (Fig. 5A). Additionally, miR-486-5p expression was remarkably down-modulated in 3 OC cells compared with IOSE80 cells (Fig. 5B). Next, miR-486-5p mimics and inhibitors were transfected into ES-2 and OVCAR-3

cells, respectively (Fig. 5C). The data of CCK-8 and BrdU experiments suggested that miR-486-5p overexpression markedly repressed the proliferation of OC cells compared with the control group (Fig. 5 D, E). The data of Transwell analysis revealed that miR-486-5p overexpression remarkably reduced

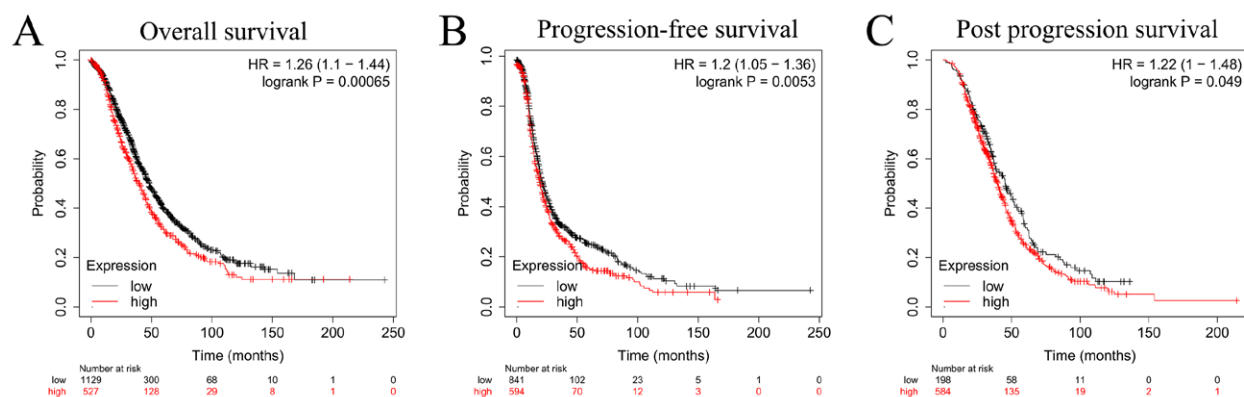


Fig 2. *BUB1B* overexpression predicted adverse prognosis in ovarian cancer patients. The relationship between *BUB1B* expression and ovarian cancer patients' (A) overall survival, (B) progression-free survival, and (C) post-progression survival was analyzed by the KM-plotter database.

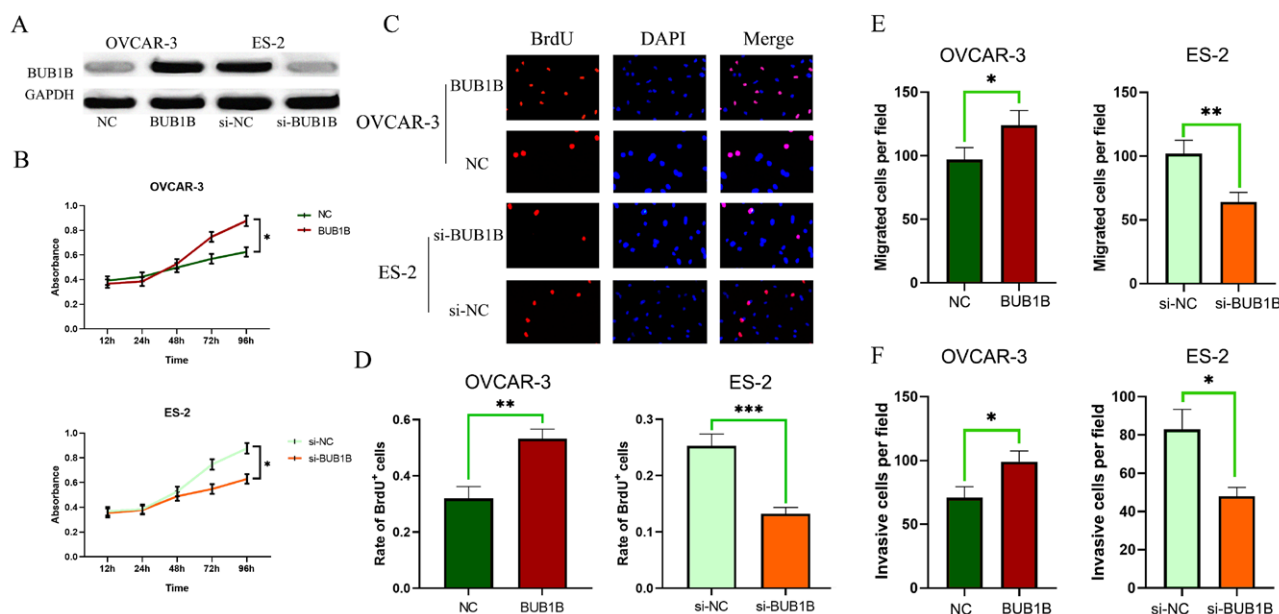


Fig 3. *BUB1B* enhanced the proliferation, migration and invasion of ovarian cancer cell lines. A) Western blot was adopted to determine the transfection efficacy of *BUB1B* overexpression plasmids and siRNA in ovarian cancer cell lines. B-D) Cell proliferation was detected by CCK-8 and BrdU assays after the transfection. E, F) Transwell was utilized to examine cell migration and invasion after the transfection.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the migration and invasion of OC cells (Fig. 5 F, G). Nonetheless, transfection with miR-486-5p inhibitors markedly enhanced the proliferation, migration and invasion of OC cells relative to the control group.

DISCUSSION

Reportedly, BUB1B is overexpressed in diverse cancers (12-16). BUB1B acts as a core component of SAC in normal tissues in humans by impeding late APC/C formation to ensure proper chromosome segregation and suppressing chromosome aneuploidy (6, 24). BUB1B overexpression enhances cancer cell viability and metastasis and is associated with cancer

progression and recurrence, implying that BUB1B is oncogenic in cancers (25-30). In this work, we report that, BUB1B, negatively modulated by miR-486-5p, enhances OC cell growth, migration and invasion.

Chromosomal aneuploidy is now recognized as an important feature of tumor cells, and modern assays have revealed the prevalence of aneuploidy in cancer (31). Aneuploidy is recognized as a dynamic role in cancer development and progression that is dependent on the environment in which it occurs, and the degree of chromosomal aneuploidy and specific karyotypes also make a difference in the prognosis of certain cancers (32). When cancer cells migrate and invade, they often depend on a specific karyotype (33-35). It is well documented that

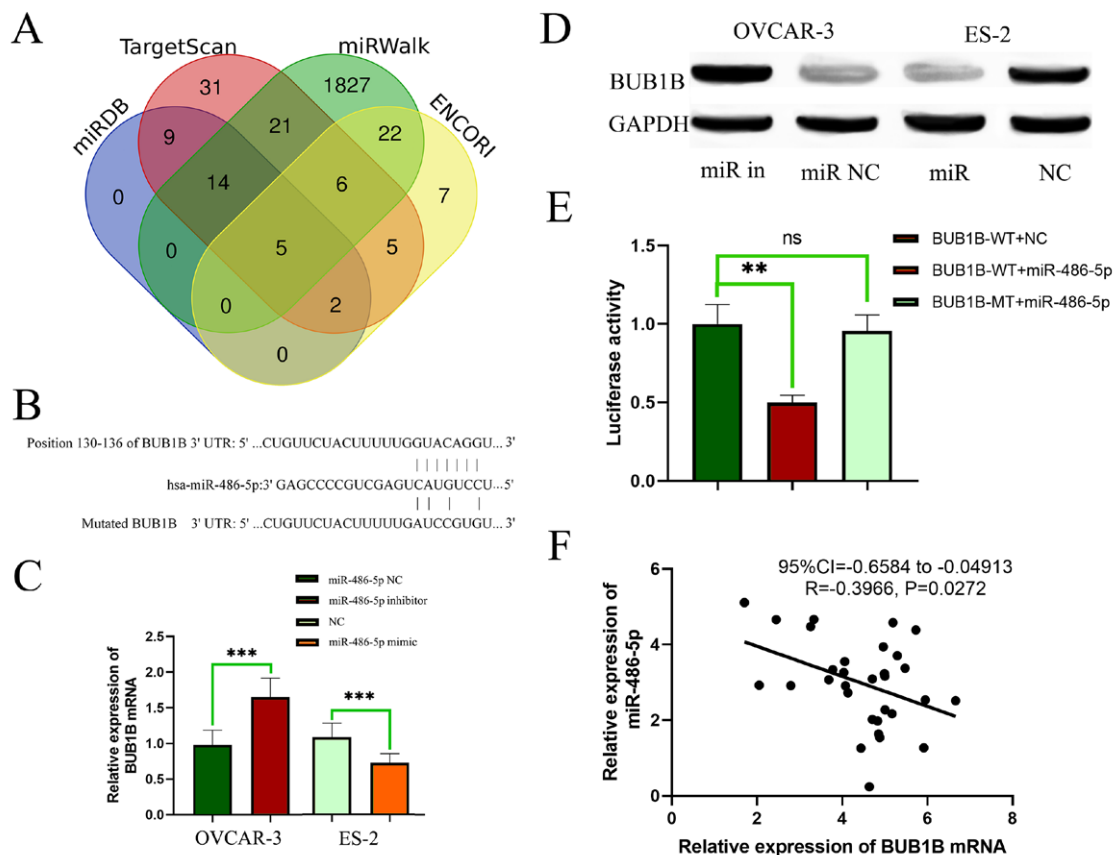


Fig. 4. BUB1B expression in ovarian cancer was modulated by miR-486-5p. **A)** Four online databases, miRDB, TargetScan, miRWalk, and ENCORI, were employed to predict the upstream miRNAs of BUB1B. **B)** Potential binding sites between miR-486-5p and BUB1B 3'UTR. **C, D)** BUB1B expression at mRNA and protein levels in ES-2 cells transfected with miR-486-5p mimics and OVCAR-3 cells transfected with miR-486-5p inhibitors was detected using qRT-PCR and Western blot, respectively. **E)** The dual-luciferase reporter experiment verified that miR-486-5p could bind to the 3'UTR of BUB1B. **F)** Correlation analysis of miR-486-5p expression and BUB1B mRNA expression in ovarian cancer tissues of 31 patients. ** $P < 0.01$; *** $P < 0.001$.

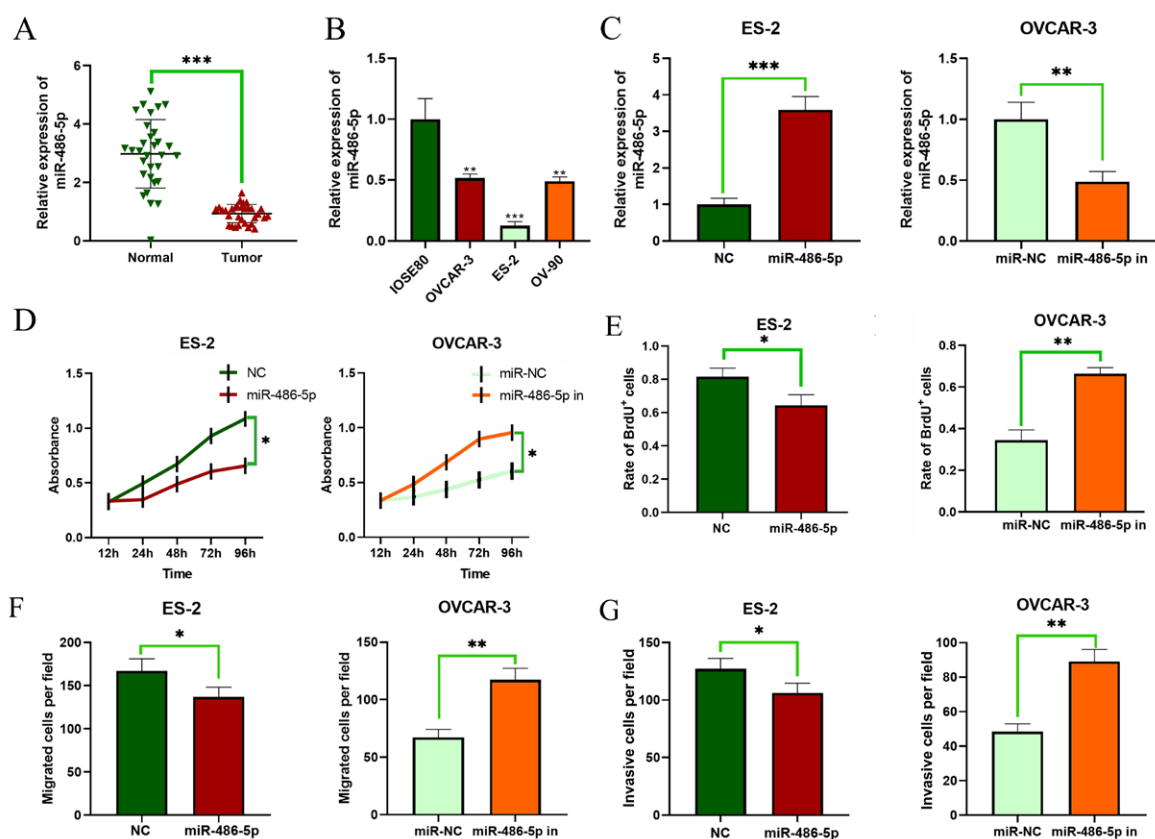


Fig. 5. miR-486-5p impeded the ovarian cancer malignant phenotype. **A)** miR-486-5p expression in cancerous and paracancerous tissues of 31 ovarian cancer patients was analyzed by qRT-PCR. **B)** miR-486-5p expression in the ovarian cancer cell line and the normal ovarian epithelial tissue cell line IOSE80 was analyzed by qRT-PCR. **C)** ES-2 cells were transfected with miR-486-5p mimics, and OVCAR-3 cells were transfected with miR-486-5p inhibitors, and the transfection efficacy was measured using qRT-PCR. **D, E)** The proliferation rates of OVCAR-3 cells transfected with miR-486-5p mimics and ES-2 cells transfected with miR-486-5p inhibitors were detected by CCK-8 and BrdU assays. **F, G)** Transwell experiments were executed to detect cell migration and invasion. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

aneuploidy in cancer tissues is driven by genes that control cell multiplication: gain of chromosomes is associated with proliferation-promoting genes, whereas chromosomes loss is associated with the genes that impede the proliferation (36). Reportedly, BUB1B exhibits an oncogenic role in some cancers. In prostate cancer, BUB1B enhances cancer cell proliferation by specifically modulating maternal embryonic leucine zipper kinase (25). BUB1B also enhances multiple myeloma cell proliferation by modulating the CDC20/CCNB axis (27). In this work, BUB1B is revealed to be more highly expressed in OC tissues than in paracancerous tissues and predicts an adverse prognosis of OC patients. *In vitro* experiments suggests that BUB1B overexpression

facilitates the proliferation and metastatic potential of OC cells. These data suggest that BUB1B participates in promoting OC progression, and is a potential therapy target for OC.

MiRNA can suppress multiple target genes, which are associated with tumorigenesis and cancer progression, and they are regarded as promising diagnostic biomarker and therapy targets. Accumulating studies show that miR-486-5p exerts tumor-suppressive effects in diverse cancers through regulating multiple pathways. For instance, miR-486-5p can inhibit cancer cell proliferation and migration in colorectal cancer, hepatocellular cancer, and non-small cell lung cancer by targeting Phosphoinositide-3-kinase regulatory subunit 1

(37-39). In breast cancer, miR-486-5p suppresses interleukin-22-induced epithelial-mesenchymal transition of cancer cells via modulating Dock1/NF- κ B/Snail pathway (40). In this work, miR-486-5p was found to be underexpressed in OC tissues compared to paracancerous tissues, and transfection of miR-486-5p mimics remarkably suppressed OC cell proliferation, migration and invasion *in vitro*. These data support that miR-486-5p is also a tumor suppressor in OC. Additionally, our study identifies BUB1B is a downstream target of miR-486-5p in OC cells, which partly explains the mechanism of BUB1B dysregulation in OC.

In conclusion, this work clarifies the expression characteristics and biological functions of BUB1B and miR-486-3p in OC, and deciphers the regulatory relationship between them. This study extends the understanding of OC progression and is promising to provide novel therapeutic targets for the clinical treatment of OC. Nonetheless, the present research is limited to *in-vitro* experiments, and we need to validate our findings with animal experiments in our following work. Additionally, further investigation is required to explore the detailed downstream mechanism by which BUB1B exerts its oncogenic effects.

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Four metastasis associated gene signatures for predicting the prognosis of osteosarcoma patients

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Osteosarcoma (OS) is a rare malignant tumor derived from bone cells. Many studies have reported that metastatic OS is associated with a poorer prognosis. However, there are no current effective methods of predicting survival. We applied gene expression data of OS from Gene Expression Omnibus (GEO) and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) to identify hub genes and construct a risk model with the help of R software. We then verified the identified signature using GEO data. Then we explored the potential relationship between the risk score and immune microenvironment in OS. After a series of analyses, we identified four hub-genes (*APBB1IP*, *UHRF2*, *PDK1* and *CORT*). Hub genes were then used to construct a model using a training data set. The model was subsequently tested using a validation data set, and performed well in sensitivity and specificity. In the immune analysis, we found the high-risk score might represent immunosuppression in OS. The identified hub genes may play a critical role in the mechanism of the metastatic OS. Moreover, the model we built was related to immunosuppression in OS. Thus, the gene signature identified could be a potential target for future clinical treatment of OS.

Osteosarcoma (OS) is a common type of sarcoma usually affecting children and teenagers. Like other sarcomas, OS originates from malignant mesenchymal cells. OS creates an osteoid matrix and fibrillary stroma, which facilitates metastasis and invasion. In most cases, OS occurs at the bones of the extremities, including the femur, tibia, fibula, and humerus. Song et al. (1) reported that OS incidence has risen 0.3% every year over the last decade. Although there are a range of treatments available for, the efficacy of these therapies are limited. . The 5-year survival rate for patients with non-metastatic

OS is 60%–70% (2, 3). There is therefore a significant difference in survival time between patients with non-metastatic and those with the metastatic OS.

To date, the mechanism of OS metastasis has not been fully elucidated. Several recent reports highlight the potential roles of molecules such as BHLH Transcription Factor (MYC) in OS metastasis(4-6). Nevertheless, there is currently no direct benefits for clinicians or patients to assess clinical outcome. Dong et al. (7) constructed a risk model, including eight genes, to predict OS metastasis, however, they ignored the prognosis, which is the primary

Key words: osteosarcoma; gene signature; prognosis; immune microenvironment

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concern of doctors. Overall, the clinical application of fundamental research results on OS is limited. Mutations or changes in the expression of single genes cannot be used to predict the prognosis of OS or guide the treatment of OS perfectly. Therefore, there is a need to discover new gene signatures or molecules associated with OS metastasis combined with clinical phenotypes to assess and predict patient prognosis accurately.

We used Gene Expression Omnibus (GEO) and Therapeutically Applicable Research to Generate Effective Treatments (TARGET) data, including mRNA expression matrix and clinical sample information. We independently analyzed the data to ensure the reliability of the results. Genes associated with OS metastasis were identified through GSE33382, GSE21257, and GSE32981. Then, clinical information from TARGET was used to identify survival-associated genes and establish a gene signature. Finally, we used both microarray data and clinical information from the GSE21257 to test the model's validity.

MATERIALS AND METHODS

Data

Data were obtained from the GEO (<http://www.ncbi.nlm.nih.gov>), a public database for genomics and high-throughput gene expression data submitted by the research community, and TARGET (<http://ocg.cancer.gov/programs/target>), a public database including clinical information and gene expression data from young adults and teenagers suffering from OS.

GSE33382, GSE21257, and GSE32981 were used to identify differentially expressed genes (DEGs). Gene expression data from TARGET were used to filter the DEGs and construct a gene signature. GSE21257 was used again to validate the genetic signature. We transformed the expression profile (FPKM values) of TARGET-OS datasets into TPMs (transcripts per kilobase million), which was identical to the results from microarrays (8).

Normalization of expression data

To deal with each GEO database's limited scale focused on OS, we applied the three datasets (GSE33382, GSE21257, and GSE32981) to identify the DEGs.

Therefore, we were unable to combine data from different databases directly. We used R 3.6.3 software with "SVA" packages to normalize the databases (9, 10).

Identification of DEGs

After normalization, the data from the different datasets were merged. Then, the R software limma package was used to identify DEGs. Non-metastatic OS and metastatic OS groups were compared. The $\text{adj.P.Value} < 0.05$ was used to correct false-positive results. A DEG heatmap was constructed using the R software package "heatmap".

Survival analysis

TARGET contained clinical information, including gene expression and survival time and was used as survival analysis. In the OS group, 86 samples had intact prognosis information. We downloaded the data matrix and clinical files for these 86 samples to verify a correlation between the GEO DEGs expression levels and survival time. Survival analysis was performed using a survival package in R software. $P < 0.05$ was used as the cut-off criterion.

Least absolute shrinkage and selection operator (LASSO) regression

To avoid over-fitting in the genetic signature, we simplified the signature. We performed LASSO regression on the DEGs that passed the survival analysis using the R package "glmnet". The λ parameter defined the complexity of the model. Λ was defined as the penalty regularisation and was determined at minimum partial likelihood deviance.

Construction and evaluation of metastasis-associated genetic signature

Hub genes were defined as the genes which remained after LASSO regression. We performed multivariate Cox analysis by combining hub gene expressions and survival times. We then established a risk model using the risk score to predict patient prognosis based on the Cox results. We also built a nomogram reflecting the prediction function of the risk model and clinical factors.

To validate the model, the OS patients' risk score was calculated in TARGET dataset and divided into high- and low-risk groups by the cut-off of the median of all risk scores. KM survival plots were generated to evaluate the prognostic value and receiver operating characteristic

(ROC) curves with different cut-off times to evaluate predictive efficiency.

Validation of the metastasis-associated genetic signature

The risk scores in the 32 OS cases from datasets in GSE21257 were calculated and divided into high- and low-risk groups as described above. KM plots and ROC curves were generated to test the risk model's prognostic value and predictive efficiency.

Gene set enrichment analysis (GSEA)

GSEA of TARGET and GSE21257 was performed using GSEA_4.0.3 software. This approach allows to explore the potential pathways associated with metastatic OS.

Generation risk-score group related gene cluster

DEGs among the high-risk and low-risk groups were defined with the cut-off that $|\text{fold-change}| > 2$ and FDR (adjusted p) < 0.05 . We made use of the “limma” R package for the DEGs.

We initially selected the unsupervised clustering “Pam” method to categorize the patients from TARGET and GSE21257 as per the DEG values through the “ConsensuClusterPlus” R package. The patients were clustered into different group clusters. In the next step, to estimate the level of immune and stromal contents, we took use of R packages “CIBERSORT” and “ESTIMATE” again. For further exploration of the influence of the immune microenvironment to prognosis. We performed KM plots for the clusters.

Consensus clustering for tumor-infiltrating immune cells

In analyzing the OS immune cells, we applied

Table II. *Multivariate cox regression analysis for hub-genes*

Gene ID	coef	exp(coef)	se(coef)	p-value
APBB1IP	-0.31535	0.729536	0.144019	0.028552
UHRF2	0.657972	1.930873	0.24753	0.007857
PDK1	0.326393	1.385959	0.152662	0.032516
CORT	0.420578	1.522841	0.113633	0.000215

“CIBERSORT” for infiltration levels for different immune cells in OS and “ESTIMATE” R packages to evaluate the immune and stromal contents. To ensure the stability of the results, we repeated the immune analysis process for 1000 times. Moreover, we performed the immune difference analysis between the low-risk group and the high-risk group. Moreover, we explored the internal relation between tumor-infiltrating immune cells.

A flowchart is shown in Fig. 1 to help better understand the complete analyses steps.

RESULTS

Datasets

We applied 3 GEO datasets (GSE33382, GSE21257, and GSE32981) for screening DEGs, and gene profiles and clinical data from TARGET were applied for the establishment of gene signatures. The baseline information is shown in Table I.

Data normalization avoid data artifacts caused by quantile normalization of low count genes

For increasing the sample volume, we matched the 3 expression matrixes. It is necessary to remove the batch differences before the data merge. A bar plot was drawn for genes expression removing the

Table I. *Baseline information of the included datasets*

Dataset		cases		deceased	p-value	female	p-value	mean	ages	p-value	mean	grade				p-value
								(years)			survival time (years)	I	II	III	IV	
GSE32981	metastasis	18	NR			8	p>0.05	21.44±9.69		p>0.05	NR	NR				
	Non-metastasis	4				2	(Fisher)	23.50±17.86		(Wilcoxon test)						
GSE33382	metastasis	33	NR			9	p<0.05	17.68±11.96		p>0.05	NR	11	9	7	3	p>0.05
	Non-metastasis	19				9	(Fisher)	19.44±12.24		(Wilcoxon test)		2	7	6	2	(Wilcoxon test)
GSE21257	metastasis	21	14		p<0.05 (Fisher)	5	p>0.05 (Fisher)	18.66±14.46		p>0.05 (Wilcoxon test)	NR	6	7	4	2	p>0.05 (Wilcoxon test)
	Non-metastasis	11	0			4		14.11±5.10				1	4	4	1	
TARGET	metastasis	21	14		p<0.05	12	p<0.05	14.21±3.87		p>0.05	NR	NR				
	Non-metastasis	65	15		(chi-square test)	15	(chi-square test)	15.47±5.20		(Wilcoxon test)						

batch differences. The gene expression baseline level significantly differed between databases using different platforms (Fig. 2A). After normalization, the gene expression baseline levels were roughly equal (Fig. 2B), making the data suitable for integration.

554 DEGs between metastatic and non-metastatic OS were identified

There were 554 DEGs in total, including 244 up-regulated genes and 310 down-regulated genes. We generated a heatmap to intuitively display the DEGs and their expression (Fig. 3A).

118 DEGs were considered with independent prognostic significance

We performed survival and univariate Cox analyses using gene expression data and clinical files in

TARGET. There were 229 genes associated with survival ($p < 0.05$), 118 of which passed univariate Cox analysis ($p < 0.05$). This means that these genes are associated with survival time and could be treated as independent factors to predict survival in univariate Cox analysis.

4 hub genes passed LASSO regression

LASSO regression was used to reduce the 118 genes identified through survival and univariate Cox analysis to be more useful for a model, and after LASSO regression with the partial likelihood deviance at a minimum resulted in only four genes - Amyloid Beta Precursor Protein Binding Family B Member 1 Interacting Protein (*APBB1IP*), Ubiquitin Like With PHD And Ring Finger Domains 2 (*UHRF2*), Pyruvate Dehydrogenase Kinase 1 (*PDK1*), and Cortistatin (*CORT*) - remaining (Fig. 3 B, C). The remained genes all showed a significant difference in the survival analysis (Fig. 4).

Multivariate Cox regression analysis and construction of a risk score signature

APBB1IP, *UHRF2*, *PDK1*, and *CORT* were used in multivariate Cox regression analysis to construct a risk signature. Multivariate Cox regression was used to weed out inappropriate genes and appropriate genes to function as best predictors for the model. The multivariate Cox regression analysis dropped no genes thus all four genes were defined as hub-genes. A risk score was calculated as hub-gene expression multiplied by the multivariate Cox regression coefficient (Table II). In our signature: Risk Score = $APBB1IP \times (-0.315) + UHRF2 \times 0.658 + PDK1 \times 0.326 + CORT \times 0.421$

Evaluation and validation of the four-gene signature

We calculated each patient's risk score with OS from the TARGET and GSE21257 data sets and divided them into high- and low-risk groups accordingly (Fig. 5 A, B). A significant difference was observed between the risk scores of patients with metastatic and non-metastatic OS. We compared hub-gene expression, survival status, and survival time in the two groups. In both databases, hub-genes expression showed the same trends. *CORT*, *UHRF2*,

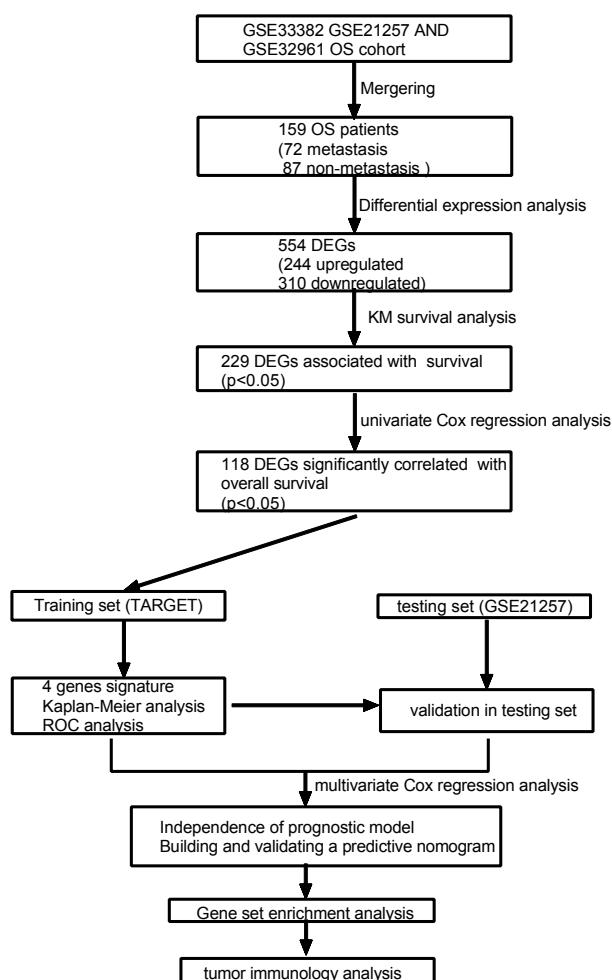


Fig. 1. Flowchart of the study.

and *PDK1* expression were up-regulated in the high-risk group, and *APBB1IP* expression was down-regulated in the high-risk group (Fig. 5 E, F). This was consistent with the multivariate Cox regression coefficients. A greater number of patients in the high-risk group died (high-risk group 24/43 vs low-risk group 5/43 in the training set; high-risk group 9/16 vs low-risk group 5/16 in testing set), and patients in the high-risk group had a shortened survival time (low-risk group 4.887 years vs high-risk group 3.349 years, $P < 0.05$ in the training set; low-risk group 9.135 years vs high-risk group 3.156 years, $P < 0.05$ in testing set) (Fig. 5 C, D).

KM survival plots revealed that patients in the high-risk groups suffered significantly worse prognoses in both databases ($P < 0.05$) (Fig. 6 A, B). In the two-group comparison, we found patients in the high-risk group suffer shorter survival, and the patients with metastatic OS scored higher risk-score (Fig. 6 C-F). ROC curves to evaluate the model's predictive effects after 3, 5, and 10 years revealed that the area under the ROC curve (AUC) for the TARGET training set was 0.839, 0.851, and 0.867 at 3, 5, and 10 years, respectively. The AUC for the

GSE test set risk signature was 0.773, 0.792, and 0.71 at 3, 5, and 10 years, respectively (Fig. 7 A-F). All AUCs in the training and test sets were more significant than 0.7, indicating that the four-gene signature could predict the prognosis.

We performed another multivariate Cox analysis of risk score and other clinical factors, including age, gender, metastasis, and primary OS site using the TARGET data. This analysis shows that risk score and metastasis are the independent prognostic factors for patients with OS (Fig. 8A). Furthermore, we established a nomogram, including risk score, and metastasis, to predict patients' survival chances with OS at 1, 3 and 5 years (Fig. 9). Calibration curves were used to evaluate the nomogram and got a C-index of 0.88, indicating that the model was suitable for clinical application (Fig. 8 B-D).

Immuno-cell infiltration of OS

CIBERSORT and ESTIMATE algorithms were employed to enrich levels of immune cells in OS based on the files from TARGET and GSE21257. The high-risk group was marked by the high plasma cells, T cell CD4 memory resting, activated dendritic

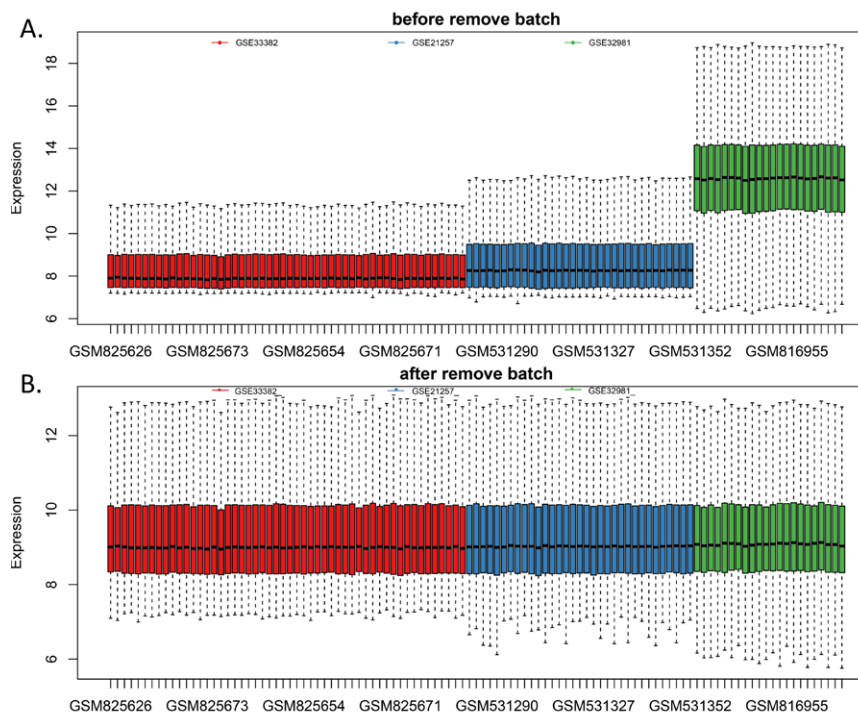


Fig. 2. mRNA expression in different databases (GSE33382, GSE21257, and GSE32981). **A)** mRNA expression before batch removal. **B)** mRNA expression after batch removal.

cells and eosinophils. The low-risk group was marked by high macrophage M2, activated mast cells and neutrophils. The low-risk group scored higher in both stromal scores and immune scores (Fig. 10A). The results revealed that the high-risk group scored in immune-score and stromal-score, which indicated differences in the microenvironment of the two risk groups. Furthermore, the correlation heatmap was applied to visualize the relationship between the immune cell in the tumor microenvironment (TME) (Fig. 10 B, C).

Identified risk-score group gene subtype

We applied the “limma” package for the DEGs

to further study risk score immune characteristics. In the merged data (TARGET and GSE21257), 933 DEGs were obtained due to the criterion that $FDR < 0.05$ and $|\text{fold-change}| > 2$. Then we regrouped samples by unsupervised clustering to the 933 DEGs. The samples were divided into gene cluster A and gene cluster B (Fig. 11). The heatmap exhibited the transcriptomic profile of the DEGs in different kinds of clusters (Fig. 12C). In the next step, we explored the prognostic value of the gene clusters with survival information. In the Kaplan-Meier plotter, we found that patients in gene cluster B had a favorable prognosis ($p=0.033$) (Fig. 12B). In the immune analysis, we found the cluster B score

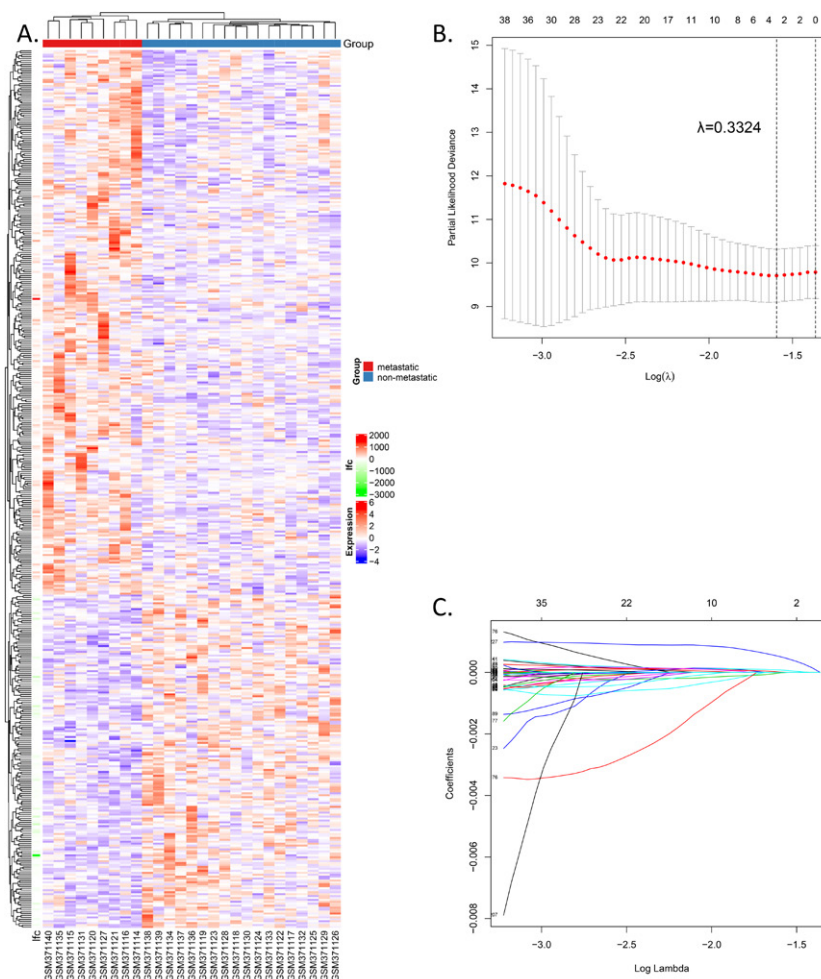


Fig. 3. A) Heatmap of DEG expression between metastatic and non-metastatic subgroups. LASSO Cox regression analysis. B) Partial likelihood deviance was plotted versus $\log(\lambda)$. The vertical dotted line indicates the λ value with the minimum error and the largest λ value in which deviance was within one standard error of the minimum. C) LASSO coefficient profiles of genes associated with survival in patients with osteosarcoma. LASSO: least absolute shrinkage and selection operator.

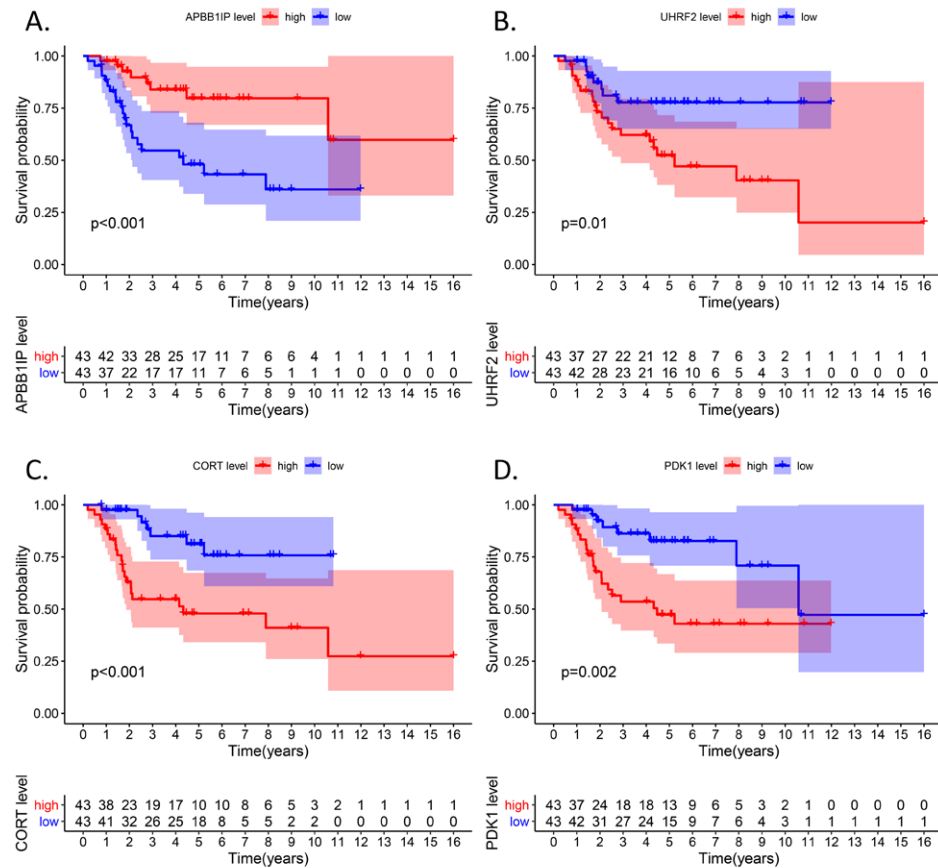


Fig. 4. Kaplan-Meier survival curves for high hub gene expression and low hub gene expression subgroups in the TARGET database. **A)** KM plots for APBB1P, **(B)** UHRF2, **(C)** CORT, and **(D)** PDK1.

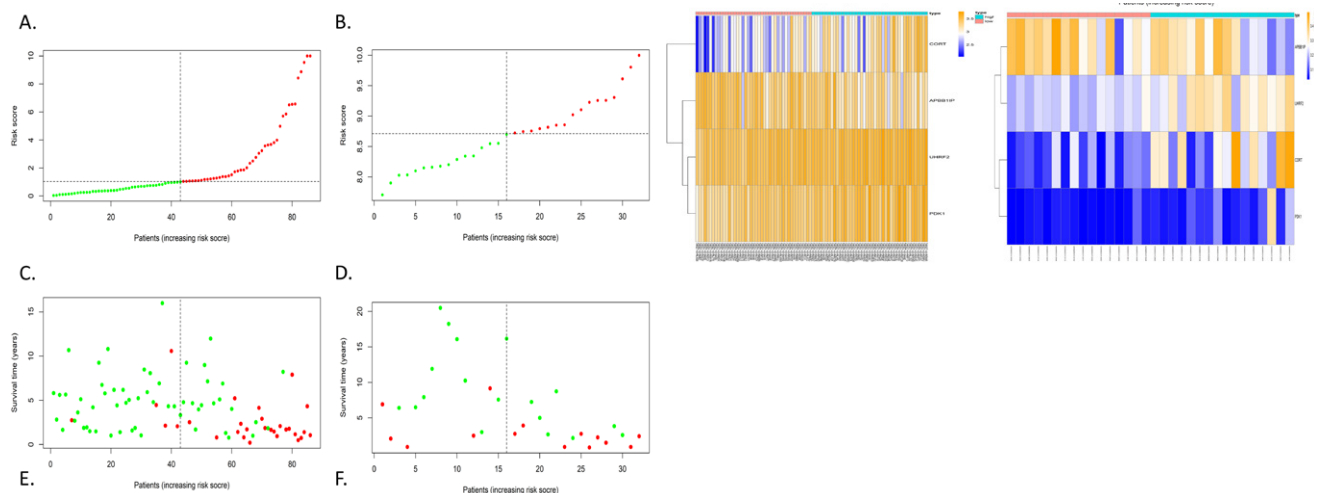


Fig. 5. Risk scores and survival status of TARGET and GSE21257 dataset patients in high- and low-risk groups. **A)** Risk scores of patients from TARGET in the high- (red) and low- (green) risk groups. **B)** Risk scores of patients from GSE21257 in the high- (red) and low- (green) risk groups. **C)** Survival status of patients from TARGET in high- and low-risk groups. Green and red represent surviving and deceased patients, respectively. **D)** Survival status of patients from GSE21257 in high- and low-risk groups. Green and red represent surviving and deceased patients, respectively. **E)** Expression of APBB1P, UHRF2, PDK1, and CORT in each patient from TARGET. **F)** Expression of APBB1P, UHRF2, PDK1, and CORT in each patient from GSE21257.

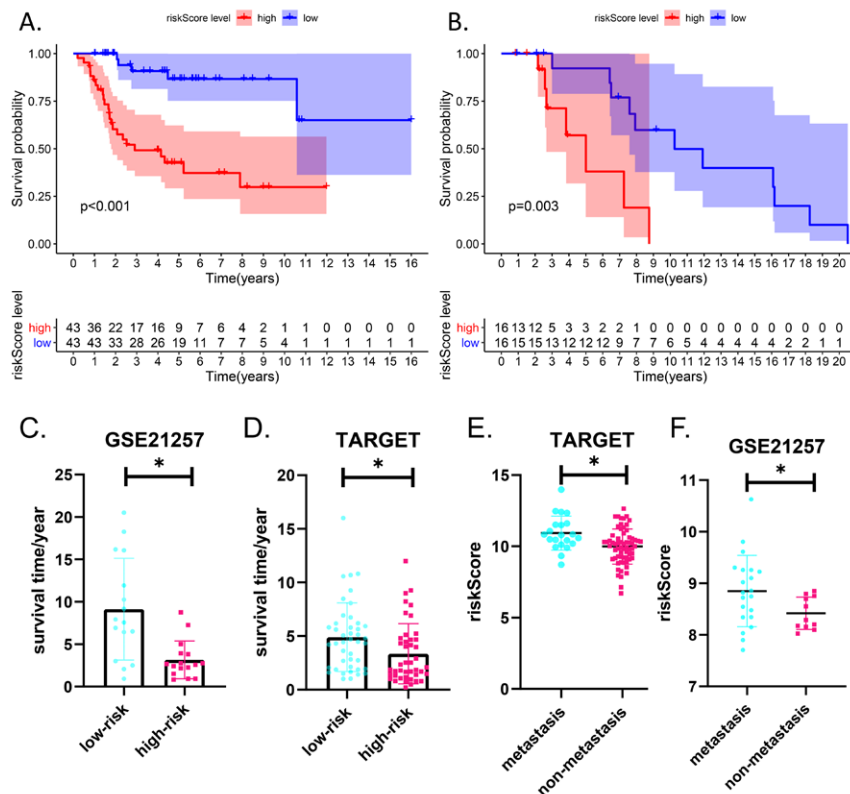


Fig. 6. *A)* Kaplan-Meier survival curve for high- and low-risk groups in the TARGET database and *(B)* GSE21257. The riskScore between metastasis and non-metastasis groups *(C)* in TARGET and *(D)* in GSE21257. The survival time between high-risk and low-risk groups *(E)* in TARGET *(F)* in GSE21257. *indicates significant difference between the two groups. * represents $p < 0.05$, Wilcoxon test.

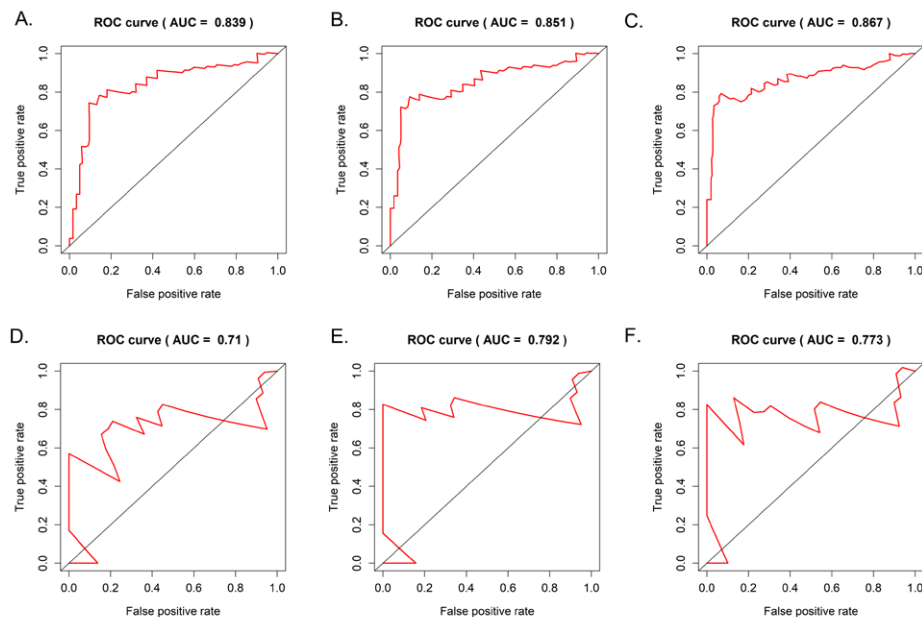


Fig. 7. Time-dependent ROC curves evaluating the prediction efficiency of the risk score for the TARGET and GSE21257 database, after three *(A)*, five *(B)*, and 10 *(C)* years for TARGET and after three *(D)*, five *(E)*, and 10 *(F)* years for GSE21257. AUC, area under the ROC curve; ROC, receiver operating characteristic; TARGET, Therapeutically Applicable Research to Generate Effective Treatment.

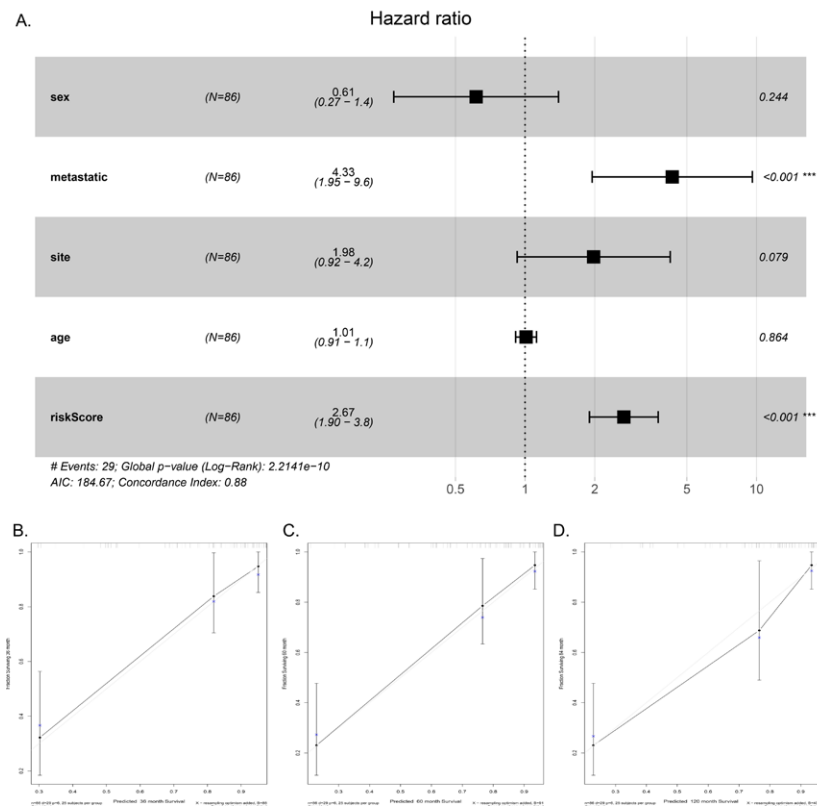


Fig. 8. A) Multivariate independent prognostic analysis of risk score and clinical factors. B, C) Calibration curve of the nomogram. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$.

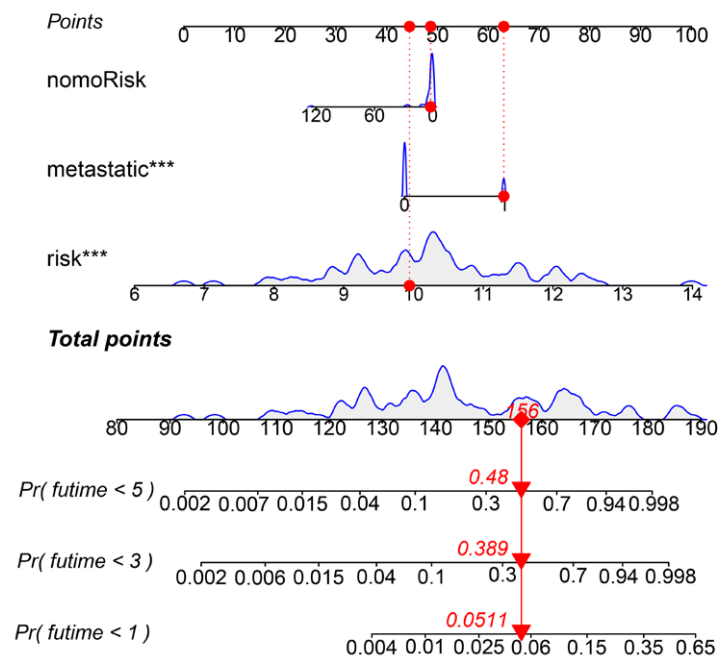


Fig. 9. Consensus matrixes of all OS samples for each (A) $k=1$ (B) $k=2$ (C) $k=3$ (D) $k=4$, displaying the clustering stability using 1000 iterations of hierarchical clustering.

higher in the immune score, which might explain the survival time diversity (Fig. 12A). The consistency between risk-score and gene clusters in the immune microenvironment and prognosis indicated that our classification method seemed effective and scientific.

Risk-score related genes and gene set enrichment analysis (GSEA)

In the GSEA, we found that several pathways involved in immune response were enriched in the low-risk group, including B cell receptor signalling

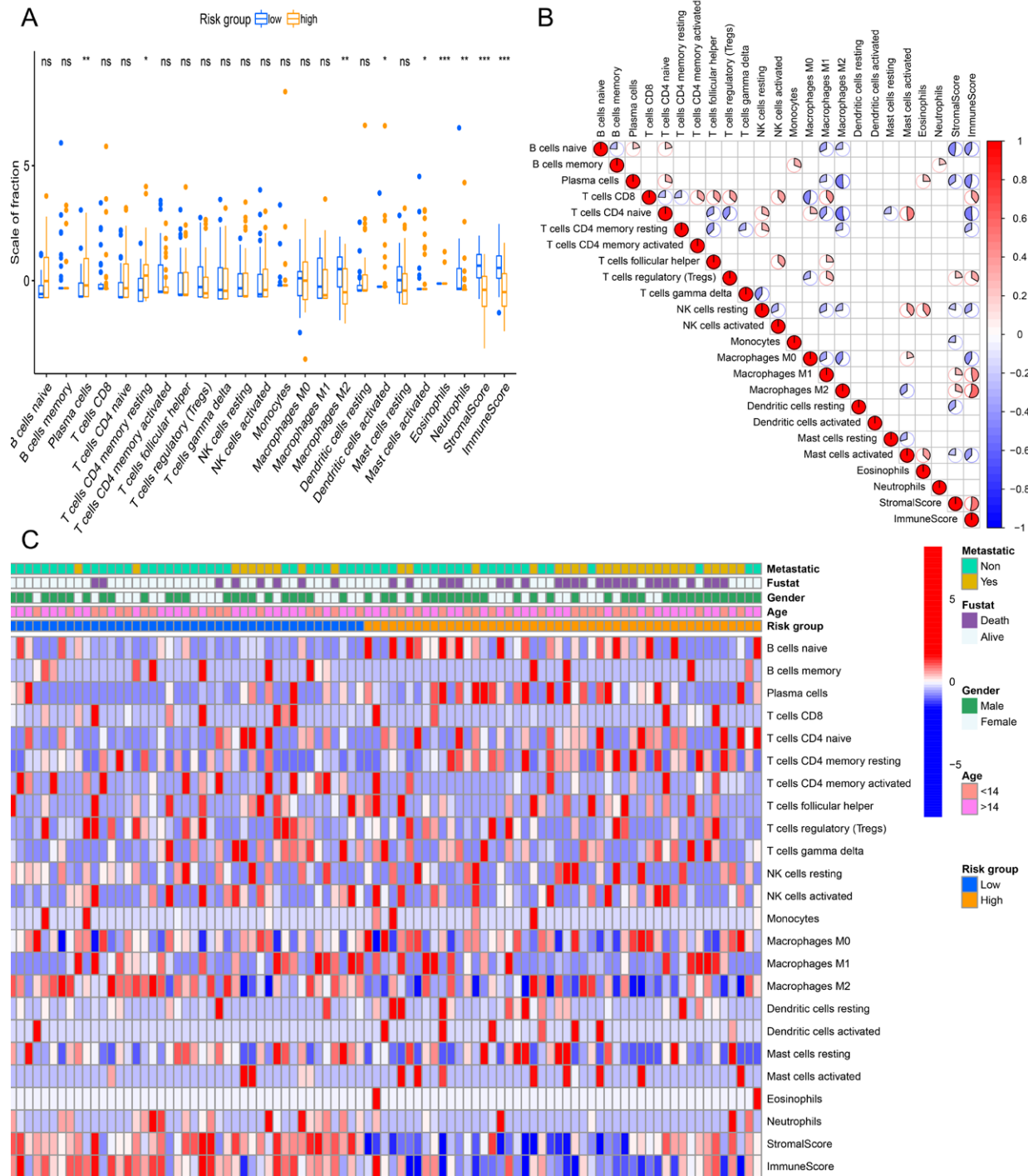


Fig. 10. Nomogram to predict the survival of patients with OS. OS, osteosarcoma. ***represents $p < 0.001$

pathway, an intestinal immune network for IGA production, natural killer cell-mediated cytotoxicity, primary immunodeficiency, T-cell receptor signalling pathway and toll-like receptor signalling pathway (Fig. 13A). This result revealed that the risk score was related to the immunity in OS. Moreover, some pathways were enriched for the high-risk group, such as cysteine and methionine metabolism, one carbon

pool by folate, pyruvate metabolism, and steroid biosynthesis and terpenoid backbone biosynthesis (Fig. 13B). More immune-related pathways were involved in the low-risk, resulting in the better prognosis patients in the low-risk group. The results indicated that the risk-score we set up might indicate the changes of TME, and the OS metastasis might be closely associated with TME and immune response.

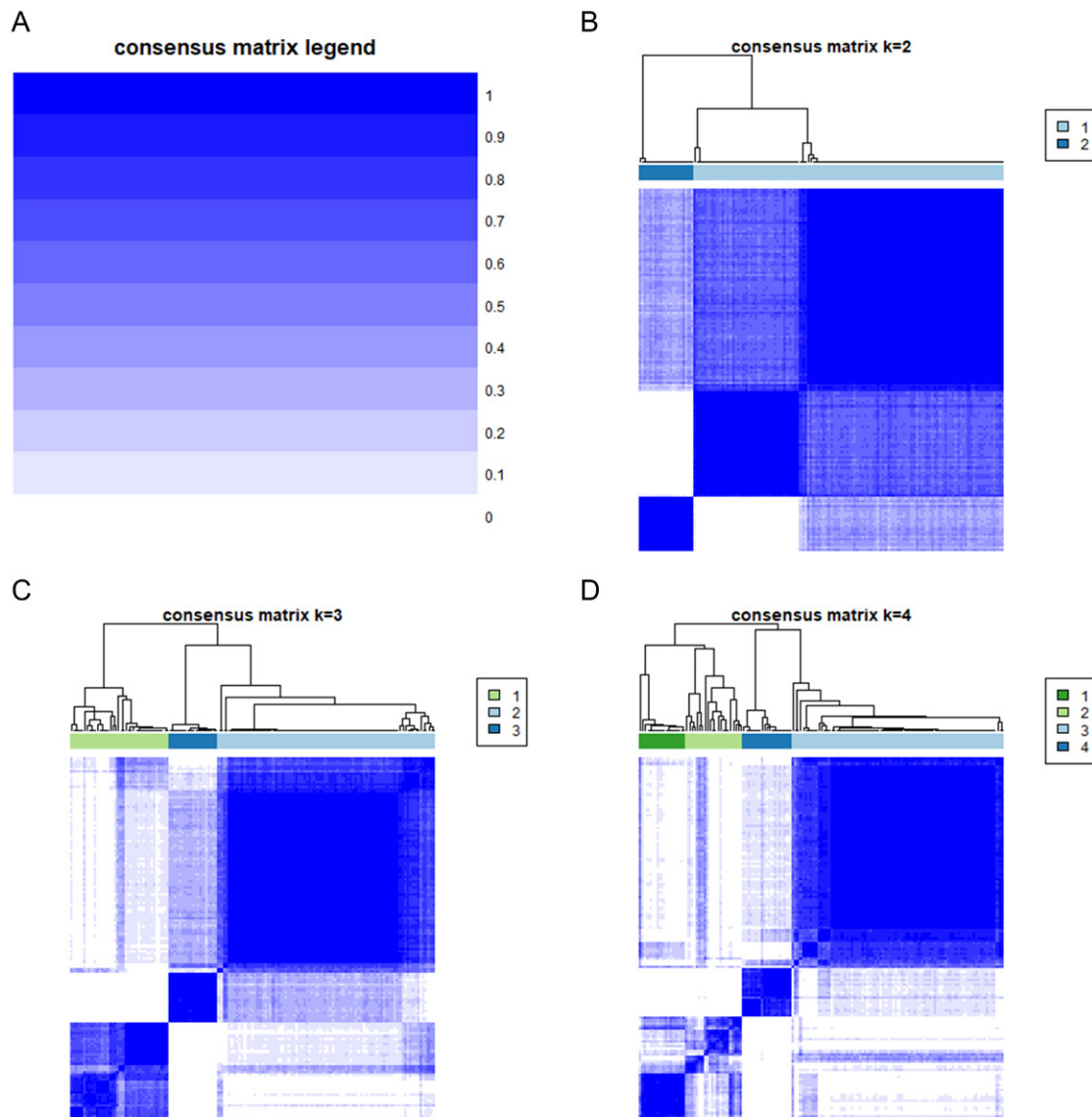
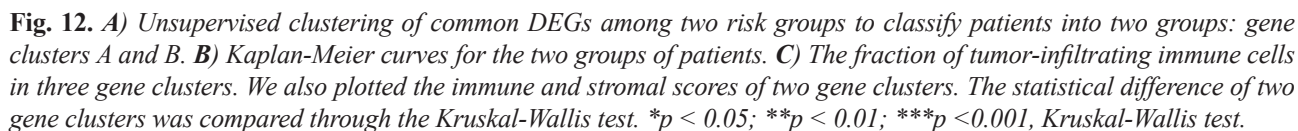


Fig. 11. *A)* The fraction of tumor-infiltrating immune cells in two risk groups. We also plotted the immune score and stromal score of two risk group. The statistical difference of two groups was compared through the Kruskal-Wallis test. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. *B)* Cellular interaction of the tumor-infiltrating immune cell types. *C)* Clustering of tumor-infiltrating immune cells in five independent HNSC cohorts. Rows represent tumor-infiltrating immune cells, and columns represent samples



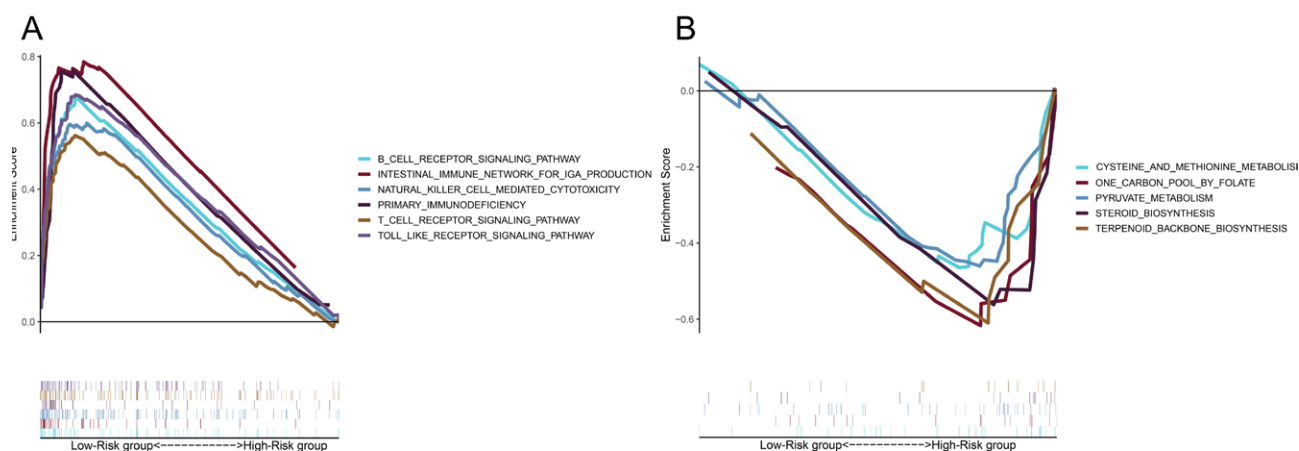


Fig. 13. *A)* Enrichment plots showing B cell receptor signaling pathway, intestinal immune network for IGA production, natural killer cell mediated cytotoxicity, primary immunodeficiency, T cell receptor signaling pathway and toll like receptor signaling pathway in the low risk subgroup. *B)* Enrichment plots showing cysteine and methionine metabolism, one carbon pool by folate, pyruvate metabolism, steroid biosynthesis and threpenoid backbone in the high risk score subgroup.

DISCUSSION

Metastasis is a distinguishing feature between malignant and benign tumors. Compared to non-metastatic OS, metastatic osteosarcoma is typically difficult to control, prone to relapse and known to indicate poor prognosis. To date, there is, however, no convincing hypothesis about OS metastasis. Therefore, there is a need to explore the potential molecules involved in OS metastasis and the construction of a genetic signature for prognosis prediction in patients with OS.

To ensure reliability, we enlarged the sample sizes with three independent datasets for the DEGs analysis. However, a simple merger was bound to produce errors due to the batch effect, so we performed batch removal before merging the data. Comparison of metastatic and non-metastatic OS revealed 554 DEGs. Examination using data from TARGET and univariate Cox analyses revealed that some of these genes were associated with survival. After LASSO regression, four genes (*APBB1IP*, *UHRF2*, *PDK1*, and *CORT*) were selected to construct a genetic signature. Multivariate Cox analysis was applied to establish and optimize the risk model. All four genes were retained following Cox regression. There were significant differences between the high- and low-

risk score groups in both the training (TARGET) and validation (GSE21257) sets, indicating that the risk model is suitable for wide application.

Therefore, this study provides clinical guidance for treatment decisions in a broad segment of the general population. In addition, surgeons can evaluate the metastasis of the OS in the diagnostic and intraoperative biopsies with the help of our signature, which is necessary for the operations and adjuvant therapies plans.

APBB1IP, also known as *RIAM* (Rap1-GTP-interacting adaptor molecule), is a signal transducer of Ras activation to actin cytoskeletal remodelling that mediates Rap1-induced adhesion (11). Coló et al. (12) reported that *RIAM* depletion leads to defective melanoma cell migration and invasion. This is consistent with our observation that this gene expression is elevated in patients in the high-risk group. Similarly, Coló et al. (12) found that *RIAM* reduces melanoma cell invasion. *RIAM* controls cell growth and survival by decreasing the activation of Erk1/2 MAPK and phosphatidylinositol 3-kinase (13). Few reports have focused on the role of *RIAM* in OS, and the role of *RIAM* in OS and OS metastasis remains unclear. Our results might provide a new area of focus for the study of OS and OS metastasis.

UHRF2 is an E3 ubiquitin-protein ligase that

regulates the cell cycle (14) and can mediate the post-translational modification of histones in tumor initiation and progression. Lu et al. (15) reported that UHRF2 is a direct target of E2F1. Moreover, tumors with high UHRF2 expression levels are more likely to promote tumor growth (16) and shorten survival time (17). This is consistent with the results of our research. Nevertheless, others have reported that UHRF2 is a tumor suppressor. This paradox might be due to differences in the kinds of tumors analyzed.

PDK1 plays a vital role in tumors. Several reports show that PDK1 expression is dysregulated in multiple cancer types. Furthermore, PDK1 has been demonstrated to play a role in cancer development, progression, and metastasis (18). PDK1 is implicated in signalling pathways through PI3K/Akt, Ras/MAPK, and Myc. Fang et al. (19) found that PDK1 inhibition could inhibit the proliferation of MG-63 OS cells, and Li et al. (20) reported that microRNA-379 suppresses OS progression by targeting PDK1. In brief, these results indicate that PDK1 is a potential target for the development of OS therapies.

CORT (cortistatin), a brain cortex peptide, structurally and functionally resembles somatostatin (SS). The relationship between CORT and cancers has been examined. Li et al. (21) revealed CORT's new function in the immune system and cancer pathogenesis and aimed to identify CORT-specific receptors. Except for this research, few studies have explored CORT's role in tumors, especially in OS. Our results indicate that CORT may play a critical role that requires more attention.

We have proved that the four highlighted genes were associated with the prognosis of OS patients, and some of them might be related to the immune system and OS-TME. Our results indicate that these genes might potentially be therapeutic targets.

According to Blomberg et al. (22), metastasis has been proved to be related to the immune microenvironment. Some researchers declared that immune cell discrepancy in the OS-TME counts a lot in tumor progression (23, 24). For further research of the model gene signature, we also paid attention to the association between risk-score and TME. The results reveal that the risk score is not only a biomarker for the prognosis but an indicator for the

OS-TME. This finding may offer a novel idea for a better understanding of the TME in OS.

Our research revealed that for OS patients, the expression levels of CD4⁺ T cells, plasma cells, M2 macrophage, dendritic cells, mast cells, eosinophils and neutrophils were correlated to the prognosis. Patients with higher stromal scores and immune scores might have a better prognosis, consistent with previous reports. The immune cells involved reflected the hosts' immune response to OS. For the verification of our conclusion, we performed unsupervised clustering. We regrouped the OS patients according to the results of unsupervised clustering for DEGs. The results confirmed that the group with a higher immune score would have a more favorable prognosis.

The GSEA revealed that pyruvate was enriched in the high-risk group. According to previous studies, immoderate pyruvate metabolism could cause the Warburg effect, necessary for tumor progression. Several immune pathways were significantly enriched in the low-risk group, such as T-cell receptor signaling, primary immunodeficiency and natural killer cell-mediated cytotoxicity. The result indicated that the immune systems of patients in the low-risk group were more activated than those in the high-risk groups. In the TME, patients in the low-risk group occupied more powerful anti-tumor ability, which might explain their more favorable prognosis.

Pyruvate is necessary for the metabolism. Most tumor cells significantly up-regulate glucose uptake and glycolysis to provide supplemental energy, resulting in pyruvate accumulation. This process could cause the Warburg effect and fuel tumor metastasis (25). Elia et al. (26) reported that breast cancer cells rely on environmental pyruvate to shape the metastatic niche. However, no studies have examined pyruvate metabolism in OS. The role of pyruvate in metastatic OS might be the same as that in breast cancer. This contention requires further experimental investigation.

Immune infiltrates play a crucial role in innate and adaptive immunity in OS (24). In the enrichment analysis, it seemed that the patients in the high-risk group suffered from immunosuppression. There were more immune-related pathways enriched in

the low-risk group. According to previous research, the inhibition of T cells, B cells, or NK cells might favor the tumor development and metastatic process (24, 27, 28). The large number of immune-related pathways involved in the low-risk group represented a favorable prognosis.

This study does have some limitations. Our results are purely from the data expression matrix and have not been proven by *in vivo* or clinical studies. Moreover, several essential genes may be overlooked in multiple continuous processes. Furthermore, we only used the simulation analysis immune score rather than the experiment in the immune analysis. Besides, we noticed that in Fig. 6, The ROC in the D E F plot has an intersection with the slash, indirectly indicating that the model may not work well sometimes. We speculated that this could be attributed to the limitation scale of size in the texting set.

In conclusion, we constructed a reliable and accurate metastasis-associated hub-gene signature based on GEO and TARGET data. In the future, this model needs to be examined and revised through additional clinical studies. The hub genes identified in this study might target OS metastasis inhibition and be a focus for future studies. Moreover, the immune infiltrates and immune-related pathways we explored might serve as the keys to OS metastasis and treatment.

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Integrated bioinformatics and validation to discover lncRNAs related to immunity and inflammation in polycystic ovary syndrome

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Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders associated with immunity and inflammation. However, the underlying immune/inflammation-related molecular characteristics of PCOS, such as miRNAs, lncRNAs remain unclear. The aim of this study was to investigate the immune-related biomarkers in PCOS based on the competitive endogenous RNA (ceRNA) mechanism. We analyzed the differential expression (DE)-mRNAs, DE-miRNAs and DE-lncRNA based on datasets GSE155489 and GSE138572. Functional enrichment analysis was performed on immune/inflammation-related pathways of DE-mRNAs. Furthermore, an immune/inflammation-related ceRNA network was constructed based on the DE-mRNAs, DE-miRNAs and DE-lncRNAs. For further validation, qRT-PCR was used to detect the expression level of the upregulated candidate lncRNAs. In addition, receiver operating characteristics (ROC) were constructed to evaluate the diagnostic value of the lncRNAs. We identified 5 lncRNAs as the potential immune/inflammation-related biomarkers of PCOS, including KCTD21-AS1, SNHG12, CRNDE, FAM157C and AC111152.2. AC111152.2 has the best performance with an area of 0.831. Our results provided novel insights into the discovery of immune/inflammation-related genetic biomarkers for PCOS. However, their specific regulatory mechanisms still need to be further studied.

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders and has been estimated to affect around 6-12% of women during their reproductive age worldwide (1). PCOS results in poor quality of life and is an economic burden to patients. The main clinical characteristics manifest as hyperandrogenism, menstrual irregularity, and polycystic ovarian morphology (2). PCOS patients usually have the additional risk of suffering from type II diabetes mellitus, metabolic diseases,

hypertension and cardiovascular events (3). As a polygenic, polyfactorial, systemic, inflammatory, autoimmune disease, the pathophysiology of PCOS is quite complex and different mechanisms could contribute to the main features of the syndrome (4, 5). Studies have shown that inflammation processes are involved in ovulation and play a key role in ovarian follicular dynamics (6, 7). Moreover, it was reported that hormones and immune cells, including both innate and adaptive immune cells,

Key words: Polycystic ovary syndrome; immune/inflammation-related biomarkers; ceRNA; lncRNA

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are reportedly a crosstalk in PCOS (8). Although research on the pathophysiology of PCOS has emerged continuously in recent years, critical issues regarding the underlying molecular characteristics of PCOS, such as miRNAs, lncRNAs remain unclear.

Competitive endogenous RNA (ceRNA) hypothesis proposed by Salmena et al. has revealed a new mechanism for RNA interaction (9). It is known that microRNA can cause gene silencing by binding to mRNA (10), and ceRNA can regulate gene expression by competitively binding to microRNA through microRNA response elements (MREs) (11). Long non-coding RNA (lncRNA) is RNA molecule greater than 200 bp in length, and is also a kind of ceRNA that can interact with the miRNA or directly regulate the transcription of mRNAs (12). The existence of a ceRNA regulatory pathway has great biological significance and functions in the progression of various diseases. Several studies have paid attention to the function of ceRNA in PCOS. For examples, Fu et al. constructed a ceRNA network to investigate the pathogenesis of PCOS (13). Ma et al. also constructed a ceRNA-based lncRNA-mRNA network to identify functional lncRNAs in PCOS. However, little is known about the role of ceRNA in PCOS immune/inflammation-related progression.

In the present study, we aimed to investigate the immune/inflammation-related ceRNA mechanisms based on pivotal lncRNAs, miRNAs, and mRNAs in PCOS. Firstly, the differential expression genes (DEGs) were identified based on datasets GSE155489 and GSE138572 and were screened for the immune/inflammation-related genes. We found that the DE-mRNAs were mainly enriched in immune/inflammation-related signaling pathways. A ceRNA network was constructed based on DE-miRNAs, DE-lncRNAs and DE-mRNAs. Finally, several lncRNAs were identified as the potential biomarkers of PCOS based on the ceRNA network. The results offer a basis for the investigation of the underlying mechanisms of immune and inflammation in PCOS.

MATERIALS AND METHODS

Clinical samples

In this study, a total of 30 PCOS patients and 30

healthy subjects (NC group) were enrolled from the Fujian Provincial Maternity and Child Health Hospital, Affiliated Hospital of Fujian Medical University. The research protocol for this study was approved by the Ethics Committee of Fujian Provincial Maternity and Children's Hospital, and informed consent was obtained from all the participants.

PCOS patients were diagnosed according to the revised Rotterdam European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine Criteria (2004). The PCOS patients who fulfilled at least two of the following criteria were included: chronic oligo-ovulation or anovulation, androgen excess and polycystic ovaries. The control group contained patients undergoing IVF or ICSI due to male factors or tubal factors of infertility, who had regular menstrual cycles, normal ovary sonographs and normal ovulating. All of the patients had no history of drugs affecting glucose or lipid metabolism and were without any known medical conditions or diseases, including Cushing's syndrome, congenital adrenal hyperplasia, androgen-secreting tumors and endometriosis. The follicular fluid was separated from the subscribers. The FF samples were stored in a refrigerator at -80°C for RNA extraction. Informed consent was obtained from all the participants.

Data collection

High-throughput datasets GSE155489 and GSE138572 of PCOS were downloaded from the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>). mRNA and lncRNA data were obtained from the dataset of GSE155489, which included 8 human ovarian granulosa cell samples, 4 PCOS and 4 normal samples. We obtained miRNA data from the dataset of GSE138572, which included 10 ovarian granulosa cell samples, 5 PCOS and 5 normal samples.

Identification of DE-mRNAs, DE-miRNA, DE-lncRNAs in PCOS

The RNA expression profile was analyzed by limma R package. The differential expression (DE) genes were identified with threshold criterion of p value < 0.05 and $|\log_2FC| > 1.5$ (14). The DE-mRNAs, DE-miRNA, and DE-lncRNAs were displayed in the form of volcano plot and heatmaps. The heatmaps and clustering of DEGs were plotted by the R ggplot2 package in the R analysis platform (15).

Functional enrichment analysis

Gene ontology (GO) functional analysis was used to analyze the biological process (BP) of differentially expressed genes. In the current study, GO analysis of DEGs was performed using DAVID (<https://david.ncifcrf.gov/conversion.jsp>). Then immune- and inflammation-related pathways were selected, and the list of immune-related genes in the pathways was obtained. Functional enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) was mainly used to analyze the signaling/metabolic pathway through which differentially expressed genes may perform their biological functions (16). In order to analyze the functional signaling pathways of differentially expressed genes, KEGG enrichment analysis of immune related genes was carried out by using clusterProfiler R package (17), in which p value < 0.05 was used as the critical value for screening significant enrichment pathway.

Construction of Protein-Protein Interaction network

The results of protein-protein interaction (PPI) were obtained by using STRING database (<https://string-db.org>) (18). The results were imported into Cytoscape (v3.8.0) for network visualization, and the protein-protein interaction network diagram was obtained. In order to ascertain the core modules of complex network, we performed MCODE plug-in analysis and identified 3 modules. The most significant module with the highest score (module score: 9.643) was selected for further investigation. Also, cytoHubba, a plug-in of Cytoscape, was used to identify hub genes based on a maximal clique centrality (MCC) algorithm.

Construction of ceRNA network

The lncRNAs corresponding to the DE-miRNAs were predicted by Starbase (<http://starbase.sysu.edu.cn/index.php>), and the intersection of the DE-miRNAs and the

Table I. Specific lncRNAs primers for qRT-PCR analysis

Gene name	Sequence 5'-3'
GAPDH	F: AAGAAGGTGGTGAAGCAGGC
	R: TCAAAGGTGGAGGAGTGGGT
KCTD21-AS1	F: AGCCTGACCTACCCTCAGAG
	R: CCTACCTACCTCACAGGGCT
MEG3	F: GGGTCCCCTGATTCTCTCCT
	R: GG TAGAGACCCATCCCCAGT
SNHG12	F: ATTCCAGTCCTCTCCTCGCT
	R: GCTGCCTGGGAGTTGTAGTT
BOLA3-AS1	F: GCACTGATCACCCAAGGTCA
	R: GCTAGAGTGCAGTGGCTCAA
CRNDE	F: GACTTGGTGTTTGGCAGCTG
	R: GGGGAAGGTGGTACATCAGC
FAM157C	F: GCCTTAGTGTTGGGAGGGAC
	R: GGTCATAGCGAGTGACGAGG
AC111152.2	F: CTCCTGCATCTATGGCGAGG
	R: AACCGCCACTCAGTACACAG

DE-lncRNAs was obtained. Then the DE-miRNAs were used to predict the target of related mRNA by the Starbase database, and gave the intersection with immune related genes. Finally, the ceRNA network was constructed based on DE-lncRNAs, DE-miRNAs and immune-related DE-mRNAs by using Cytoscape visualization software.

RNA separation and quantitative PCR

Total RNA was extracted from the follicular fluid using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Next, RNA was quantified via SYBR Green using the Roche Light-Cycler 480 Real Time PCR system (Roche, Germany). GADPH was used as the internal control. Subsequently, quantitative PCR (qPCR) was performed using a final volume of 20 μ L of SYBR Green PCRMasterMix. Primer sequences are summarized in Table I. Target values were assessed according to the $2^{-\Delta\Delta CT}$ method through normalization to an internal control, where the mean of the control samples is used as a calibrator.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8), p -value < 0.05 was considered statistically significant. Receiver operating characteristic

(ROC) curve was established by GraphPad Prism (version 8) according to lncRNA expression, and it was used to analyze the diagnostic value, including the area under the curve (AUC), sensitivity, and specificity.

RESULTS

Identification of DE-mRNAs in PCOS

The influence of coding genes is usually the most intuitive and fundamental change in molecules, because many coding genes play an important role in human diseases. To intuitively demonstrate the expression changes of mRNAs in PCOS, gene expression profiles of 4 PCOS and 4 normal samples from GEO database were carried out to identify the DE-mRNAs. As shown in Fig. 1A, clustering of 1688 (739 upregulated and 949 downregulated) DE-mRNAs of high or low expression level were identified (p value < 0.05, $|\log_2FC| > 1.5$). The volcano plot in Fig. 1B shows distinct cluster distribution of these DE-mRNAs in normal and PCOS samples.

Functional enrichment analysis

We first performed the GO functional enrichment analysis to investigate the function of the 1688 DE-

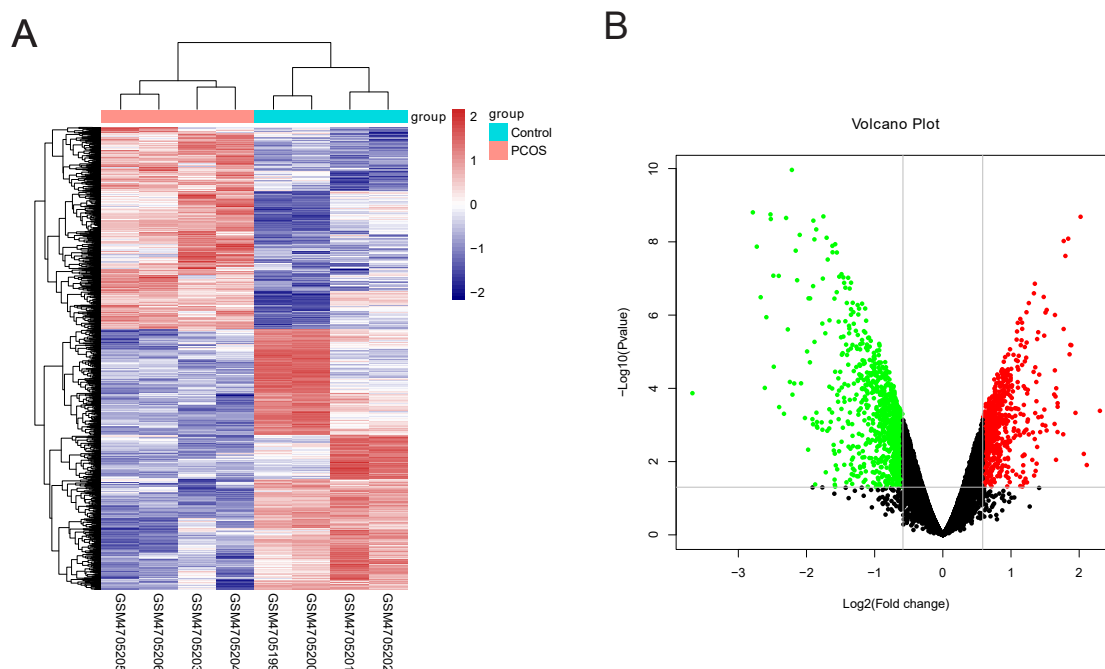


Fig. 1. Identification of differentially expressed mRNAs. **A)** The heatmap and **(B)** volcano plot of DE-mRNAs identified in datasets GSE155489. Up-regulated and down-regulated genes are colored with red and blue/green, respectively.

mRNAs. As seen in Fig. 2A, we selected the top 15 GO terms that were significantly enriched related to immunity and inflammation, such as neutrophil-mediated immunity, positive regulation of immune effector process/lymphocyte-mediated immunity, inflammation-related terms such as regulation of inflammatory response, cytokine production involved in inflammatory response. Then, we identified 199 DE-mRNAs enriched in the immune and inflammation-related terms (Fig. 2B). To further verify the functions of 199 DE-mRNAs in the biological processes in PCOS, we performed KEGG enrichment analysis. As seen in Fig. 2C, they were involved in inflammation and immune regulation

signaling pathways. The results of functional enrichment analysis indicated that these DE-mRNAs were necessary and immune-related in PCOS.

Construction of PPI network of immune-related DE-mRNAs

In the next step, we imported 199 immune-related genes into the string database to obtain protein interaction data, then constructed the PPI network in the Cytoscape software. The network, which consisted of 177 nodes and 842 edges is shown in Fig. 3A. Because of the complexity of network, we performed MCODE plug-in to identify the most significant module, which is shown in Fig. 3B. In

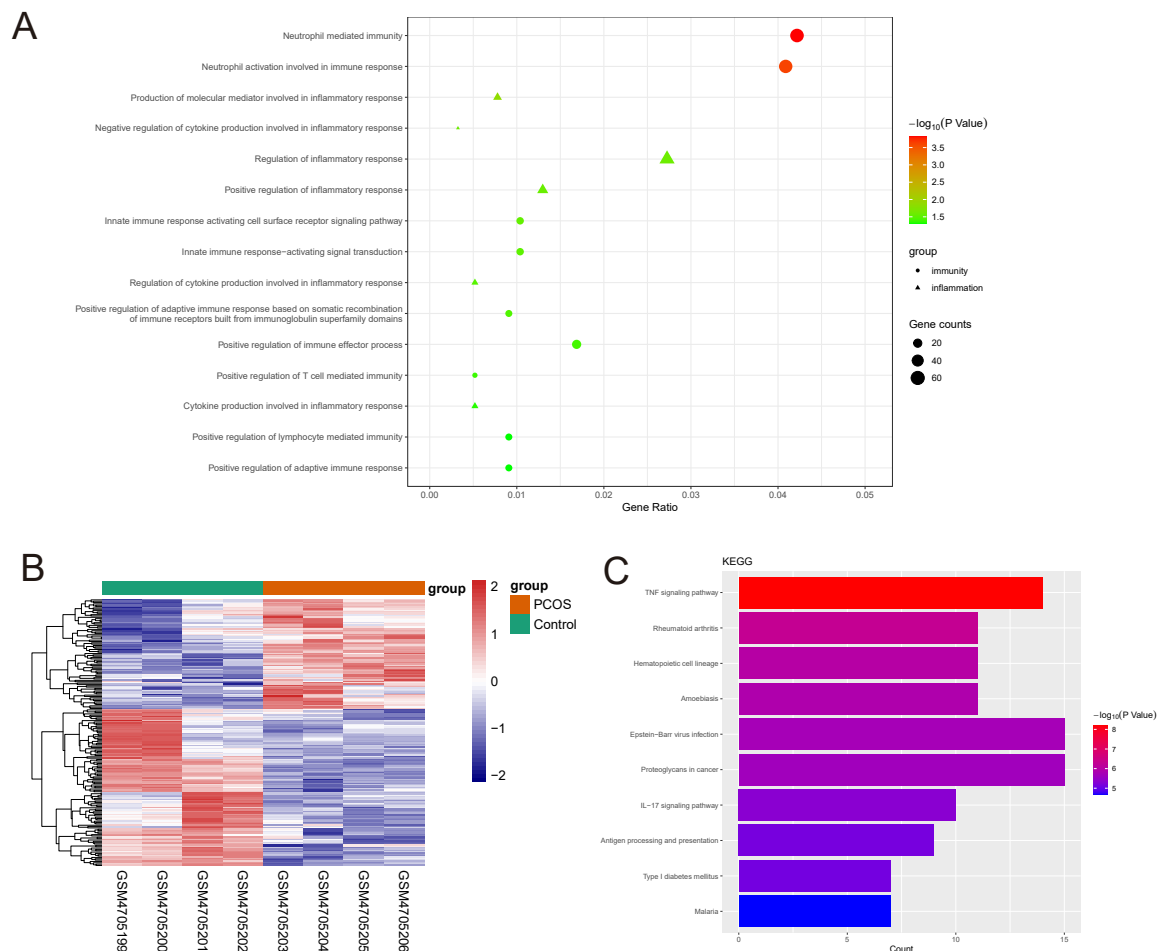


Fig. 2. Functional enrichment analysis of immunity and inflammation related DEGs. **A)** The top 15 significantly enriched Gene ontology (GO) terms that related to immunity or inflammation. **B)** The heat map shows the 199 DE-mRNAs that enriched in the top 15 significantly GO terms related to immunity or inflammation. Blue shows down-regulated genes, red shows up-regulated genes. **C)** The top 10 significantly Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

addition, we used the MCC algorithm of cytoHubba to analyze the hub genes of the PPI network. The top 10 genes are shown in Fig. 3C.

Construction of immune/inflammation-related ceRNA network

In order to explore the underlying mechanism and interaction of DE-genes in PCOS, a ceRNA network of lncRNA-miRNA-mRNA was constructed. A total of 37 DE-miRNAs in normal and PCOS groups were also identified (p value <0.05 , $|\log_2FC| > 1.5$).

The results of heatmap demonstrated that these DE-miRNAs were hierarchical clustering in each group (Fig. 4 A, B). Furthermore, 231 DE-lncRNAs were also selected (p value <0.05 , $|\log_2FC| > 1.5$) (Fig. 4 C, D). Next, 37 DE-miRNAs were used to predict the targeted lncRNAs through Starbase database. Then the targeting relationship of lncRNA-miRNA and miRNA-mRNA, respectively, were predicted by using the Starbase database. We intersected the predicted-lncRNAs and DE-lncRNAs, predicted-mRNAs and DE-mRNAs. Only immune-related

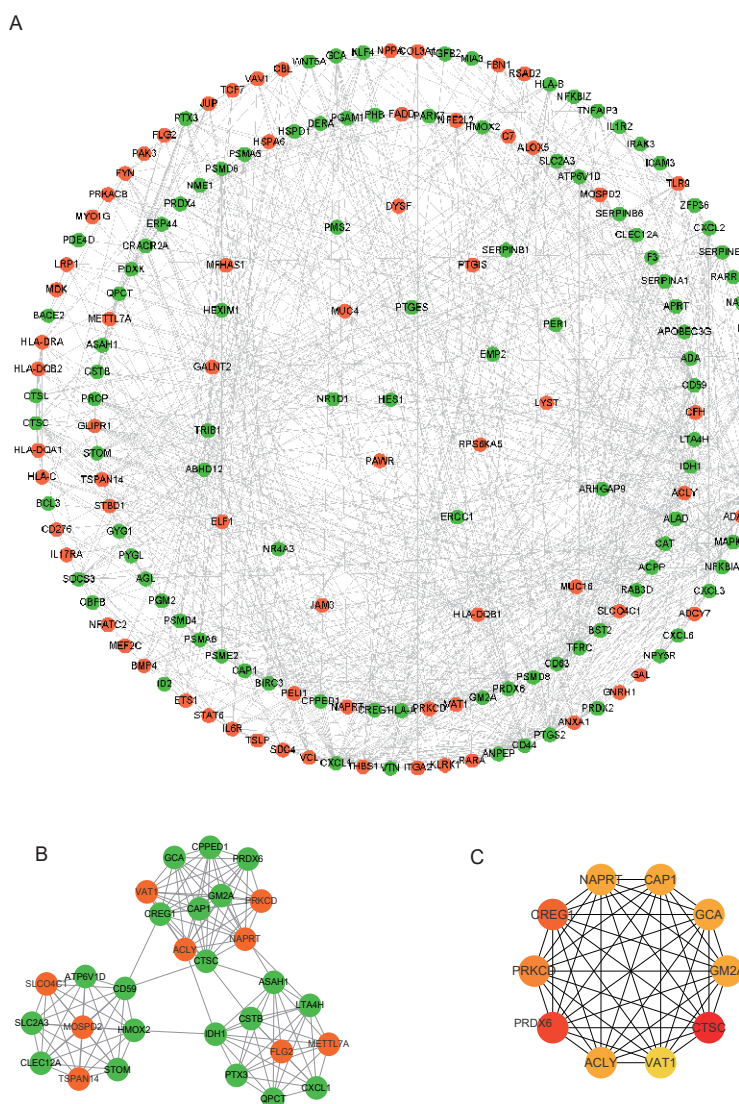


Fig. 3. Construction of Protein-Protein Interaction (PPI) network. **A)** The PPI network of the immunity- and inflammation-related DEGs. Red represents up-regulation, and green represents down-regulation. **B)** The top three core sub-networks: each point represents a gene, the connection between the two points represents the interaction between the two proteins. Red represents up-regulation, and green represents down-regulation. **C)** The hub gene of the PPI network analyzed by the cytoHubba, top 10 genes were displayed by MCC algorithm. The redder the color, the higher the score.

lncRNAs and mRNAs that co-target miRNAs were eligible for the construction of ceRNA networks, and the expression trends of lncRNAs and mRNAs were opposite to co-target miRNAs. Finally, 18 relationship pairs were obtained and the immune-related ceRNA network shown in Fig. 5 was constructed. This network contained 8 DE-mRNAs, 4 DE-miRNAs, and 9 DE-lncRNAs. Up-regulated immune-related lncRNAs, including KCTD21-

AS1, MEG3, SNHG12, BOLA3-AS1, CRNDE, FAM157C and AC111152.2 were selected for the following validation.

Validation of lncRNAs as biomarkers in PCOS by qRT-PCR

To further verify whether the lncRNAs (KCTD21-AS1, MEG3, SNHG12, BOLA3-AS1, CRNDE, FAM157C and AC111152.2) could act

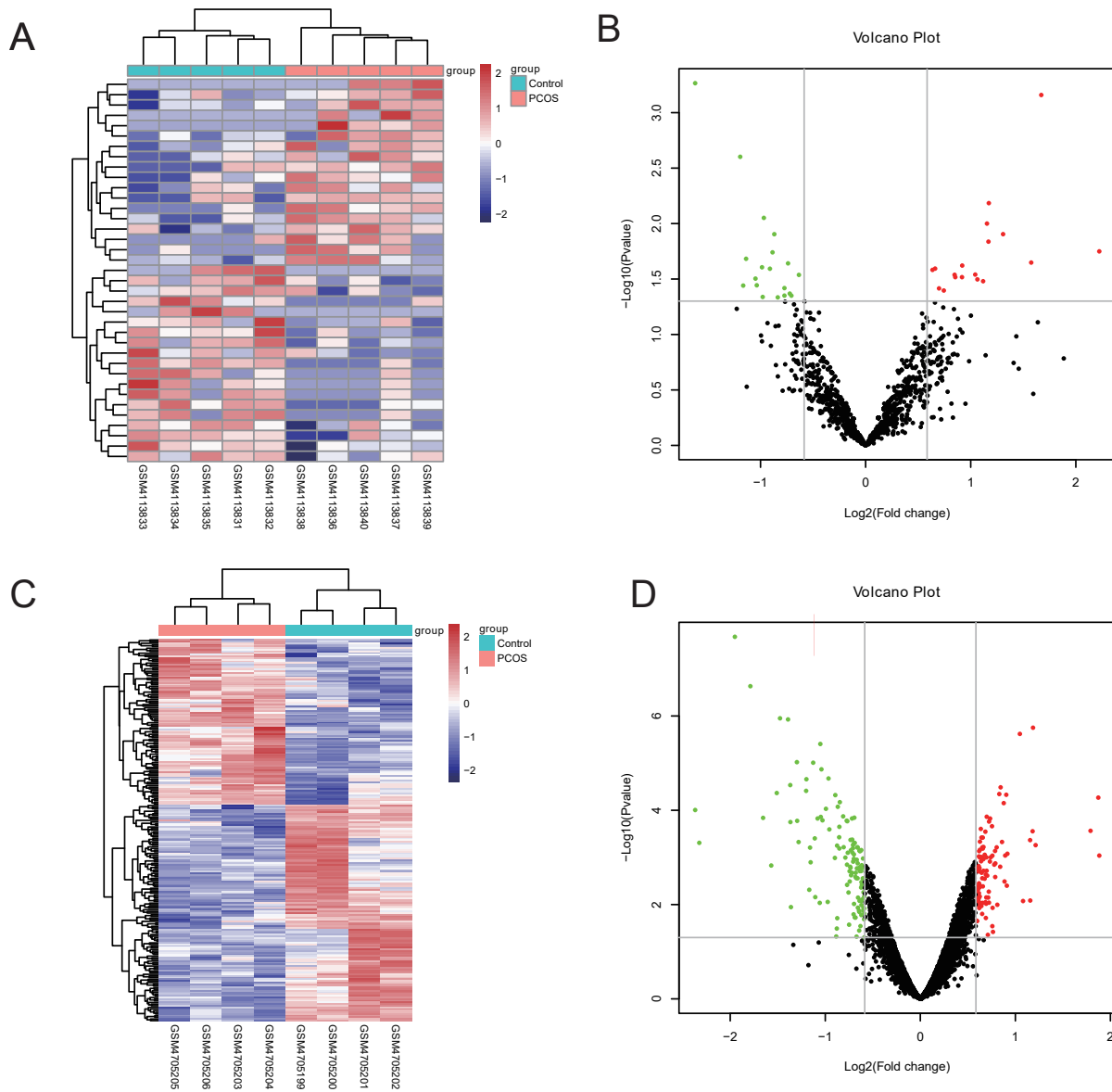


Fig. 4. Identification of DE-lncRNAs and DE-miRNAs. **A)** The heat map and **(B)** the volcano plot of DE-lncRNAs identified in GSE155489 datasets. **C)** The heat map and **(D)** the volcano plot of DE-miRNAs identified in GSE138572 datasets. Up-regulated and down-regulated genes are colored with red and blue/green, respectively.

as independent biomarkers of PCOS patients, we carried out qRT-PCR to validate the expression levels of the genes. Consistent with the omics data, KCTD21-AS1, SNHG12, CRNDE, FAM157C and AC111152.2 were significantly upregulated between PCOS and healthy controls (Fig. 6A). To assess the potential diagnosis value of the five lncRNAs for PCOS, we further implemented a ROC curve analysis. As shown in Fig. 6 B-F, the AUC of the candidate genes are 0.793 (KCTD21-AS1, 95%CI: 0.674-0.913, $p < 0.001$, Fig. 6B), 0.691 (SNHG12, 95%CI: 0.557-0.826, $p < 0.05$, Fig. 6C), 0.677 (CRNDE, 95%CI: 0.542-0.811, $p < 0.05$, Fig. 6D), 0.689 (FAM157C, 95%CI: 0.554-0.824, $p < 0.05$, Fig. 6E) and 0.831 (AC111152.2, 95%CI: 0.729-0.934, $p < 0.001$, Fig. 6F). Among these genes, we found that KCTD21-AS1 and AC111152.2 performed best. These indicated that the two lncRNAs could be candidate biomarkers for PCOS diagnosis.

DISCUSSION

PCOS is a heterogeneous condition which is associated with an endocrine reproductive disorder of females. Although the etiology is unclear, emerging evidence revealed that the epigenetics might be a contributing factor (19). Chronic inflammation is considered to be the cause of ovarian dysfunction (20). In addition, immune cells are included in the ovarian stroma, which is an essential part in ovarian biology (21). Chen et al. suggested that women with PCOS have altered endometrial immune cells, which may reflect a state of chronic low grade inflammation (22). For example, Albaghdadi reported that anomalous activation of ovarian macrophages and numerical and functional deficits in the Th17 (CD4+IL17A+) and the CD4+CD25+CD127 low Tregs play crucial role in PCOS (23). Lakomy et al. indicated the pathogenesis of PCOS is related to

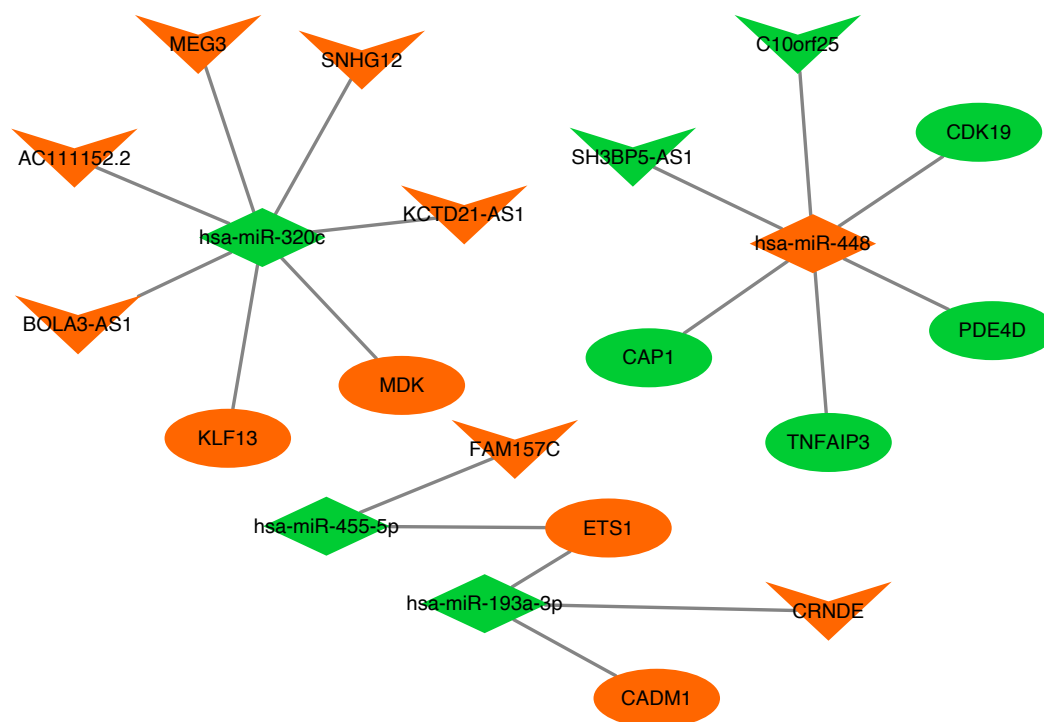


Fig. 5. Construction of ceRNA network. The ceRNA network was constructed based on the immune/inflammation-related DE-mRNAs, DE-lncRNAs and DE-miRNAs. The circle represents mRNA, the diamond represents miRNA, and the arrow represents lncRNA. Red represents the up-regulation of the RNA expression, and green represents the down-regulation of the RNA expression.

immune cells including T, CD4+T, and NK cells. NK cells are likely to be a potential predictive factor for PCOS women with infertility (24). There are limited studies on the specific endometrial immune status of PCOS patients (22). In the present study, we identified the DEGs of PCOS and found them significantly enriched in GO terms that significantly related to immunity and inflammation. In addition, they were involved in inflammation and immune regulation signaling pathway such as TNF signaling pathway. Despite its immunity and inflammation relevance, the exact molecular mechanism underlying PCOS remains poorly understood.

Emerging works on lncRNA as key regulator in complex immune and inflammation have been reported (25). Moreover, lncRNAs not only regulate gene expression, but also can be used as biomarkers for diagnosis and prognosis. Recent studies demonstrated that lncRNA served as ceRNA

that competitively combined to microRNA through microRNA response elements (MREs) to regulate gene expression in PCOS. For example, Fu et al. constructed a ceRNA network involved in the PCOS model to investigate the pathogenesis of PCOS and clues to find key diagnostic and therapeutic roles of lncRNA in PCOS (13). Ma et al. also constructed a ceRNA-based lncRNA-mRNA network to identify functional lncRNAs in PCOS. However, little is known about the role of the ceRNA in PCOS immune/inflammation-related progression. We mainly focused on the mechanisms of lncRNAs in our study and firstly conducted an immune/inflammation-associated ceRNA network. The immune/inflammation-related DE-mRNAs, DE-miRNAs and DE-lncRNAs were identified based on datasets and bioinformatics analysis, and a ceRNA network was constructed to explore lncRNAs as potential biomarkers involved in the PCOS. These

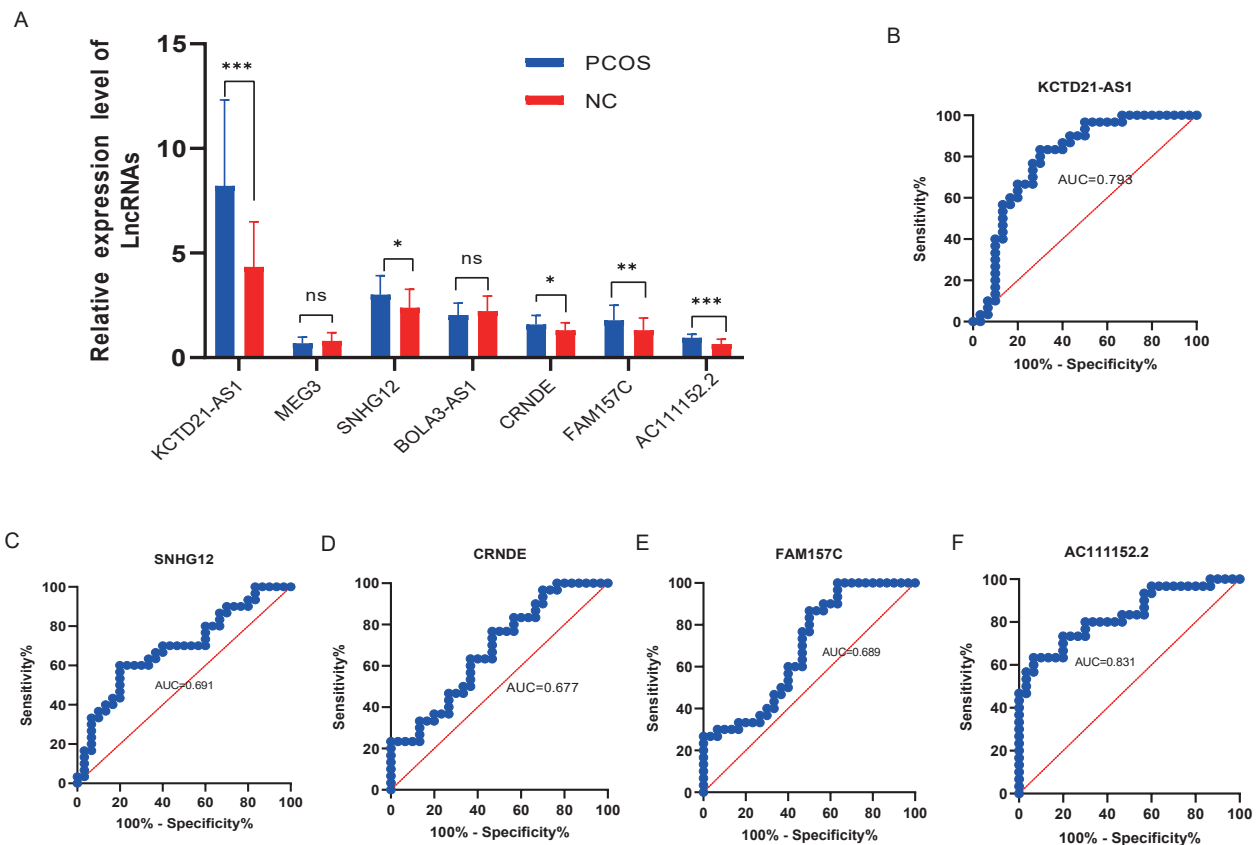


Fig. 6. Validation of lncRNAs as potential biomarkers in PCOS by qRT-PCR. **A)** Validation of KCTD21-AS1, MEG3, SNHG12, BOLA3-AS1, CRNDE, FAM157C and AC111152.2. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: no significance). **B-F)** The ROC curve of KCTD21-AS1, SNHG12, CRNDE, FAM157C and AC111152.2 in distinguishing PCOS and normal controls.

results partly explained the immune/inflammation-associated pathogenesis of PCOS and provided a basis for finding the key diagnostic and therapeutic roles of lncRNA in PCOS patients.

In our study, seven lncRNAs were selected for the following validation. However, only KCTD21-AS1, MEG3, SNHG12, BOLA3-AS1, CRNDE can be used as the candidate biomarkers. LncRNA KCTD21-AS1 was found to be hub nodes in the ceRNA regulatory network and as biomarkers of hypertensive nephropathy (26). It was reported that lncRNA MEG3 serve as a ceRNA that affects various cell processes such as proliferation, apoptosis, and angiogenesis by sponging miRNAs (27). For example, lncRNA MEG3 can sponge microRNA-17 as a ceRNA in asthma (28). SNHG12 have been reported to function in many kinds of tumor through absorbing miRNAs (29). Liang et al. suggested that lncRNA SNHG12 suppresses esophageal squamous cell carcinoma progression through competing endogenous RNA networks (30). In addition, many tumor cells can not only escape immune-mediated attack, but also enhance the polarization of effector immune cells through SNHG12 (31). BOLA3-AS1 was reported to be prognostic signature glycolysis-related lncRNAs in endometrial cancer (EC) (32). CRNDE regulates the development of tumor by regulating microRNA (33, 34), and also promotes cell apoptosis by suppressing miR-495 in inflammatory bowel disease (35). By integrated analysis, these five lncRNAs may serve as potential biomarkers in PCOS. Also, QRT-PCR and ROC analyses showed that KCTD21-AS1 and AC111152.2 performed best, therefore they could be candidate biomarkers for PCOS diagnosis.

In conclusion, this study aimed to screen out the lncRNAs as the potential biomarkers in PCOS based on the ceRNA mechanism. The immune/inflammation-related DE-mRNAs, DE-miRNAs and DE-lncRNAs were identified based on datasets and bioinformatics analysis. Functional enrichment analysis indicated that the DE-mRNAs were enriched in inflammation and the immune regulation signaling pathway. A ceRNA network was constructed and we found that these lncRNAs may serve as potential biomarkers involved in the

PCOS. Our results provided novel insights into the discovery of immune-related genetic biomarkers for PCOS. However, further detailed research is needed to illustrate the molecular mechanism of these lncRNAs as novel diagnostic markers and therapeutic targets for PCOS.

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Inhibition of miR-138-5p protects against oxidized LDL-induced endothelial apoptosis and inflammation through the SIRT1/NF- κ B signaling pathway

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This research aims to probe the effect and mechanism of microRNA-138-5p (miR-138-5p) in regulating oxidized low-density lipoprotein (Ox-LDL)-induced lipid peroxidation of vascular endothelial cells (VECs). The miR-138-5p profile in the serum of 50 atherosclerosis (AS) patients and 50 healthy donors was compared by reverse transcription-polymerase chain reaction (RT-PCR). VECs were treated with Ox-LDL to construct a model of lipid peroxidation injury *in vitro*. miR-138-5p mimics and inhibitors or the corresponding negative controls were transfected into VECs. CCK-8 and BrdU methods were used to detect the viability of VECs. RT-PCR and Western blot (WB) were implemented to test inflammatory cytokines (TNF- α , IL-1 β , IL-6), SIRT1, COX2, iNOS, STAT6, NF- κ B, Caspase3, Bcl2, Bax, MMP3, MMP9, ICAM1, VCAM1, and LOX1, respectively. Moreover, the relevancy between miR-138-5p and SIRT1 was validated by bioinformatics analysis and further testified by dual-luciferase reporter assay. As the data indicated, miR-138-5p was up-regulated in the serum of AS patients and exhibited a positive correlation with the plasma LDL level. *In vitro*, overexpressing miR-138-5p promoted Ox-LDL-mediated VECs apoptosis, inhibited cell viability and angiogenesis, aggravated inflammation, attenuated the expression of STAT6 and promoted NF- κ B pathway activation, whereas miR-138-5p inhibition exerted the opposite effects. Mechanistically, miR-138-5p targeted the 3' untranslated region (3' UTR) of SIRT1 and negatively regulated SIRT1. Moreover, knocking down SIRT1 distinctly reversed miR-138-5p inhibitor-mediated anti-apoptosis and anti-inflammatory effects against Ox-LDL. Collectively, inhibiting miR-138-5p attenuated Ox-LDL-modulated VEC injury and inflammation by regulating the SIRT1/NF- κ B pathway.

Atherosclerosis (AS) is a chronic inflammatory vascular disease that causes atheromatous plaque, a focal lesion located in the intima of large and medium-sized arteries (1). Oxidized low-density lipoprotein (Ox-LDL) aggravates AS through a variety of mechanisms, including inducing endothelial cell (EC) activation and dysfunction, macrophage foam

cell formation, and smooth muscle cell metastasis and proliferation (2). Vascular endothelial cells (VECs) are attached to the lining of blood vessels and can engulf foreign bodies, bacteria, and necrotic and aging tissues. Also, they contribute to herd immunity, and their damage and dysfunction are the initiating factors that lead to AS (3).

Key words: miR-138-5p; SIRT1; vascular endothelial cell; oxidized low-density lipoprotein; inflammatory response

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MicroRNAs (miRNAs), a type of non-coding RNAs about 18-24 nucleotides long, are widely found in cells. Although miRNAs have no protein-coding function, they can modulate genes at the post-transcriptional level by binding the 3'untranslated region (3'UTR) of messenger RNAs (mRNAs) (4). In recent years, increasing studies have revealed that miRNAs are implicated in regulating Ox-LDL-mediated VEC damage. For example, miR-106b (5), and miR-590 (6), are down-regulated in Ox-LDL-induced VECs. Up-regulating miR-106b and miR-590 attenuates Ox-LDL-mediated cell viability reduction and apoptosis. Nevertheless, the influence of miR-138-5p on Ox-LDL-mediated cell damage remains elusive.

Sirtuin1 (SIRT1) is an epigenetic enzyme involved in the deacetylation of histones and non-histones. It is mainly used as a metabolic sensor to respond to energy state changes via key transcription factors and cofactor deacetylation. In this way, sirtuin modulates mitochondrial function, biogenesis, oxidative stress, inflammation, cell death, and cellular senescence (7). It is worth noting that SIRT1 directly dampens the NF- κ B signaling by deacetylating the p65 subunit of the NF- κ B complex (8). On the other hand, knocking down CPEB1 lessens Ox-LDL-induced EC apoptosis and inflammation by activating the SIRT1 pathway (9).

Here, we tested the miR-138-5p expression in the plasma of AS patients and probed the role of miR-138-5p in regulating Ox-LDL-mediated VECs damage through *in-vitro* experiments. Our findings substantiate that the miR-138-5p expression is enhanced in AS patients and Ox-LDL-induced VECs. Moreover, miR-138-5p hampers SIRT1 and activates the NF- κ B pathway, which is an essential transcription factor controlling inflammation (10-11). Consequently, we hypothesized that miR-138-5p exerts a vital role in Ox-LDL-induced VEC damage.

MATERIALS AND METHODS

Clinical sample collection

The blood of 50 AS patients and 50 healthy donors who visited the Foshan Hospital of TCM from January 2019 to July 2019 were collected, and the plasma was separated and stored at -80°C for later use. In addition, the general

clinical data of patients, LDL, HDL, total cholesterol, triglyceride and LDH levels in the plasma were collected. These AS patients all had acute myocardial infarction (AMI), unstable angina (UA) or stable angina (SA) and were diagnosed with atherosclerotic cardiovascular disease (ASCVD) by coronary angiography with at least 50% stenosis of the main left coronary artery or 75% stenosis of the major epicardial artery (12). The exclusion criteria included diabetes, cancer, or infection. The ethics committee of the Foshan Hospital of TCM authorized this study, and all patients signed an informed consent.

Cell culture and treatment

EC-304 cells were bought from the American Type Culture Collection (ATCC) and grown in a complete Dulbecco's modified Eagle's medium-F12 (DMEM-F12, Invitrogen Life Technologies, Carlsbad, CA, USA) comprising 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Beyotime technology, Shanghai, China) in a constant temperature incubator (5% CO₂, 37°C). miR-138-5p mimics, inhibitors, and the relevant negative controls (miR-NC and miR-in) were all acquired from Guangzhou Ribo Biotechnology Co., Ltd (Guangzhou, China). SIRT1 siRNAs and its negative control oligo (si-NC) were all from GenePharma (Shanghai, China). Human Ox-LDL was provided by Guangzhou Yiyuan Biotechnology Co., Ltd (Guangzhou, China). Ox-LDL was dissolved in PBS buffer and then applied to treat EC-304 cells (25 to 200 μ g/mL ox-LDL, 24 h) for subsequent experiments.

Cell transfection

EC-304 cells (1 $\times$ 10⁵ cell/well) in the logarithmic growth phase were seeded in 24-well plates. Twenty-four h later, when the cells covered 70% of the well, the culture medium was discarded, and the cells were transfected with miR-138-5p mimics, or inhibitors, or si-SIRT-1 using the Lipofectamine 3000 (Catalog No. L3000-015, Invitrogen, California, USA) following the manufacturer's standard protocol. Opti-MEM®. Low serum medium (Catalog No. 31958-062, Invitrogen, California, USA) was adopted for the dilution of Lipofectamine 3000, miR-138-5p mimics, inhibitors, and si-SIRT-1. After 24 h, the miR-138-5p or SIRT1 mRNA level was checked by reverse transcription-polymerase chain reaction (RT-PCR) to verify the transfection validity.

Cell counting kit-8 (CCK-8) assay

EC-304 cells were subjected to inoculation in 96-well plates (5×10^3 cells/well) and further culturing for 24 h. Afterward, the primary medium was discarded, and 10 μ L CCK-8 solution (Beyotime Biotechnology Co., Ltd., Shanghai, China) and 100 μ L DMEM-F12 medium (the ratio of CCK-8 to the medium was 1:10) was added. After 24 h of incubation in a standard incubator, each well's absorbance value at 450 nm was measured. Five repetitive wells were set for each experiment, and the experiment was repeated three times.

RT-PCR

EC-304 cells and the patient plasma were collected. The TRIzol lysis buffer (Invitrogen, California, USA) was adopted to lyse the cells on ice. The total RNA was extracted with chloroform-isopropanol-75% ethanol and dissolved with double distilled water containing 1% Diethyl pyrocarbonate (DEPC). The RNA concentration and purity were monitored by GENESYS™ 30 Visible Spectrophotometer (Thermo, Waltham, MA, USA). By observing the instructions of the RevertAid First Strand cDNA Synthesis Kit (Catalog No.K1621, Thermo, Waltham, MA, USA), the reverse transcription reaction was performed at 70°C for 10 min, 5 min on ice, 42°C for 60 min, 95°C for 5 min, and 0°C for 5 min. Then, the obtained cDNA was amplified. The volume of the RT-PCR system was 25 μ L, comprising 500 ng cDNA template, 250 nmol/L forward and reverse primers, and 12.5 μ L of 2 $\times$ SYBR Green PCR Master mixture (Thermo, Waltham, MA, USA). The primer sequences were as follows:

TNF- α , forward, 5'-ATGAGCACTGAAAGCATGATC-3', reverse 3'-TCACAGGGCAATGATCCCAAAGT-5'; IL-1 β , forward, 5'-ATGGCAGAAGTACCTAAGCTC-3', reverse, 3'-TTAGGAAGACACAAATTGCATGGTGAAGTCACT-5'; IL-6, forward, 5'-ATGAACTCCTTCTCCACAAGC-3', reverse, 3'-CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG-5'; SIRT1, forward, 5'-GCAGATTAGTAGGCGGCTTG, 3', reverse, 3'-TCTGGCATGTCCCACTATCA-5'; miR-138-5p, forward, 5'-AACCGGAGCTGGTGTGTGA-3', forward, 5'-CAGTGCAGGGTCCGAGGT-3'; β -actin served as the endogenous control of TNF- α , IL-1 β , IL-6 and SIRT1, while U6 was that of miR-138-5p. The value of $2^{-\Delta\Delta Ct}$ signified the relative gene expression.

Western blot (WB)

After the treatment, the EC-304 cells were harvested and lysed on ice with RIPA lysis buffer (Beyotime

Biotechnology Co., Ltd., Shanghai, China). After fully lysing for 30 min, the cells were centrifuged (14,000 rpm, 4°C, 15 min). Subsequently, 5 μ g of total protein were loaded on 12% polyacrylamide gel for electrophoresis at 100 V for 2 h. The protein was transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% skimmed milk at room temperature (RT) for one h and flushed three times (10 min/time) with TBST. Afterward, the membranes were maintained with the primary antibodies, including anti-SIRT1 (1:1500, ab), anti-Caspase3 (1:1500, ab32351), anti-Bcl2 (1:2000, ab32124), anti-Bax (1:2000, ab32503), anti-p-NF- κ B p65 (1:1000, ab76302), anti-NF- κ B (1:1000, ab32536), anti-p-STAT6 (1:1000, ab18808), anti-STAT6 (1:1500, ab217998), anti-COX2 (1:1000, ab179800), anti-iNOS (1:1000, ab178945), anti-MMP3 (1:1000, ab52915), anti-MMP9 (1:1500, ab76003), anti-ICAM1 (1:1000, ab109631), anti-VCAM1 (1:1000, ab134047), and anti-LOX1 (1:1000, ab214427) (Abcam, USA) overnight at 4°C. After the membranes were cleared with TBST, they were subjected to incubation with the horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG (concentration 1:3000) for 1 h at RT. Next, the membranes were rinsed three times with TBST (10 min each time). The Pierce ECL Western blot Substrate kit (Catalog No. 32109 Thermo Pierce) was utilized for X-ray development, and Image J was employed to determine each protein's gray intensity.

Dual-luciferase reporter assay

The SIRT1 sequences were amplified and transferred to the downstream luciferase gene of pmirGLO plasmids (Promega, Fitchburg, WI, USA) to construct PMIR-SIRT1-WT. At the same time, SIRT1 fragments containing the mutant target region (pMIR-SIRT1-MT) were designed based on the binding sites between SIRT1 and miR-138-5p presumed by Targetscan (http://www.targetscan.org/vert_72/). Afterward, pMIR-SIRT1-WT, pMIR-SIRT1-MT, miR-138-5p mimics and miR-NC were co-transfected into EC-304 cells with lipofectamine®3000 (Catalog No. L3000-015, Invitrogen, California, USA). Forty-eight h following the transfection, the relevant luciferase activity was tested in the Promega dual-luciferase reporter gene system (Fitchburg, WI, USA).

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

The BrdU method was adopted to test cell viability.

EC-304 cells were seeded in 24-well plates (1×10^5 cells/well) and transfected with miR-138-5p mimics, inhibitors, and si-SIRT-1 for 24 h. Following treatment with Ox-LDL (100 $\mu\text{g/mL}$) for 24 h, the cells were incubated with 10 μM BrdU (Catalog. No. 40204ES60, Yeasen, Guangzhou, China) for 60 min at 37°C . Next, the complete medium was discarded, and the cells were rinsed with PBS three times and secured with 4% paraformaldehyde at 4°C for 24 h. Subsequently, the fixative was removed, and the cells were cleared with PBS (three times, 5 min each time), followed by blocking with 5% BSA at RT for 1 h and incubation with the rabbit anti-BrdU antibody (1:200, ab6326, Abcam) at 4°C for 12 h. The next day, the cells were flushed three times with PBS (5 min each time) and incubated with the Goat Anti-Rabbit IgG H&L (FITC) for 2 h at RT. Afterward, they were cleaned with PBS three times (5 min each time). For nucleus staining, 200 μL DAPI stain solution (Catalog.No. C1005, Beyotime Biotechnology Co., Ltd., Shanghai, China) was added to the plates, incubated at RT for 5 min, then the cells were washed with PBS three times (5 min each time). Finally, an Olympus fluorescence microscope was applied to observe and photographed BrdU-positive cells. Image J was employed for BrdU positive cell counting. Five representative fields of vision under high magnification were randomly selected. The proportion of the BrdU-positive cell number to the total cell number was taken as the positive rate of BrdU. The average value of three

repetitive wells was taken to indicate the proliferative ability of tumor cells.

Flow cytometry (FCM)

The Annexin V-FITC/PI Apoptosis Detection Kit (Catalog. No. 40302ES60, Yeasen, Guangzhou, China) was used for testing cell apoptosis. EC-304 cells from different treatment groups were taken, and the cell suspension was inoculated into 6-well plates (2000 μL /well, 1×10^5 cells/mL). Following trypsinization with EDTA-free trypsin, the cells were harvested. Then, they were cleaned twice with pre-cooled PBS, and 1×10^5 of the cells were harvested. Afterward, 100 μL 1 $\times$ Binding Buffer was added for cell resuspension, and 5 μL AnnexinV-FITC and 5 μL PI staining solution was supplemented and mixed gently and reacted away from light at RT for 10 min. After that, 400 μL 1 $\times$ Binding Buffer was added and mixed well. The sample was tested with CytoFLEX flow cytometry (Beckman Coulter, USA) three times within 1 h, and the average was taken.

Tube formation experiment

Ninety-six-well plates were coated with Matrigel (Catalog. No. 356234, BD Company) and incubated at 37°C for 30 min. Then, 10,000 EC cells were seeded in the 96-well plates and maintained at 37°C . After 6 h, the culture was terminated. The cell status was observed using an Olympus IX51 microscope (Olympus, Japan),

Table I. Clinical features of healthy donors and AS sufferers

Clinical parameters	Healthy controls (n = 50)	AS patients (n = 50)	P value
Age (years)	53.65 $\pm$ 7.98	53.98 $\pm$ 7.28	0.829
Gender (male/female)	24/26	29/21	0.316
BMI (kg/m^2)	24.49 $\pm$ 1.69	24.82 $\pm$ 1.81	0.348
Total cholesterol (mol/L)	3.989 $\pm$ 0.565	4.265 $\pm$ 0.768	0.043*
HDL (mol/L)	1.29 $\pm$ 0.181	0.957 $\pm$ 0.152	<0.0001*
LDL (mol/L)	2.86 $\pm$ 0.453	3.45 $\pm$ 0.796	<0.0001*
Triglyceride (mg/dl)	1.71 $\pm$ 0.212	2.12 $\pm$ 0.328	<0.0001*
LDH (U/L)	187.4 $\pm$ 32.6	297.6 $\pm$ 42.7	<0.0001*

* $p < 0.05$. AS: atherosclerosis; BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; LDH: lactic dehydrogenase

and the tube length was analyzed using Image J-pro (v6.0, National Institutes of Health, USA).

Statistical analysis

Data were analyzed with SPSS 17.0 and expressed as mean $\pm$ deviation ($\bar{x}\pm s$). One-way analysis of variance and LSD test was used for the comparison of mean differences between multiple groups, and Student's *t*-test was employed for comparing two-group data. Pearson linear regression was adopted to dissect the correlation between miR-138-5p and LDL levels in AS patients. $P<0.05$ indicated a statistical difference.

RESULTS

Expression characteristics of miR-138-5p in AS patients and Ox-LDL-treated VECs

We implemented RT-PCR to monitor the miR-138-5p profile in the plasma of AS sufferers and healthy controls. The outcomes disclosed that miR-138-5p was up-regulated in AS patients (vs healthy

controls) (Fig. 1A). The total cholesterol, HDL, LDL, Triglyceride, and LDH levels in the serum were tested, and the data confirmed that AS patients had a higher level of those blood lipid indexes (compared with healthy controls, Table I, Fig. 1 B, C). Further linear regression uncovered that the miR-138-5p profile was positively related to the LDL level but reversely linked to the HDL contents in the plasma of AS patients ($P<0.001$, Fig. 1 D, E). Furthermore, we tested the miR-138-5p profile in VECs under the treatment of different concentrations of Ox-LDL. As a result, Ox-LDL up-regulated miR-138-5p dose-dependently (Fig. 1F). Thus, miR-138-5p potentially contributes to Ox-LDL-mediated EC dysfunction.

Impact of miR-138-5p on Ox-LDL-mediated VECs viability and angiogenesis

VECs were transfected with miR-138-5p mimics and inhibitors to clarify the role of miR-138-5p in Ox-LDL-mediated VECs injury. Then, cell viability was examined by the CCK-8 method and

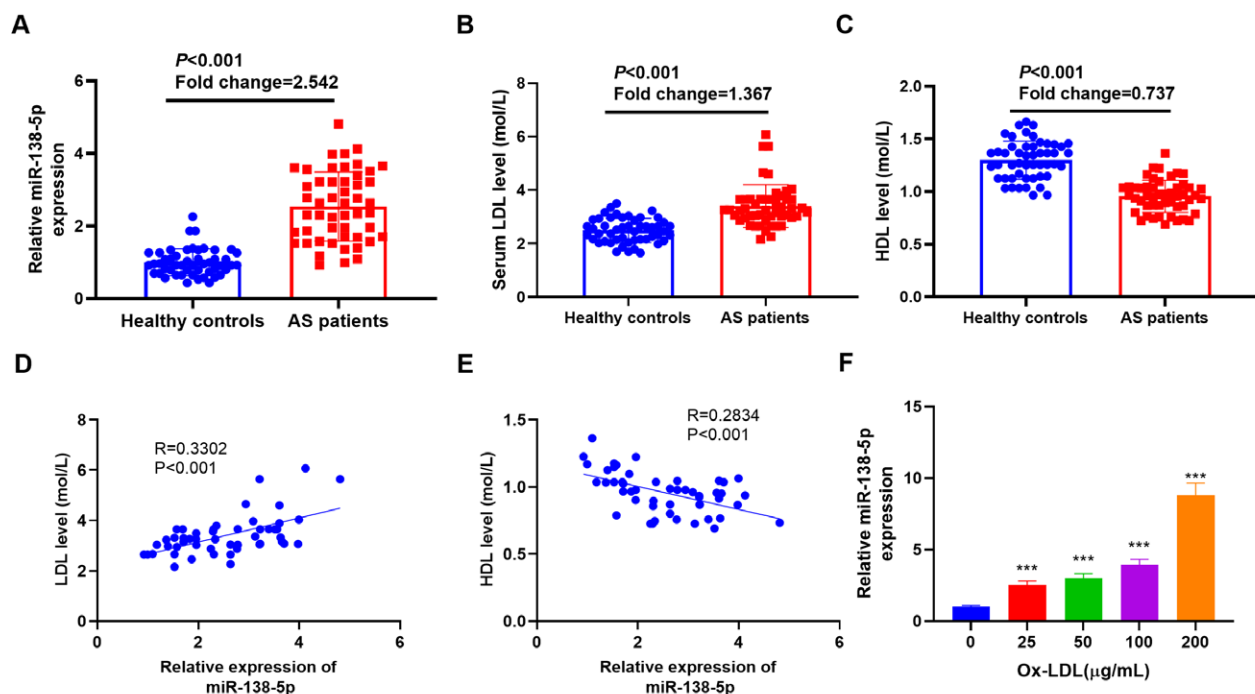


Fig. 1. The miR-138-5p level in patients' serum and VECs. **A)** The miR-138-5p level in 50 healthy controls and 50 AS patients was tested by RT-PCR; **B, C)** The levels of LDL and HDL in the serum of 50 healthy controls and 50 AS patients. **D, E)** The correlation between miR-138-5p and LDL levels, as well as between miR-138-5p and HDL level in AS patients; **F)** The miR-138-5p profile in EC-304 cells treated with Ox-LDL (25-200 $\mu\text{g/mL}$) for 24 h. *** represents $P<0.001$.

BrdU assay. Results displayed that Ox-LDL abated VECs viability (*vs* the control group) (Fig. 2 A, B). Meanwhile, in contrast to the Ox-LDL+miR-NC group, the miR-138-5p mimics' transfection curbed VEC viability, while miR-138-5p inhibitors enhanced VEC viability (Fig. 2 A, B). Next, the tube formation experiment was conducted to test the tube formation of VECs. Compared with the control group, Ox-LDL mediated a substantial reduction in the tube-forming ability of VECs, and this effect was enhanced by miR-138-5p overexpression. However, miR-138-5p inhibitors elevated the tube-forming ability of VECs compared with that of the Ox-LDL group (Fig. 2C). In addition, the MMP3, MMP9, ICAM1, VCAM1 and LOX1 in VECs were tested by WB. It was revealed that Ox-LDL up-regulated MMP3, MMP9, ICAM1, VCAM1 and LOX1 (*vs* the control group). Forced up-regulation of miR-138-5p further aggravated those protein levels (*vs* Ox-

LDL+miR-NC group). On the contrary, inhibition of miR-138-5p restrained those mediators (compared with the Ox-LDL+miR-in group) (Fig. 2 E, F). These data confirmed that miR-138-5p aggravated Ox-LDL-mediated VEC damage and dysfunction.

miR-138-5p aggravated Ox-LDL-mediated VEC apoptosis

We tested the efficacy of miR-138-5p in Ox-LDL-mediated VEC apoptosis. The results confirmed that in comparison to the control group, Ox-LDL increased the apoptotic rate of VECs (Fig. 3A), repressed the Bcl2 expression, and elevated the levels of Caspase3 and Bax in VECs (Fig. 3 B, C), indicating that Ox-LDL facilitated VECs apoptosis. Besides, miR-138-5p mimics enhanced VECs apoptosis (*vs* the Ox-LDL+miR-NC group), while miR-138-5p inhibitors bridled VECs apoptosis (*vs* the Ox-LDL+miR-in group) (Fig. 3 A-C), illustrating that miR-138-5p aggravated Ox-LDL-mediated VEC apoptosis.

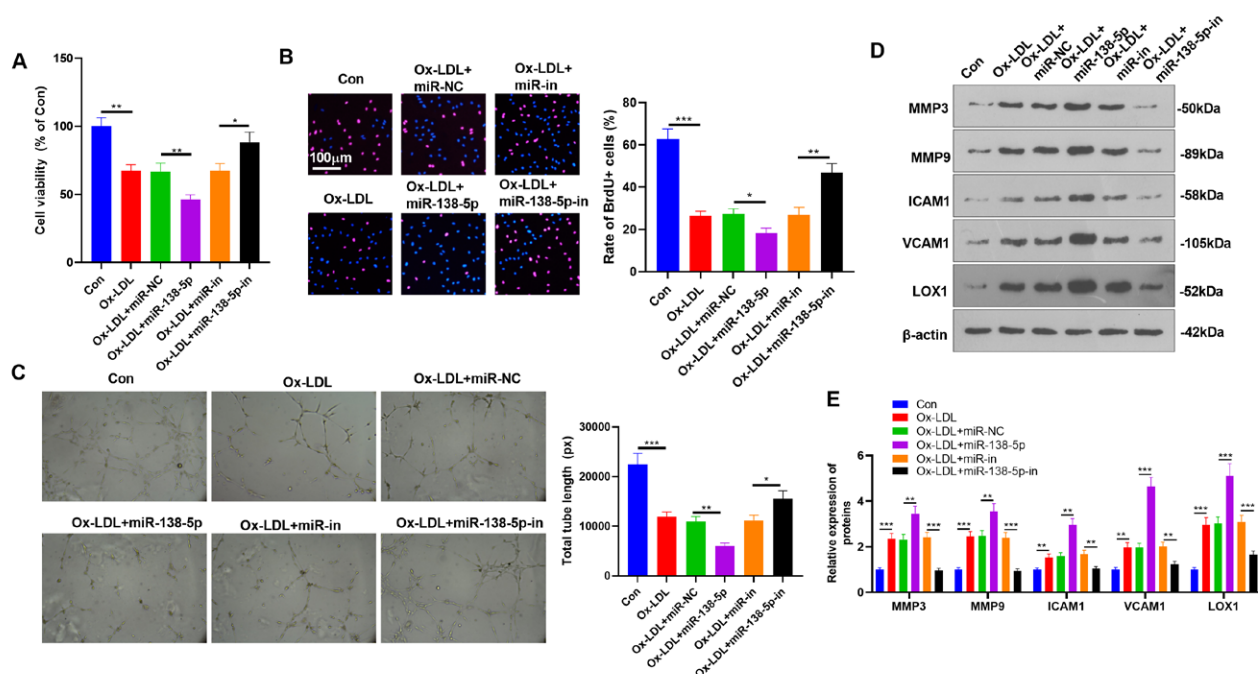


Fig. 2. The function of miR-138-5p in Ox-LDL-induced EC damage. The EC-304 cells were treated with Ox-LDL (100 μg/mL) and transfected with miR-138-5p mimics or inhibitors. **A, B)** Cell viability was monitored by the CCK-8 experiment and BrdU assay, respectively. **C)** The tube formation assay was conducted to examine the angiogenesis of EC-304 cells. **D, E)** WB was used for testing MMP3, MMP9, ICAM1, VCAM1 and LOX1 in EC-304 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Function of miR-138-5p in Ox-LDL-mediated VEC inflammation

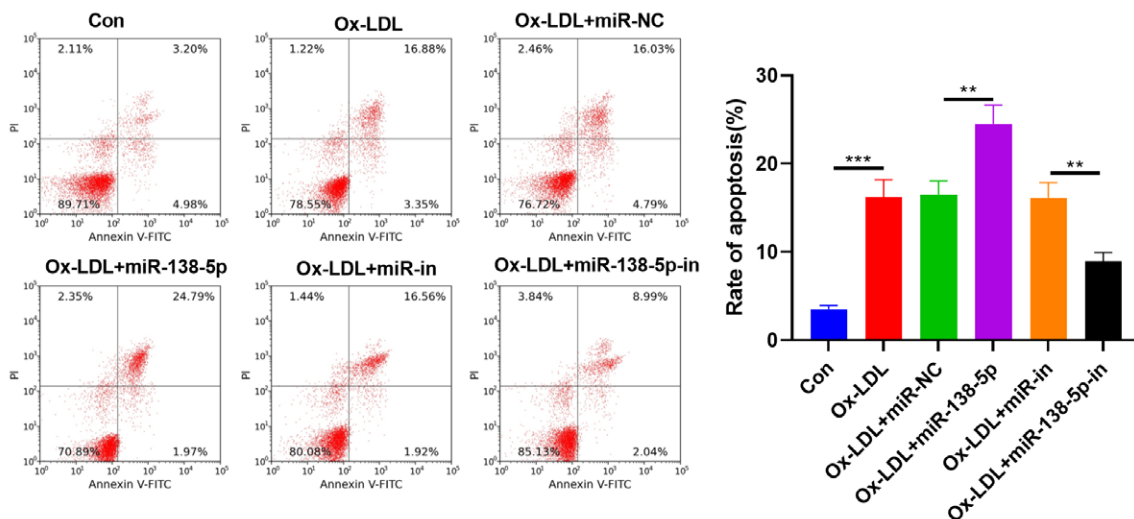
We gauged the expression of inflammatory factors and signaling pathways in VECs to probe the mechanism of miR-138-5p in regulating VEC damage. As a result, Ox-LDL facilitated the inflammatory factors (TNF- α , IL-1 β , and IL-6), inflammatory proteins (COX2 and iNOS), and NF- κ B but down-regulated STAT6 phosphorylation in VECs. Meanwhile, miR-138-5p mimics heightened the levels of TNF- α , IL-1 β , IL-6, COX2, iNOS and NF- κ B and down-regulated p-STAT6 compared

with the Ox-LDL+miR-NC group. On the contrary, the miR-138-5p inhibitors' transfection exerted the reverse effect (*vs* the Ox-LDL+miR-in group) (Fig. 4 A-C). These outcomes confirmed that miR-138-5p aggravated Ox-LDL-mediated VEC inflammation.

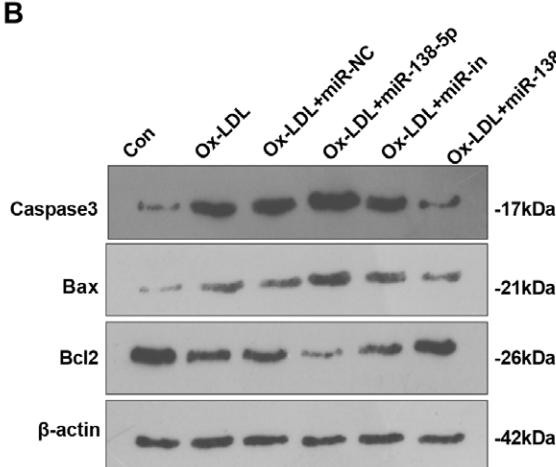
SIRT1 was the functional target of miR-138-5p

The bioinformatics databases miRmap, microT, miRanda, Pictar, and Targetscan were browsed to analyze the targets of miR-138-5p (Fig. 5A). Interestingly, there were 85 gene candidates, among which SIRT1 is important (Fig. 5B). Furthermore,

A



B



C

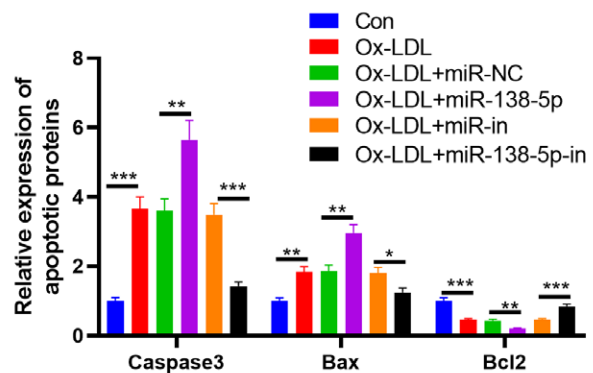


Fig. 3. The function of miR-138-5p in Ox-LDL-induced VECs apoptosis. The EC-304 cells were treated with Ox-LDL (100 μ g/mL) and transfected with miR-138-5p mimics or inhibitors. **A)** FCM was conducted to evaluate the apoptotic levels of ECs. **B)** WB was employed to gauge apoptotic markers (including Caspase3, Bcl2, and Bax); **C)** The gray intensity of the bands was analyzed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

we implemented a dual-luciferase reporter assay to verify the targeting association between miR-138-5p and SIRT1. The results demonstrated that miR-138-5p mimics declined the luciferase activity of cells transfected with SIRT1-WT reporter gene vectors but had no significant impact on that of SIRT1-MT (Fig. 5C). We verified the impact of miR-138-5p's selective regulation on the SIRT1 expression with RT-PCR and WB to testify the role of miR-138-5p-mediated inflammation. It was manifested that overexpressing miR-138-5p hindered the mRNA profile of SIRT1 (Fig. 5D). Meanwhile, Ox-LDL treatment led to the SIRT1 pathway inactivation (vs the control group). Furthermore, overexpressing miR-138-5p impeded the SIRT1 expression, while

knocking down miR-138-5p exerted the reverse effect (vs the corresponding control group) (Fig. 5E). These findings suggested that SIRT1 was targeted by miR-138-5p.

SIRT1 knockdown reversed miR-138-5p inhibitor-mediated protective effects against Ox-LDL

Aiming at confirming SIRT1's role in miR-138-5p, we transfected VECs with miR-138-5p inhibitors or si-SIRT1. WB outcomes exhibited that inhibiting miR-138-5p enhanced the SIRT1 level, which was repressed after si-SIRT1 transfection (vs the Ox-LDL+miR-in group, Fig. 6A). Next, we tested cell viability and apoptosis. It was revealed that down-regulating SIRT1 reduced

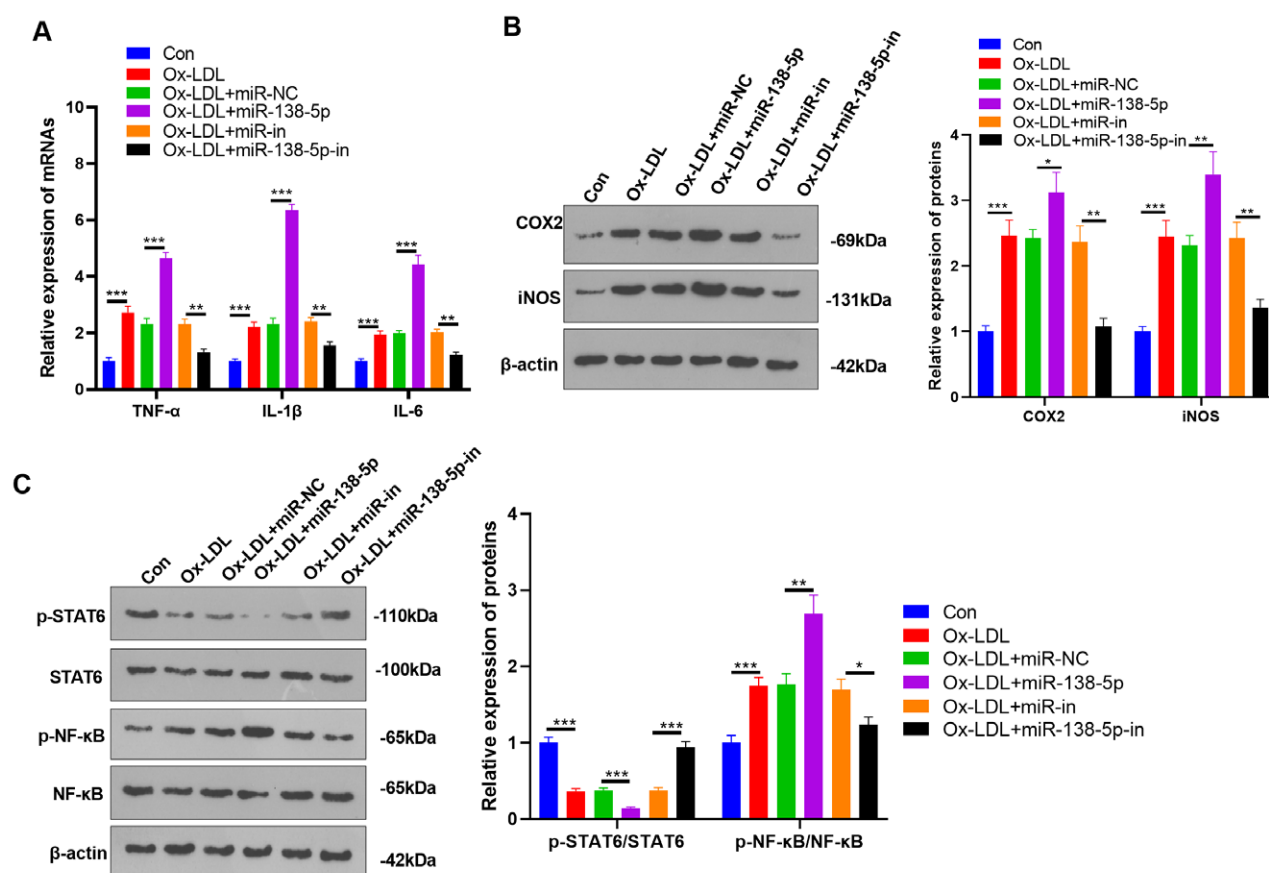


Fig. 4. The impact of miR-138-5p on Ox-LDL-induced VEC inflammation. The EC-304 cells were treated with Ox-LDL (100 µg/mL) and transfected with miR-138-5p mimics or inhibitors. **A)** RT-PCR was adopted to verify TNF-α, IL-1β, and IL-6; **B)** WB was adopted to determine the contents of COX2 and iNOS in the cells. **C).** WB was conducted to determine the expression of STAT6 and NF-κB and their phosphorylated levels in the cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

cell viability and the Bcl2 level while boosted the Caspase3 and Bax expression (vs Ox-LDL+miR-in group, Fig. 6 B, C). The inflammatory reactions of VECs were evaluated. The data suggested that following si-SIRT1 addition, levels of MMP3,

MMP9, ICAM1, VCAM1, LOX1, TNF- α , IL-1 β , IL-6, COX2, and iNOS were all enhanced (vs the Ox-LDL+miR-in group, Fig. 6 D-F). Moreover, SIRT1 knockdown reduced STAT6 levels but enhanced NF- κ B phosphorylation (compared with

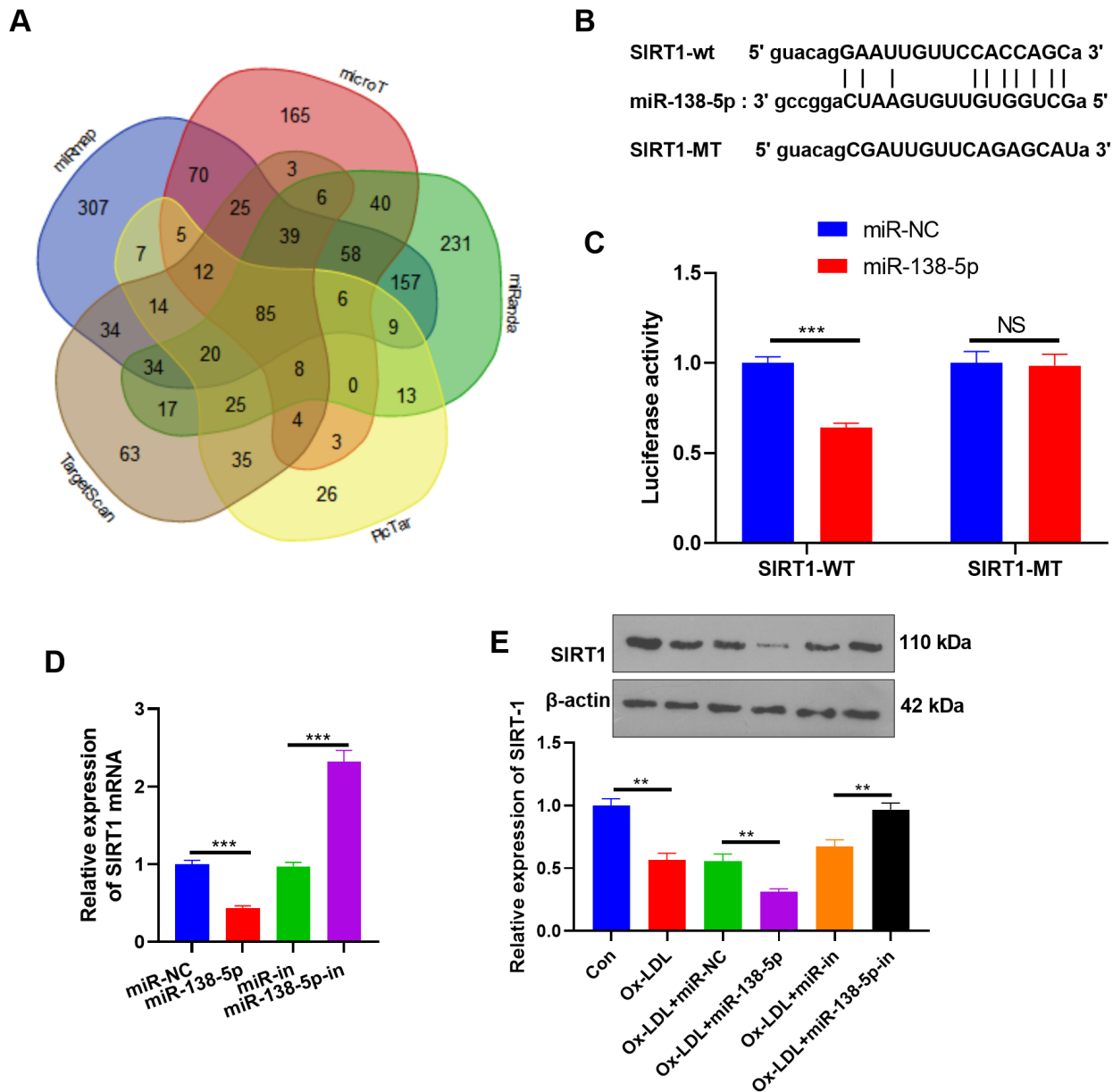


Fig. 5. *SIRT1* was targeted by miR-138-5p. **A)** The candidate targets of miR-138-5p were predicted via miRmap, microT, miRanda, Pictar and Targetscan, and the co-targeted genes were analyzed by Venn Diagram. **B)** The binding sites between *SIRT1* and miR-138-5p. **C)** Dual-luciferase reporter assay was implemented to examine the association between *SIRT1* and miR-138-5p. **D)** The EC-304 cells were transfected with miR-138-5p mimics or inhibitors, and the *SIRT1* mRNA expression was monitored by RT-PCR. **E)** The EC-304 cells were treated with Ox-LDL (100 μ g/mL) and transfected with miR-138-5p mimics or inhibitors. The *SIRT1* expression was monitored by WB. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

the Ox-LDL+miR-in group, Fig. 6G). Therefore, SIRT1 knockdown reversed miR-138-5p inhibition-induced protective effects by enhancing apoptosis and inflammation.

DISCUSSION

This article explores the function of miR-138-5p in Ox-LDL-mediated VEC injury. Our results manifest that down-regulating miR-138-5p

attenuates VEC damage by hindering Ox-LDL-mediated inflammation and oxidative stress through mediating the SIRT1/NF- κ B pathway.

As an important barrier to blood vessel inner walls, VECs contribute to protecting blood vessels. The up-regulated Ox-LDL-mediated VEC damage in AS patients has been confirmed by increasing studies (13). Diversified scavenger receptors that mediate Ox-LDL cell effects are expressed in VECs. Lectin-like oxidized low-density lipoprotein receptor

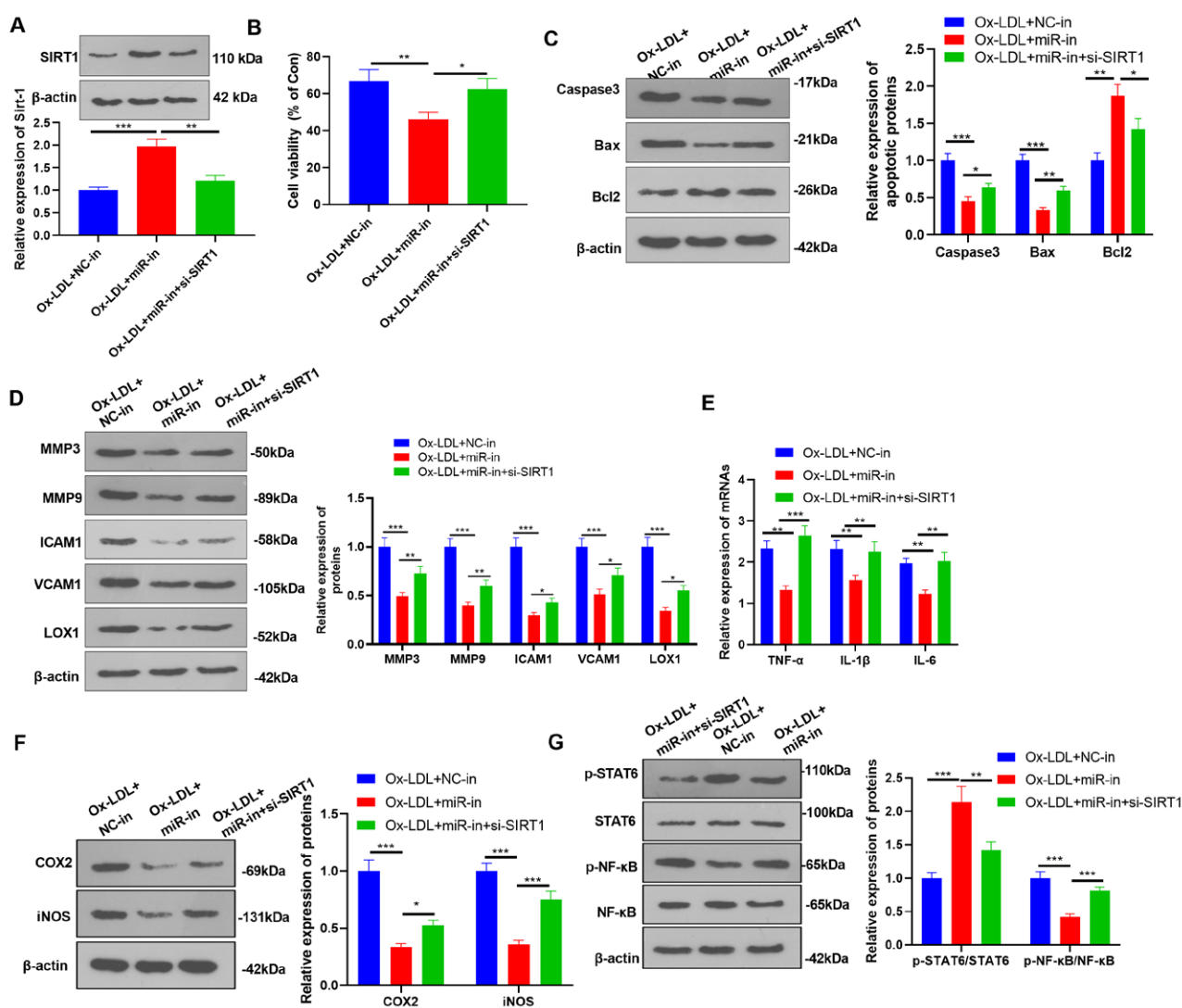


Fig. 6. The effects of SIRT1 knocking down on miR-138-5p inhibitor-mediated protective effects against Ox-LDL. EC-304 cells were transfected with miR-138-5p inhibitor (miR-in), the negative control (NC-in), or si-SIRT1, and then treated with Ox-LDL (100 μ g/ml). **A)** WB was implemented for gauging the SIRT1 level. **B)** CCK-8 was adopted for testing cell viability. **C, D)** WB tested apoptosis-related proteins (including Caspase3, Bax, and Bcl2), MMP3, MMP9, ICAM1, VCAM1, LOX1. **E)** RT-PCR was adopted for evaluating TNF- α , IL-1 β , and IL-6 in EC-304 cells. **F, G)** The COX2, iNOS, STAT6, and NF- κ B levels were assessed by WB. * P <0.05, ** P <0.01, *** P <0.001

1 (LOX-1) is the dominating Ox-LDL receptor on VECs, and it is also expressed in macrophages and smooth muscle cells. LOX-1 is up-regulated after exposure to several pro-inflammatory and pro-atherosclerotic stimulation, and it aggravates VEC damage by activating multiple inflammation signaling pathways (14). Here, we tested the LOX-1 expression in VECs after Ox-LDL and discovered that Ox-LDL signally uplifted the LOX-1 level.

During AS, many damage-associated molecular patterns (DAMPs) are released into the circulation. Those DAMPs have the potential to motivate multiple inflammatory pathways, such as the toll-like receptor signaling, neutrophil extracellular trap formation and inflammasome formation (15-18). NF- κ B is a vital transcription factor with inflammation-regulating function in cells. Its activation is often induced by the DAMPs-mediated TLR4 signaling pathway. NF- κ B is phosphorylated and translocated into the nucleus, thus up-regulating diversified inflammatory cytokines, which further aggravate endothelial damage and apoptosis (19-20). For instance, MMP3 and MMP9, the vital members of MMPs, are augmented by NF- κ B p65 transcription. MMP3 and MMP9 are released into the extracellular matrix and induce endothelial cell apoptosis and tight junction disruption (21-22). In addition, as key proteins in the inflammatory response and oxidative stress, ICAM1, VCAM1, COX2 and iNOS are up-regulated in Ox-LDL-induced EC damage, and hampering their expression reduces EC damage (23-24). In this study, we found that VEC apoptosis increased and cell viability decreased after Ox-LDL treatment. At the same time, TNF- α , IL-1 β , IL-6, MMP3, MMP9, ICAM1, VCAM1, COX2, iNOS, and NF- κ B levels increased in VECs, which further confirmed the overactivated inflammation and oxidative stress were implicated in EC damage.

Multiple miRNAs contribute to Ox-LDL-regulated VEC damage. As one miRNA, miR-138-5p regulates various cellular biological processes. For example, miR-138-5p exerts a tumor-suppressive effect in lung adenocarcinoma (25) and glioma (26), showing inhibition on cell proliferation, metastasis, epithelial-mesenchymal transition, etc. Of special note, miR-138-5p modulates cell damage and

inflammation by targeting SIRT1. For instance, miR-138-5p is overexpressed in myocardial ischemia-reperfusion injury, which intensifies cardiomyocyte apoptosis through targeted inhibition of SIRT1-PGC-1 α (27). Additionally, the up-regulated miR-138-5p regulates PTEN/PI3K/Akt by targeting SIRT1 and boosts the nucleus pulposus cell apoptosis of the intervertebral disc in TNF- α -induced intervertebral disc degeneration cells, thereby accelerating the intervertebral disc degeneration (28). Here, we discovered that the miR-138-5p expression was augmented in the plasma of patients with coronary heart disease and positively correlated with the plasma LDL level. *In vitro*, up-regulating miR-138-5p aggravated Ox-LDL-mediated VEC damage, apoptosis, and inflammation, while down-regulating miR-138-5p attenuated Ox-LDL-mediated VEC damage by weakening inflammation, which revealed the underlying effect of miR-138-5p in AS treatment.

As a key regulatory enzyme in cells, SIRT1 is implicated in inflammation-mediated VEC damage (29). Indeed, SIRT1 has exhibited outstanding values in preventing vascular injury, both through pharmacological intervention and through targeted molecular modulation (30-31). Here, we verified that miR-138-5p is one of the upstream regulatory molecules of SIRT1, and its down-regulation elevates the SIRT1 expression, thus attenuating the profiles of NF- κ B, COX2, and iNOS. Besides, we discovered that the STAT6 phosphorylation was curbed with the up-regulation of SIRT1. Previous studies have demonstrated that Sirt1 changes the polarization of macrophages through the PI3K/Akt/STAT6 pathway and alleviates the crystalline inflammation of monosodium urate (32), indicating that the anti-inflammatory effect of SIRT1 is closely related to STAT6. STAT6, as an essential transcriptional regulator in the JAK-STAT pathway, has been confirmed by multiple studies to ease inflammation by abating the NF- κ B phosphorylation (33-34). These results affirmed that the anti-inflammatory effect of SIRT1 was achieved through up-regulation of STAT6 to hinder NF- κ B-mediated inflammation and oxidative stress in Ox-LDL-mediated VEC injury.

Overall, this study revealed a new regulatory pathway, namely the miR-138-5p-SIRT1-STAT6-

NF- κ B axis. Under the treatment of Ox-LDL, the up-regulated miR-138-5p attenuated the SIRT1 profile, resulting in decreased STAT6 phosphorylation and activated NF- κ B. Subsequently, the intensified inflammation and oxidative stress aggravated the VEC damage. In conclusion, this study explores the molecular mechanism of VEC damage in AS, which is expected to bring a new theoretical reference for treating AS. Nonetheless, further experiments are needed to investigate the functions of miR-138-5p in regulating AS progression *in vivo*.

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Molecular basis of therapeutic effects of β -hydroxybutyrate on cardiac hypertrophy

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This study was aimed to explore the molecular basis underlying the therapeutic effects of β -hydroxybutyrate in cardiac hypertrophy. We established a model of cardiomyocyte hypertrophy in H9c2 cells induced by angiotensin II (AngII). The anti-hypertrophic effects of β -hydroxybutyrate were evaluated by assessing cell apoptosis and expression levels of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and nuclear factor of activated T-cells 1 (NFATc1). The miRNA-mRNA network and associated functional analyses were performed based on miRNA sequencing data. Expression levels of six miRNAs involved in the miRNA-mRNA network were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). Eight mM β -hydroxybutyrate significantly attenuated AngII-induced cell apoptosis and significantly decreased the AngII-induced upregulation of ANP ($p < 0.01$), BNP ($p < 0.01$), and NFATc1 ($p < 0.01$). Based on the miRNA sequencing data, 1327 miRNA-mRNA pairs were screened, including miR-672-3p, miR-760-5p, miR-6325, miR-3583-3p, miR-3065-3p, and miR-3541. Functional analysis revealed that target genes were significantly enriched in four KEGG pathways, including the Wnt signaling pathway, tumor necrosis factor (TNF) signaling pathway, adherens junction, and Hippo signaling pathway. These genes were significantly enriched in eight biological processes, including protein deneddylation, amino acid transmembrane transport, and amino acid transport. Following treatment with β -hydroxybutyrate, the expression levels of miR-672-3p, miR-760-5p, miR-3583-3p, miR-3065-3p, and miR-3541 were significantly upregulated, and miR-6325 was significantly downregulated when compared with AngII treatment. Our data confirmed the protective role of β -hydroxybutyrate on cardiac hypertrophy, providing insights into observed therapeutic effects.

Cardiac hypertrophy is a threat to human health and primarily occurs in patients with end-stage heart disease. This disease is characterized by cardiomyocyte enlargement and cardiac muscle thickening, resulting in a reduced workload pressure on the ventricular walls. Several therapeutic approaches have been developed for treatment,

including stem cell and cardiac regeneration, dietary supplements, and pharmacological interventions. In recent years, RNA-based therapy using microRNAs (miRNAs) has gained considerable momentum.

Reportedly, miRNAs are vital regulators involved in several crucial intracellular signaling pathways in various diseases, including physiological and

Key words: β -hydroxybutyrate; cardiac hypertrophy; cell apoptosis

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pathological cardiac remodeling. For example, Yin et al. reported a decrease in miR-1 expression in patients with cardiac hypertrophy. Furthermore, they revealed that increased miR1 expression could inhibit the hypertrophic remodeling of cardiomyocytes by targeting nuclear factor of activated T-cells (NFAT). Heymans et al. demonstrated that miR-155 knockout markedly reduced cardiac hypertrophy and dysfunction in mice. Recent evidence has shown that miR-365 promotes the progression of cardiac hypertrophy by downregulating the expression of genes such as S-phase and kinase-associated protein 2. Moreover, the critical roles of other miRNAs such as miR-217, miR-29, and miR-200c have been reported. β -hydroxybutyrate can potentially inhibit lipolysis stimulation, which has been implicated in prolonging myocardial injury by upregulating cardiac mitochondrial uncoupling protein expression and downregulating glucose transporter 4 expression. Some miRNAs afford protection against cardiac hypertrophy; however, limited evidence is currently available. Further research is needed to clarify the roles of miRNAs in cardiac hypertrophy in association with β -hydroxybutyrate.

Thus, we explored the molecular basis underlying the therapeutic effects of β -hydroxybutyrate on cardiac hypertrophy. Accordingly, a model of cardiomyocyte hypertrophy, induced by angiotensin II (AngII), was constructed using H9C2 cells. Additionally, we evaluated the antihypertrophic effect of β -hydroxybutyrate based on miRNA sequencing data.

MATERIALS AND METHODS

Cell culture

In the present study, we used cardiac hypertrophy H9C2 cells (ATCC, CRL-1446TM), obtained from the Cell Center of Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA). The culture medium was supplemented with 1% penicillin, streptomycin, and 10% fetal bovine serum (FBS) at 37°C.

Construction of cardiac hypertrophy model

In brief, cells used for constructing the cardiac hypertrophy model were seeded at 3500 cells/well in 96-

well plates and incubated overnight at 37°C. Then, to select the optimal AngII concentration, AngII at different concentrations (0, 5, 8, 10, 12, and 15 μ M) were added to the cells. At 24 h and 48 h, fresh serum-free medium containing 10% CCK8 solution (C0039, Beyotime Biotechnology, Shanghai, China) was added to each well. The cells were further incubated for 1 h. Finally, the OD₄₅₀ value was detected using a microplate reader (Epoch, BioTek, USA), and the cell survival rate was calculated to determine the optimal AngII concentration.

Next, to determine the optimal concentrations of AngII and β -hydroxybutyric acid sodium salt (hereafter, β -hydroxybutyrate), the cells were divided into six groups: AngII treatment group, AngII combined with 2 mM β -hydroxybutyrate, AngII combined with 4 mM β -hydroxybutyrate, AngII combined with 8 mM β -hydroxybutyrate, AngII combined with 16 mM β -hydroxybutyrate, and a control group. After 24 and 48 h of culture, 10% CCK8 solution was added. After 1 h of incubation, the OD₄₅₀ value was determined, and the cell survival rate was calculated.

Apoptosis assay

The percentage of apoptotic cells was detected after drug treatment for 48 h using an Annexin V-FITC/PI Apoptosis Detection Kit (556547, BD, USA). Firstly, cells were collected in a 1.5 mL centrifuge tube and resuspended in 1 $\times$ binding buffer. Cells in the treatment groups were detected by dual staining with 5 μ L of FITC-Annexin V and 5 μ L of propidium iodide (PI, 50 μ g/mL). For the control group, cells were divided into no staining, double staining with FITC-Annexin V and PI, single staining PI, and single staining FITC-Annexin V. After incubation for 15 min in the dark at 25°C, data measurements were performed at 488 nm and 530 nm, and further analyzed using a flow cytometer (FACSCanto II, BD, USA).

High-throughput sequencing

Total RNA from samples was extracted using TRIzol reagent (9109, TaKaRa, Japan) in accordance with the manufacturer's instructions. RNA quality was detected using the Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, DE, USA), qualifying for library construction when the amount of RNA exceeded 1 μ g and OD₂₆₀/OD₂₈₀ ranged between 1.8-2.0.

RNA was first purified and subjected to fragmentation using the Truseq™ Small RNA sample prep Kit (Illumina, USA). Next, we synthesized the first cDNA strand using random hexamer primers. Finally, the library was

constructed after performing 3-end adenylation and adapter ligation. Next, the library was amplified (12 cycles of polymerase chain reaction [PCR]), purified using 6% Novex TBE PAGE gel and quantified using Picogreen on

Table I. The primer sequence for atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP), and activated T-cell nuclear factor 1 (NFATc1)

Name	Sequence (5'-3')
ANP-ratF	AAAGGCTGAGAGAGAAACCAGAG
ANP-ratR	CTCCTCCAGGTGGTCTAGCA
BNP-ratF	CTGCCCCAGATGATTCTGCTC
BNP-ratR	CAAAGCAGCTTGAACATATGTGCC
NFATc1-ratF	ATGTCTGGGAGATGGAAGCG
NFATc1-ratR	ATTGGCAGGAAGGTACGTGAA
GAPDH-ratF	AGACAGCCGCATCTTCTTGT
GAPDH-ratR	CTTGCCGTGGGTAGAGTCAT

Table II. The primer sequence for miRNAs

Name	Sequence (5'-3')
rno-miR-672-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGAAG
JH-rno-miR-672-3p-F	GACCACACACAGTCGCCAT
rno-miR-760-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGGCT
JH-rno-miR-760-5p-F	GACCCCTCAGGCCACCAG
rno-miR-6325-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCAG
JH-rno-miR-6325-F	GCCGAGAAAGTCAGACAGAGACT
rno-miR-3583-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCAT
JH-rno-miR-3583-3p-F	GCCGAGCCTGACTTCTCTCTTC
rno-miR-3065-3p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCCCA
JH-rno-miR-3065-3p-F	GCCGTCAGCACCAGGATATTGT
rno-miR-3541-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAGT
JH-rno-miR-3541-F	GCCGAGTCCCTCCCCCTC
rat-U6-RT	CGCTTCACGAATTTGCGTGTCAT
JH-R	GTGCAGGGTCCGAGGT
rat-U6-F	GCTTCGGCAGCACATATACTAAAAT
rat-U6-R	CGCTTCACGAATTTGCGTGTCAT

TBS380. Bridge PCR and high-throughput sequencing were conducted on the cBot and Illumina HiSeq 4000 platform, respectively.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the TRIzol reagent. After quantification and confirmation of RNA integrity, RNA samples were transcribed into cDNA according to the manufacturer's instructions. Then, cDNA was amplified using SYBR Green qPCR Master Mix (A25742, Thermo, Rockford, IL, USA). The relative RNA levels of genes associated with oxidative stress and apoptosis were calculated using the $2^{-\Delta\Delta C_t}$ method. The C_t value was defined as the number of cycles in which the fluorescent signal in the reaction tube reached the set threshold. For mRNA (atrial natriuretic peptide [ANP],

brain natriuretic peptide [BNP], and nuclear factor of activated T-cells 1 [NFATc1]), *GAPDH* was used as the reference gene, and the primer sequences are shown in Table I. For miRNA (rno-miR-672-3p, rno-miR-760-5p, rno-miR-6325, rno-miR-3583-3p, rno-miR-3065-3p, and rno-miR-3541), U6 was used as the reference gene, and the primer sequences are shown in Table II.

Bioinformatics analysis

The miRNA-target regulatory relationship was constructed using the miRanda software. ClusterProfiler (version 3.16.0, <http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) was used to analyze the enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, as well as Gene Ontology (GO) of miRNAs involved in the miRNA-target regulatory relationship. The functional

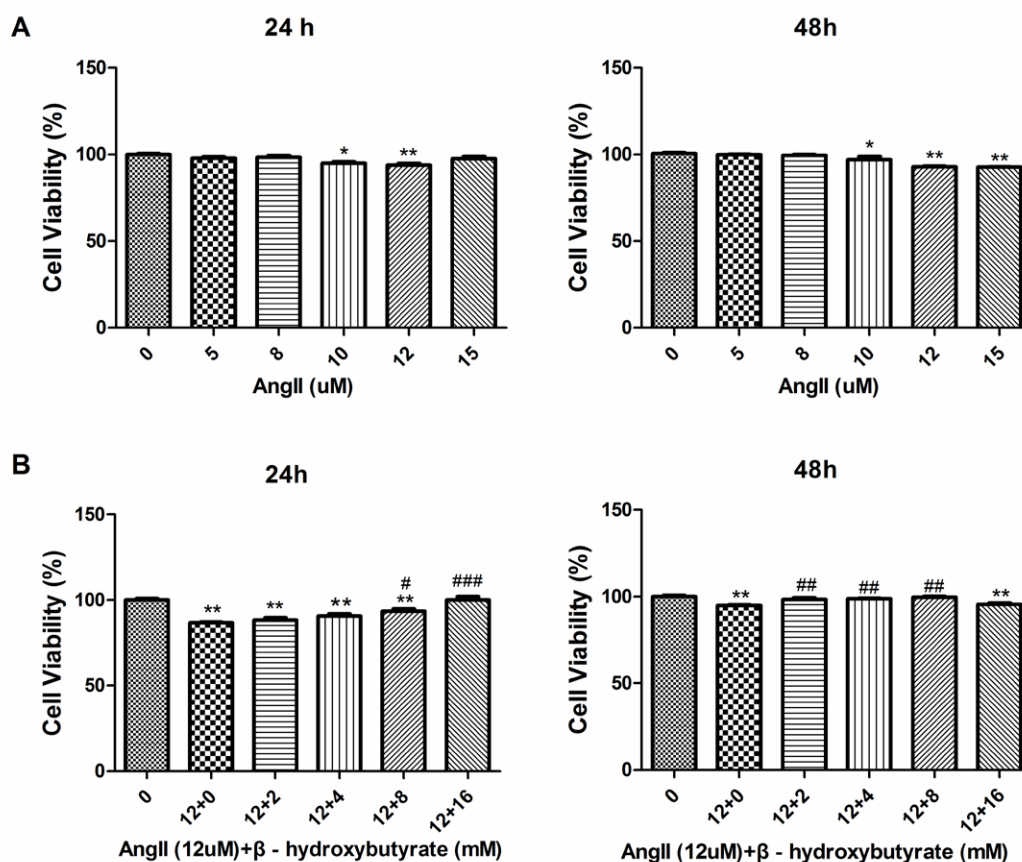


Fig. 1. CCK8 assay detects the effect of AngII and β -hydroxybutyrate treatment on the viability of rat cardiomyocytes H9C2. **A)** the viability of cardiomyocytes H9C2 treated with different concentrations of AngII for 24 h and 48 h. **B)** the viability of cardiomyocytes H9C2 (with 12 μ M AngII) treated with different concentrations of β -hydroxybutyrate for 24 h and 48 h. ** $p < 0.01$, * $p < 0.05$ vs control (H9C2 cells); # $p < 0.05$, ### $p < 0.01$ vs cardiomyocytes H9C2 (with 12 μ M AngII). CCK8: Cell Counting Kit-8; AngII: angiotensin II.

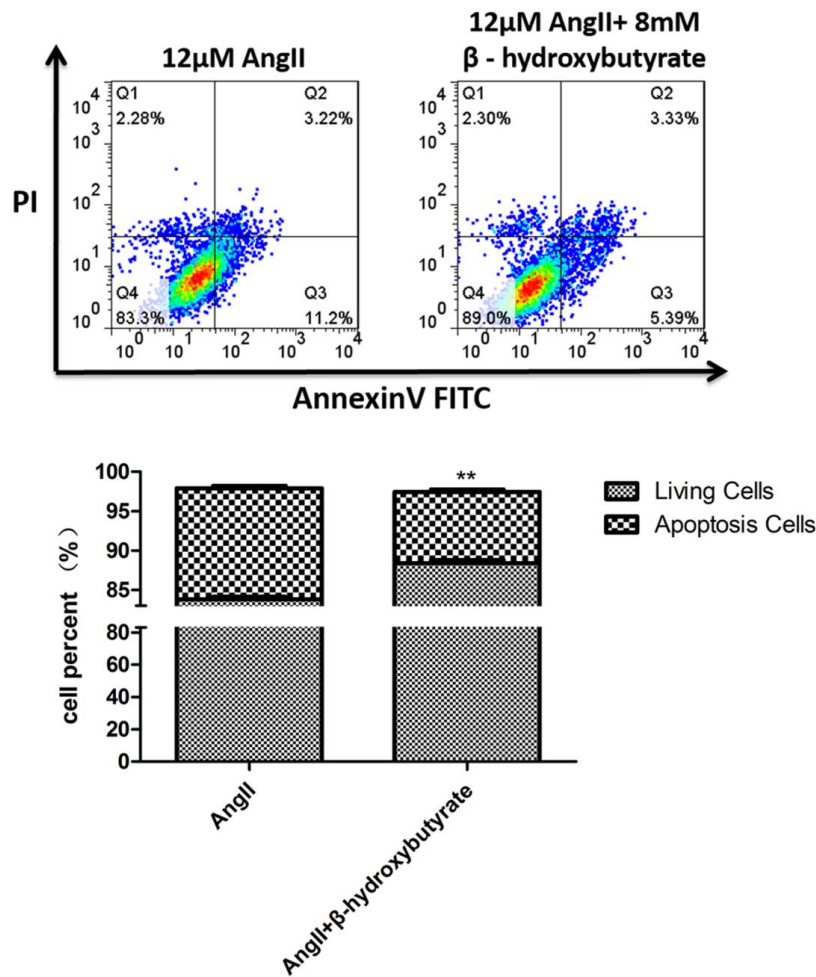


Fig. 2. H9C2 cells apoptosis treated with 12 μ M AngII and 12 μ M AngII + 8 mM β -hydroxybutyrate were analyzed by flow cytometry, respectively. ** p < 0.01 vs 12 μ M AngII treatment. AngII: angiotensin II.

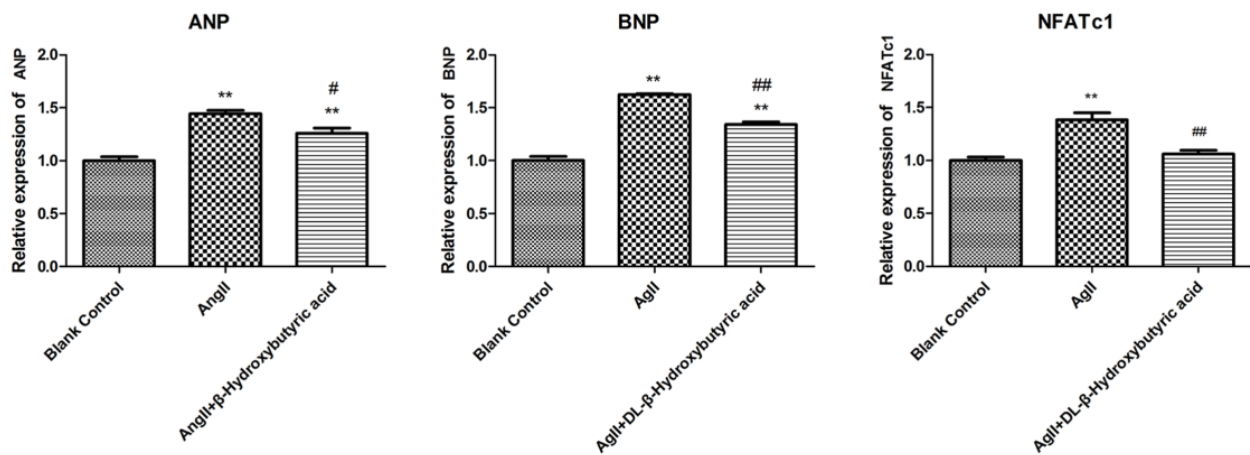


Fig. 3. The expression levels of ANP, BNP, and NFATc1 in blank control, AngII-induced model, and β -hydroxybutyrate treatment group. ** P < 0.01 vs blank control (H9C2 cells); # P < 0.05, ## p < 0.01 vs AngII-induced model. AngII: angiotensin II; ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; NFATc1: nuclear factor of activated T-cells 1.

pathways with parameter enrichment gene count ≥ 2 and P-value < 0.05 , after BH correction, were considered as a significant enrichment result.

The protein-protein interaction (PPI) network was generated using STRING (Version 11.0, <http://string-db.org/>), and Cytoscape (version 3.6.1, <http://www.cytoscape.org/>) was employed to illustrate the network. For the analysis, the PPI score was set at 0.9. Furthermore, transcription factor (TF) families associated with target genes were screened using the AnimalTFDB (<http://bioinfo.life.hust.edu.cn/AnimalTFDB#!/>).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). All data are presented as the mean $\pm$ standard deviation. One-way ANOVA and Newman-Keuls multiple comparison tests were used to detect differences between groups. $P < 0.05$ and $P < 0.01$ were set as the screening criteria for significant and highly significant differences.

RESULTS

Cardiac hypertrophy model and treatment concentration of β -hydroxybutyrate

The effect of different AngII concentrations and AngII combined with β -hydroxybutyrate on cell viability are shown in Fig. 1. After 24 h of Ang II treatment, H9C2 cell viability was significantly inhibited at the 10 μ M and 12 μ M concentration ($P < 0.05$). After AngII treatment for 48 h, H9C2 cell viability was significantly inhibited at the 10 μ M, 12 μ M, and 15 μ M, concentration ($P < 0.05$). Thus, 12 μ M AngII was selected as the appropriate concentration for establishing the cardiac hypertrophy model.

Furthermore, to determine the appropriate concentration of β -hydroxybutyrate, a series of β -hydroxybutyrate concentrations (2 mM, 4 mM, 8 mM, and 16 mM) were added to AngII-treated H9C2 cells; then, the effect of combination treatment on cell viability was assessed. After 24 h treatments of H9C2 cells (with 12 μ M AngII) with different concentrations of β -hydroxybutyrate, the viability of H9C2 cells treated with 2, 4, and 8 mM β -hydroxybutyrate was significantly lower than that of the control cells (H9C2 cells without AngII) ($P < 0.01$). In addition, after 24 h treatments of H9C2 cells (with 12 μ M AngII) with 8 and 12 mM of β -hydroxybutyrate, the viability of H9C2 cells was significantly higher than that of the 0 mM β -hydroxybutyrate group ($P < 0.05$). We also detect the viability of H9C2 cells (with 12 μ M AngII) treated with different concentration of β -hydroxybutyrate for 48 h. The results indicated that the viability of H9C2 cells treated with 2, 4 and 8 mM β -hydroxybutyrate was significantly higher than that treated without butyrate ($P < 0.01$), and there was no significant difference between the three concentration treated groups and control group.

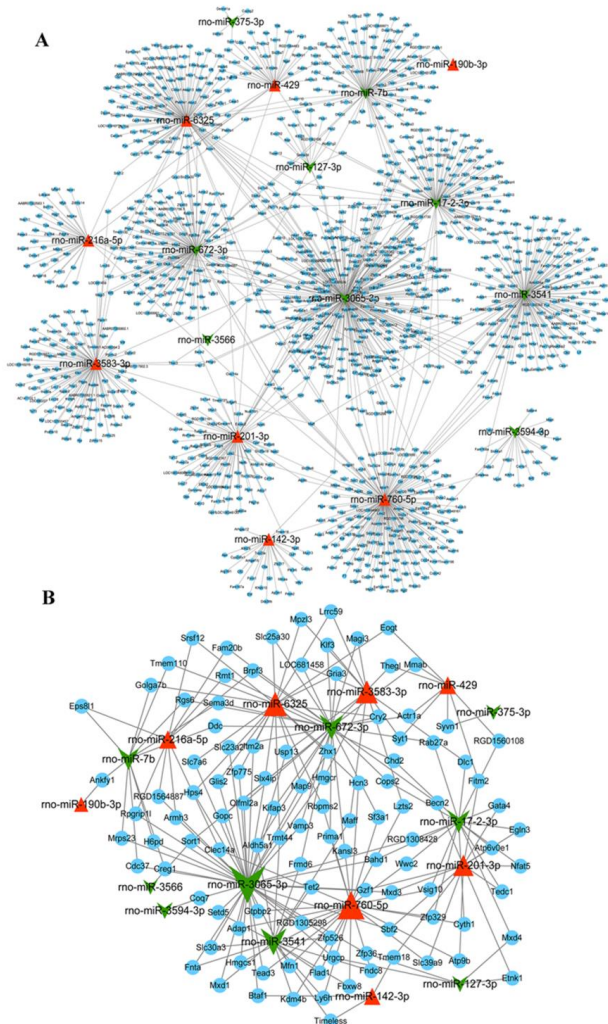


Fig. 4. miRNA-mRNA network and sub-network of miRNA-mRNA. A: miRNA-mRNA network; B: miRNA-mRNA sub-network. The red triangles represent upregulated miRNAs, the green inverted triangles represent downregulated miRNAs, and the blue circles represent Target genes.

Finally, 8 mM β -hydroxybutyrate was selected as the appropriate concentration for treatment the cardiac hypertrophy model.

Cell apoptosis level assessment

Fig. 2 presents the apoptosis levels in cells treated with AngII vs those treated with AngII combined with β -hydroxybutyrate. β -Hydroxybutyrate significantly attenuated Ang II-induced apoptosis ($P < 0.01$).

mRNA expression levels assessment

The expression levels of *ANP*, *BNP*, and *NFATc1* are shown in Fig. 3. Compared with the blank control, AngII treatment upregulated the expression levels of mRNAs, including *ANP* ($P < 0.01$), *BNP* ($P < 0.01$), and *NFATc1* ($P < 0.01$). Notably, following treatment with β -hydroxybutyrate, the expression levels of *ANP* ($P < 0.05$), *BNP* ($P < 0.01$), and *NFATc1* ($P < 0.01$) were significantly downregulated when compared with AngII treatment alone (Fig. 3).

miRNA-mRNA network and functional analysis

In total, 1327 miRNA-mRNA pairs were screened, including 1236 nodes (17 miRNAs and 1219 target genes). The miRNA-mRNA network and the sub-network with a degree greater than 1 are shown in Fig. 4, including miR-672-3p, miR-760-5p, miR-6325, miR-3583-3p, miR-3065-3p, and miR-3541.

Furthermore, functional analysis revealed that target genes were significantly enriched in four KEGG pathways, including the Wnt signaling pathway, the tumor necrosis factor (TNF) signaling pathway, adherens junction, and the Hippo signaling pathway (Fig. 5A). Additionally, these genes were significantly enriched in eight biological processes, including protein deneddylation, amino acid transmembrane transport, and amino acid transport (Fig. 5B).

The PPI network included 1074 pairs associated with 391 mRNAs, and the network with the top 50 degrees is shown in Fig. 5C. Moreover, the network of TF-target genes, including 32 TFs, is shown in Fig. 5D. The network revealed that zfc-C2H2 could regulate 39 target genes.

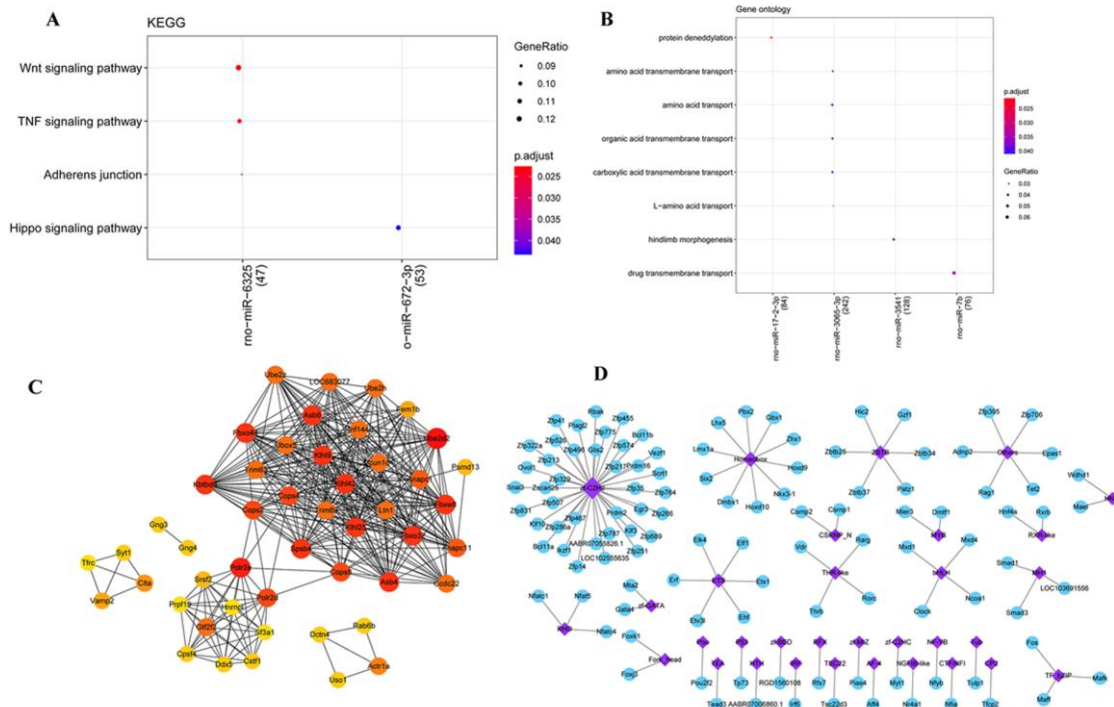


Fig. 5. The functional analysis of target genes. **A)** KEGG enrichment; **B)** biological process enrichment; **C)** protein-protein interaction network of genes with top 50 degrees. The color ranges from red to yellow, representing the degree; **D)** transcription factor-target network; purple represents TF, blue represents target gene. KEGG, Kyoto Encyclopedia of Genes and Genomes.

miRNA expression levels evaluation using qRT-PCR

The expression levels of miR-672-3p, miR-760-5p, miR-6325, miR-3583-3p, miR-3065-3p, and miR-3541 were detected using qRT-PCR. Compared with AngII treatment, expression levels of miR-672-3p, miR-760-5p, miR-3583-3p, miR-3065-3p, and miR-3541 were significantly upregulated following treatment with the β -hydroxybutyrate. Additionally, treatment with β -hydroxybutyrate significantly downregulated the expression of miR-6325 when compared with AngII treatment (Fig. 6).

DISCUSSION

Hypertrophic growth poses a significant threat to human health, subsequently resulting in cardiovascular

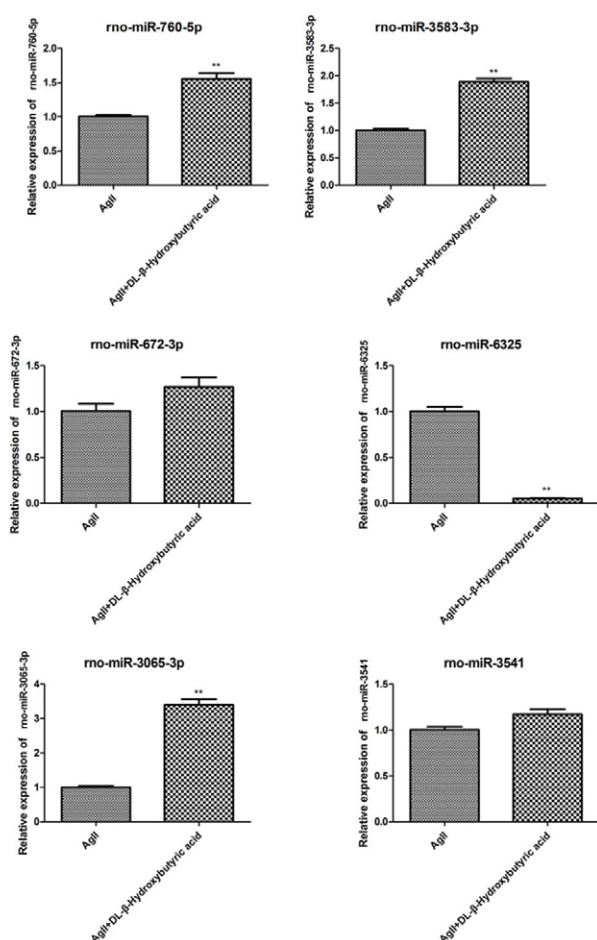


Fig. 6. Expression levels of miR-760-5p, miR-3583-3p, miR-672-3p, miR-6325, miR-3065-3p, miR-3541 after β -hydroxybutyrate treatment. ** $P < 0.01$ vs AngII-induced model. AngII: angiotensin II.

diseases. The protective role of β -hydroxybutyrate in cardiac hypertrophy has been previously reported. In this study, we used rat cardiomyocytes (H9C2) to construct an Ang II induced-cardiac hypertrophy model. AngII is a degradable peptide from the renin-angiotensin system and is one of the most powerful stimuli to induce cardiac hypertrophy and fibrosis. This model has been proven by previous studies to be a good alternative to animal-free products. After directly comparing the hypertrophic response of H9C2 cells with the hypertrophic response of primary rat neonatal cardiomyocytes stimulated by hypertrophic factors, the researchers found that H9C2 cells showed almost the same hypertrophic response as observed in primary cardiomyocytes.

Our study revealed that the β -hydroxybutyric significantly attenuated AngII-induced cell apoptosis, and significantly decreased AngII-induced upregulation of *ANP*, *BNP*, and *NFATc1*. It is widely known that *ANP*, *BNP*, and *NFATc1* are associated with hypertrophy, thus, we assessed the expression levels of these genes in cells induced by Ang II, with or without β -hydroxybutyrate treatment. ANP is a hypertrophy marker, which was found to increase both the mRNA and protein levels of the human hypertrophic heart. Pathological cardiac hypertrophy is often accompanied by interstitial and perivascular fibrosis, as well as apoptosis and the release of ANP/BNP. The above-mentioned cascade expression is triggered by NFAT which causes cardiac hypertrophy through nuclear ectopic. NFAT is considered to be an important mediator of multiple signal transduction pathways involved in the coordination of pathological stimuli for cardiac hypertrophy. As a member of the NFAT family, NFATc1 is an important transcription factor that is essential for the morphological development of heart valves. We demonstrated that treatment with the β -hydroxybutyrate significantly decreased AngII-induced upregulation of *ANP*, *BNP*, and *NFATc1*, suggesting the protective role of β -hydroxybutyrate in patients with cardiac hypertrophy.

Functional analysis based on miRNA sequencing data showed target genes mainly associated with the Wnt signaling pathway, the TNF signaling pathway, adherens junction, and the Hippo signaling pathway.

Furthermore, we revealed that genes associated with the β -hydroxybutyrate treatment were significantly enriched in four KEGG pathways, including the Wnt signaling pathway, the TNF signaling pathway, adherens junction, and the Hippo signaling pathway. Previously, it has been reported that various cellular and biological processes could be controlled via Wnt signaling, including cell adhesion, cancer development, and cell proliferation. Moreover, Rezvani et al. demonstrated the regulatory role of Wnt signaling in the development of cardiac hypertrophy. Similarly, the Hippo signaling pathway is reportedly involved in cell proliferation, survival, and differentiation, with a significant role proposed in cardiac development, homeostasis, and injury. Meanwhile, a critical pathogenic role in heart failure and cardiac hypertrophy has been widely reported, with anti-TNF therapy confirmed as a useful treatment strategy in these patients. Based on these findings, treatment with β -hydroxybutyrate could potentially afford protection against hypertrophy, due to the regulation of identified pathways. However, further experiments using rodent models are needed to clarify the possible underlying mechanisms.

miRNAs are vital regulators of various intracellular signaling pathways. By employing qRT-PCR analysis, we confirmed the upregulation of miR-672-3p, miR-760-5p, miR-3583-3p, miR-3065-3p, miR-3541, and downregulation of miR-6325 after β -hydroxybutyrate treatment. To date, various miRNAs have been proposed as attractive pharmaco-targets in cardiac hypertrophy, including miR-1, miR-133, and miR-10. Although the six miRNAs investigated in the present study have not been previously reported, we recommend that the potential role of these miRNAs in cardiac hypertrophy should be further investigated.

Previous study has shown that β -hydroxybutyrate increases in mice with heart failure and can attenuate ROS production, further reducing oxidative stress-induced apoptosis. Exercise has been shown to increase β -hydroxybutyrate levels, further increasing antioxidant capacity, therefore exercise-based cardiac rehabilitation programs may also be feasible. Another study also found that 3-hydroxybutyrate injected into the plasma of patients with heart failure has a beneficial effect on its hemodynamics, which further

proves that β -hydroxybutyrate may be used as a new therapeutic principle for the treatment of cardiac hypertrophy. There were also some limitations in this study. Firstly, this study was based on the results of *in vitro* cell line experiments, and more accurate rodent *in vivo* experiments will be necessary in the future. Secondly, when H9C2 cells were treated with high concentration β -hydroxybutyrate, it was found that the processing effect was opposite between 24 h and 48 h. This indicates that the action of β -hydroxybutyrate on H9C2 cells may have more complex mechanisms in different time periods, thus more experiments are needed in future studies.

In conclusion, we revealed that treatment with β -hydroxybutyrate significantly attenuates AngII-induced cell apoptosis and decreases hypertrophy-associated genes, including *ANP*, *BNP*, and *NFATc1*. Furthermore, our data highlighted the therapeutic effects of β -hydroxybutyrate in cardiac hypertrophy. Additional in-depth investigations are needed to clarify molecular mechanisms underlying the effects of β -hydroxybutyrate in patients with cardiac hypertrophy.

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Elevation of serum betatrophin in pregnant women with gestational diabetes mellitus

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The present study aimed to explore changes in serum betatrophin concentration in women with gestational diabetes mellitus (GDM) and its relationship with clinical metabolic indicators. The research also aimed to provide a basis for the in-depth study of the pathogenesis of GDM, together with a new target for the treatment of the condition and improving the methods for its prediction and diagnosis. A total of 60 participants were included of whom 30 were enrolled in the pregnancy with normal glucose regulation (P-NGR) group, and 30 in the GDM group. The clinical metabolic indicators in the two groups were measured and body mass index (BMI) was calculated. Additionally, two models were established, i.e., homeostasis assessment of insulin resistance (HOMA-IR), and homeostasis assessment of beta-cell function (HOMA- β). An enzyme-linked immunosorbent assay (ELISA) was used to detect the serum betatrophin concentrations in the two groups. The differences in the clinical metabolic indicators and betatrophin concentrations between the two groups were compared, and the correlation between betatrophin and clinical metabolic indicators was analyzed. The receiver operating characteristic (ROC) curve was used to predict GDM using the serum betatrophin concentration. The levels of triglyceride (TG) ($P = 0.001$), total cholesterol (TC) ($P = 0.004$), low-density lipoprotein (LDL) ($P < 0.001$), fasting blood glucose (FBG) ($P < 0.001$), fasting serum insulin (FSI) ($P = 0.028$), HOMA-IR ($P < 0.001$), HOMA- β ($P < 0.001$), and the concentration of betatrophin ($p < 0.001$) were significantly higher in the GDM compared with the P-NGR group, and the difference was statistically significant. The concentration of betatrophin was positively correlated with BMI ($P = 0.017$), TG ($P = 0.005$), TC ($P = 0.018$), FSI ($P = 0.023$), and HOMA-IR ($P = 0.022$) in the GDM group. Multiple linear regression analysis showed that HOMA-IR and TG could affect the serum betatrophin concentration. The best cut-off point of the serum betatrophin concentration predicted by the ROC curve in the case of GDM was 800.1pg/ml. The serum concentration of betatrophin in the GDM group was significantly increased and positively correlated with BMI, TG, TC, FSI, and HOMA-IR. Insulin resistance in the GDM population may be a mechanism involved in the overexpression of serum betatrophin; therefore, serum betatrophin could potentially be used as a biochemical indicator for predicting GDM.

Gestational diabetes mellitus (GDM) is a metabolic disorder that occurs among approximately 9.5–26.6% of global pregnancy cases (1). There are obvious differences in its incidence in various countries, based on differences related to geography,

race, and the living habits of the GDM population. The condition is closely correlated with increased fetal/maternal morbidity and complications for both the mother and her offspring (2).

In recent years, studies generally indicated that the

Key words: betatrophin; gestational diabetes mellitus (GDM); insulin resistance; triglyceride

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pathogenesis of GDM is primarily insulin resistance (IR). Existing studies reported that physiological IR during pregnancy was mainly due to the increased secretion of placental hormones (3, 4). However, more recent studies showed that the pathogenesis of GDM also included genetic (5, 6), inflammatory (7–9), and lipid factors (10, 11), as well as liver secretions (12).

Betatrophin is a multifunctional active polypeptide that is expressed in the liver and/or adipocytes. Early in 2004, the gene was submitted to the GenBank database (13) as “hepatocellular carcinoma-associated protein” (TD 26). Subsequently, the peptide (also known as angiopoietin-like protein 8 [ANGPTL8] (14, 15)) was discovered by various laboratories, re-feeding induced factors by fat and liver cells (RIFL) (13).

The primary sensor of blood glucose level is pancreatic β -cells, which, based on blood glucose level, can secrete insulin to regulate homeostasis (16). Although the pathogenesis of type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) is different, the recovery of pancreatic β -cells may be a possible treatment for various types of diabetes. Therefore, the proliferation of pancreatic β -cells is considered a new approach for the treatment of all types of diabetes and has aroused significant interest among researchers. Several studies have been conducted on changes in serum betatrophin concentration among T1DM and T2DM populations, and the results showed that, compared with the normal glucose metabolism populations, the serum betatrophin concentration was significantly increased in diabetic populations (17–20). Additional research has shown that the serum betatrophin concentration was significantly positively correlated with blood lipid levels and fasting serum insulin (FSI). However, scholars have suggested that the results of studies involving people with obvious IR are not consistent (21, 22). Although betatrophin is closely correlated with glucose metabolism, to date, no reports have been published on betatrophin deficiency as a cause of diabetes.

Most recently, studies chiefly focused on serum betatrophin expression in a T1DM or T2DM population; however, few reports exist on the

correlation between serum betatrophin and GDM. It remains unclear how the serum betatrophin concentration may change within the GDM population, and whether such a change may be correlated with HOMA-IR or HOMA- β . Only a few studies have been conducted on the correlation between the above two aspects, and their results are contradictory.

Accordingly, the present study aimed to investigate changes in the serum betatrophin concentration within the GDM population and the relationship of such changes with clinical metabolic indicators as a whole. It also aimed to clarify the correlation between the serum betatrophin concentration and IR/the pancreatic β -cell function, explore the pathogenesis of GDM, and find new therapeutic methods for its treatment. The study aimed to provide a theoretical basis for the correlation between serum betatrophin concentration and GDM and to present new ideas for the in-depth study of the pathogenesis and treatment of this condition.

MATERIALS AND METHODS

Subjects

The research participants in the present study were Chinese females with a gestational age of 24–28 weeks who attended the Obstetrics and Gynecology Clinic of Hongqi Hospital, affiliated to Mudanjiang Medical College (China) between September 2016 and September 2017.

All of the participants were asked about their medical history and living habits to exclude those who did not meet the inclusion criteria. Participants' medical history included their current (up-to-date) medical history, past history, family history of genetic disease, history of heart disease, and surgical and medication histories. Living habits included smoking and/or alcohol abuse. Medical staff conducted the relevant physical examinations on the participants to exclude other illnesses.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of the Anhui No. 2 Provincial People's Hospital (NO. 2016-385) and signed informed consent was given by all participants for their inclusion in the study.

The inclusion criteria for the current study were as follows. For the P-NGR group, women had to be in weeks 24–28 of pregnancy, aged 20–40 years, and of Han nationality. Participants also had to meet the normal glucose tolerance as measured by the 75 g oral glucose tolerance test (OGTT), i.e., fasting blood glucose (FBG) <5.1 mmol/L, 1-h postload plasma glucose (1 h-PG) <10.0 mmol/L after taking glucose, and 2-h postload plasma glucose (2h-PG) <8.5 mmol/L after taking glucose. For the GDM group, women had to be in weeks 24–28 of pregnancy, aged 20–40 years, and of Han nationality. Participants also had to meet the abnormal glucose tolerance as measured by the 75 g OGTT, i.e., FBG $\geq$ 5.1 mmol/L, 1 h-PG $\geq$ 10.0 mmol/L after taking glucose, and 2h-PG $\geq$ 8.5 mmol/L after taking glucose.

The current study rejected prospective participants

below the age of 20 and those older than 40 years. Patients with a history of DM or complicated with other diseases (hyperthyroidism, hepatitis, pancreatitis) that could affect blood glucose levels were also excluded.

Detection of clinical metabolic indicators

A total of 60 participants were included in the study (30 each in the P-NGR and GDM groups). The clinical metabolic indicators in the P-NGR and GDM groups were measured at 24–28 weeks of gestation.

Experimental principle of the study

Double antibody sandwich enzyme immunoassay was used to detect the serum betatrophin concentration of the participants. A microplate was coated with a purified antibody to create a solid-phase antibody. The

Table I. Comparison of the clinical metabolic indicators between the P-NGR group and the GDM group

Variate	P-NGR group	GDM group	P
Age (year)	29.20 $\pm$ 2.22	28.50 $\pm$ 1.91	0.195
Height (cm)	164.97 $\pm$ 3.10	164.57 $\pm$ 2.45	0.581
Body weight (kg)	67.50(64.00–70.00)	68.00(65.00–71.5)	0.267
BMI (kg/m ²)	24.81 $\pm$ 1.93	25.33 $\pm$ 1.42	0.242
Gestational age (week)	26.56 $\pm$ 1.00	26.32 $\pm$ 1.05	0.372
SBP (mm Hg)	130.30 $\pm$ 6.80	132.17 $\pm$ 8.10	0.338
DBP (mm Hg)	77.30 $\pm$ 7.45	78.87 $\pm$ 7.52	0.421
TG (mmol/L)	2.40(1.66–2.77)	2.99(2.03–4.40)	0.001*
TC (mmol/L)	5.02(4.60–6.01)	5.71(5.24–7.47)	0.004*
LDL (mmol/L)	2.97(2.88–3.17)	3.48(3.31–3.61)	<0.001*
HDL (mmol/L)	1.53(1.49–1.59)	1.53(1.48–1.62)	0.824
FBG (mmol/L)	4.35(4.22–4.49)	5.22(5.14–5.32)	<0.001*
FSI (μ IU/mL)	7.60(6.88–8.53)	8.85(7.18–9.50)	0.028*
HOMA-IR	1.50 $\pm$ 0.23	1.99 $\pm$ 0.40	<0.001*
HOMA- β	188.30(155.23–199.88)	89.56(77.23–104.06)	<0.001*

* $P < 0.05$, compared with P-NGR group

P: Pearson correlation; SBP: systolic blood pressure; DBP: diastolic blood pressure; TG: triglyceride; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; FBG: fasting blood glucose; FSI: fasting serum insulin

experimental standard or serum sample, biotinylated betatrophin antibody, and horseradish peroxidase (HRP) were added to the microplate coated with the solid-phase antibody HRP labeled avidin was washed thoroughly and then colored with 3,3',5,5'-tetramethylbenzidine (TMB). After catalyzing TMB using peroxidase, it turns a blue color that is visible to the naked eye; it subsequently turns yellow following acid treatment. During the experiment, the serum betatrophin concentration was higher if the color was dark, and lower if the color was light. The optical density was measured at 450 ± 2 nm and the concentration of serum betatrophin was calculated.

Detection of biochemical indicators

The participants in the two groups (women in stage 24–28 weeks of pregnancy) fasted for 8–12 h and were instructed to abstain from alcohol for 24–48 h. Fasting blood was collected from the elbow vein in the early morning of the following day to determine the FBG, FSI, triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels. Subsequently, 2 mL of serum was separated from all the samples following centrifugation and stored at -80°C .

Participants' BMI were measured as $(\text{kg}/\text{m}^2) = \text{weight} (\text{kg}) / \text{height}^2 (\text{m}^2)$. Insulin resistance was derived using the following equation: $\text{HOMA-IR} = \text{FSI} (\text{mU}/\text{L}) \times \text{FBG} (\text{mmol}/\text{L}) / 22.5$, $\text{HOMA-}\beta = 20 \times \text{FSI} (\text{mU}/\text{L}) / [\text{FBG} (\text{mmol}/\text{L}) - 3.5]$.

Detection of serum betatrophin concentration

After re-thawing the stored serum, the betatrophin concentration was determined by enzyme-linked immunosorbent assay (ELISA) kit for human hepatocellular carcinoma-associated protein TD26, E11644H (Wuhan Yiaibo Company).

Statistical analysis

All the data were entered into an Excel document, and the SPSS Statistics 24.0 software was used for conducting data analysis. The measurement data that obeyed the normal distribution were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$), and an independent sample *t*-test was used for comparison between groups. The Mann–Whitney U-test was employed for comparison between the non-normally distributed variables. Spearman's rank correlation coefficient was used to analyze the correlation between the

serum betatrophin concentration and the clinical metabolic indicators, and the difference was statistically significant ($P < 0.05$). Multiple linear regression analyses were performed with betatrophin as the dependent variable, and the relevant meaningful clinical metabolic indicators were used in the single-factor correlation analysis as independent variables. The receiver operating characteristic (ROC) curve was adopted for analyzing the predictive effect of serum betatrophin on GDM.

RESULTS

Comparison of the clinical metabolic indicators between groups

The levels of TG ($P = 0.001$), TC ($P = 0.004$), LDL ($P < 0.001$), FBG ($P < 0.001$), FSI ($P = 0.028$), HOMA-IR ($P < 0.001$), HOMA- β ($P < 0.001$), and betatrophin were significantly higher in the GDM compared with the P-NGR group, and the differences were statistically significant. There was no statistical significance in age difference ($P = 0.195$), height ($P = 0.581$), body weight ($P = 0.267$), BMI ($P = 0.242$), gestational age ($P = 0.372$), systolic blood pressure (SBP, $P = 0.338$), diastolic blood pressure (DBP, $P = 0.421$), and HDL ($P = 0.824$) between the two groups (see Table I).

Comparison of the serum betatrophin concentration between groups

The serum betatrophin concentration in the GDM group was significantly higher compared with the P-NGR group (861.32 ± 63.99 pg/mL vs 725.61 ± 152.80 pg/mL, $P < 0.001$), and the difference was statistically significant (Fig. 1).

Comparison of the correlation between the serum betatrophin concentration and the clinical metabolic indicators between groups

The serum betatrophin concentration was positively correlated with BMI ($r = 0.432$, $P = 0.017$), TG ($r = 0.504$, $P = 0.005$), TC ($r = 0.430$, $P = 0.018$), FSI ($r = 0.415$, $P = 0.023$), and HOMA-IR ($r = 0.417$, $P = 0.022$) in the GDM group. There was no correlation between the serum betatrophin concentration and other clinical metabolic indicators in the P-NGR group (Table II). The detailed correlations of serum betatrophin concentration with each factor are illustrated in Figs. 2–6.

Multiple linear regression analyses

Multiple linear regression analyses were performed using the serum betatrophin concentrations that were used as dependent variables in the P-NGR and GDM groups, and using BMI, TG, TC, FSI, and HOMA-IR, which were used as the independent variables, respectively. In the GDM group, TG and HOMA-IR entered into the multiple linear regression equation, and the results suggested that these two variables could affect the serum betatrophin concentration ($P < 0.05$). None of the above indicators entered into the equation ($P > 0.05$) in the P-NGR group (see Table III for details).

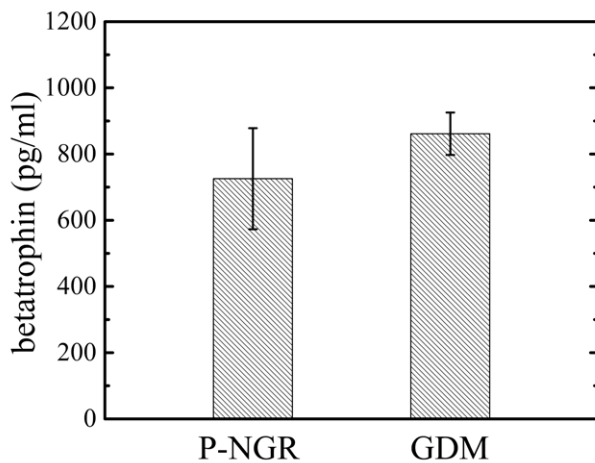


Fig. 1. Comparison of the serum concentration of betatrophin between the P-NGR group and the GDM group

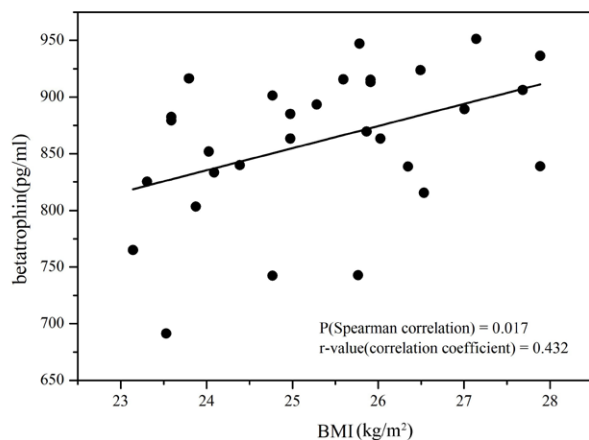


Fig. 2. Correlation between BMI and the serum concentration of betatrophin

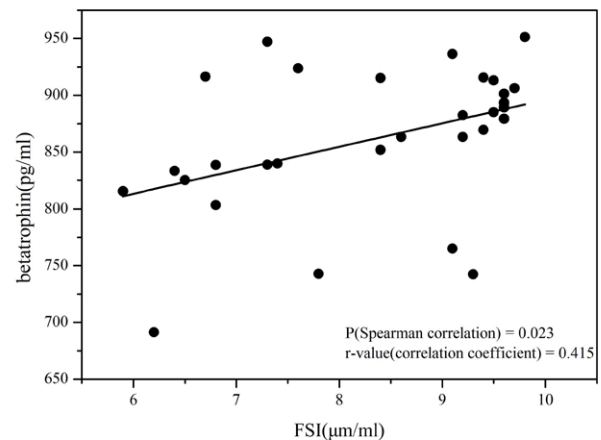


Fig. 3. Correlation between FSI and the serum concentration of betatrophin

The predictive effect of serum betatrophin on gestational diabetes mellitus

As shown in Fig. 7, the best cut-off point of serum betatrophin concentration for predicting GDM was 800.1 pg/ml, and the area under the ROC curve was 0.748 with a sensitivity of 86.7%, and specificity of 65%.

DISCUSSION

Few studies have investigated the correlation between serum betatrophin concentration and GDM. Researchers (21, 23) have different opinions regarding the correlations between serum betatrophin concentration and diabetes, BMI, and lipid metabolism. Betatrophin serum expression in the GDM population has not yet been clarified, and the relationship between serum betatrophin concentration and FBG, blood lipids, HOMA-IR, and various metabolic indicators has not been clearly explained. Consequently, further research is needed to determine whether serum betatrophin can effectively predict GDM and provide new treatments for this condition. Accordingly, by comparing the serum betatrophin concentration between the GDM and P-NGR groups in this study at the gestational age of 24–28 weeks, the correlation between the serum betatrophin concentration and the clinical metabolic indicators of GDM in the Chinese population was

investigated to explore the pathogenesis of GDM, as well as the predictive effect of serum betatrophin on the condition.

A total of 60 participants who met the inclusion criteria were enrolled in the present study (30 each in the GDM and P-NGR groups). The serum betatrophin concentration was significantly higher in the GDM group compared with the P-NGR group, and the difference was statistically significant. This result was consistent with that of a study conducted by Kong et al. (24). Since few studies have been completed on changes in serum betatrophin concentration in the GDM population, further

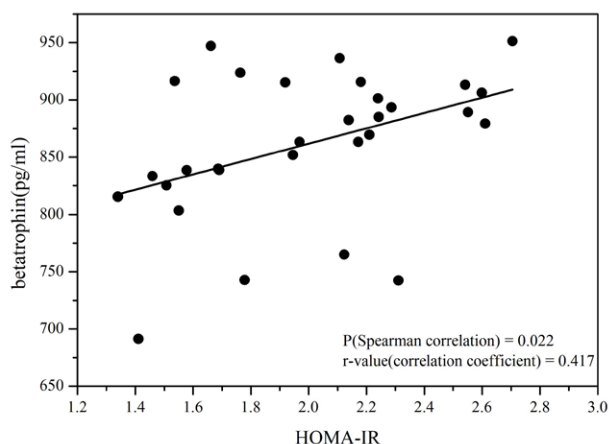


Fig. 4. Correlation between HOMA-IR and the serum concentration of betatrophin

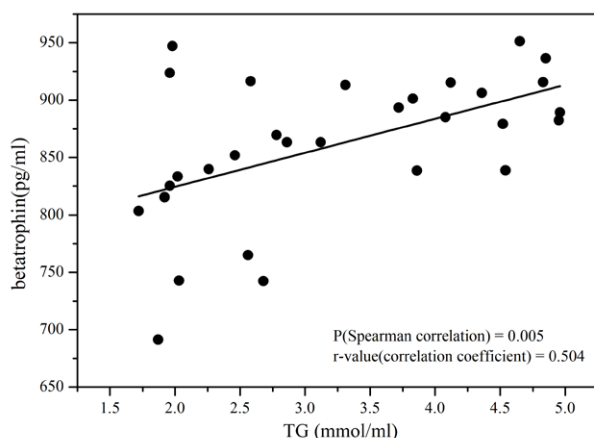


Fig. 5. Correlation between TG and the serum concentration of betatrophin

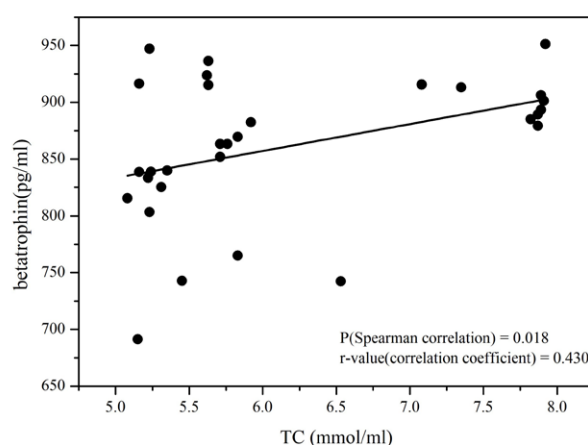


Fig. 6. Correlation between TC and the serum concentration of betatrophin

research is needed to clarify these changes.

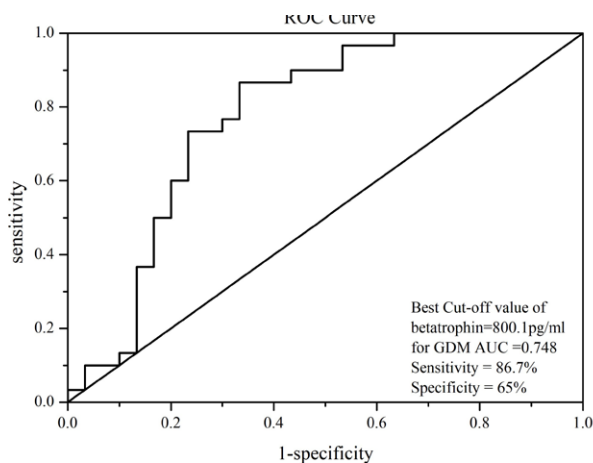
In the present study, the correlation analysis showed that serum betatrophin concentration in the GDM group was positively correlated with BMI. As shown in Table II, there was no difference in BMI between the two groups. Moreover, the correlation analysis showed that there was no correlation between the serum betatrophin concentration and BMI in the P-NGR group. This lack of correlation in the P-NGR group was consistent with the results of the above-noted studies, while the correlation between the two factors in the GDM group differed from the results of studies in other countries. Some studies (25–28) tested the content of visceral fat in European and Asian populations. After adjusting the factors affecting age and total fat content, Asian females in the United States reflected a higher visceral fat content compared with white females. However, the results showed no difference in visceral fat content among males. Moreover, some differences were found in the fat distribution between females in European countries and females in China. Compared with Europeans, Asians appeared more likely to accumulate visceral fat, while serum betatrophin was mainly secreted by the liver and adipose tissue. An increasing number of studies have shown that it is necessary to consider the impact of ethnic differences when using BMI to diagnose

Table II. The correlation analysis of the serum concentration of betatrophin with each clinical metabolic indicator between the P-NGR group and the GDM group

Variate	P-NGR		GDM	
	<i>r</i> value	<i>P</i> value	<i>r</i> value	<i>P</i> value
Age (year)	0.091	0.633	−0.256	0.171
Height (cm)	−0.168	0.374	−0.325	0.057
Body weight (kg)	0.029	0.879	0.233	0.215
BMI	0.106	0.578	0.432	0.017*
Gestational age (week)	−0.195	0.302	−0.055	0.771
SBP	0.018	0.924	0.136	0.473
DBP	0.353	0.056	−0.094	0.620
TG	0.224	0.233	0.504	0.005*
TC	0.038	0.841	0.430	0.018*
LDL	0.191	0.312	−0.174	0.359
HDL	−0.148	0.437	0.057	0.767
FBG	0.112	0.556	0.304	0.102
FSI	0.232	0.217	0.415	0.023*
HOMA-IR	0.188	0.319	0.417	0.022*
HOMA-β	0.052	0.785	0.143	0.449

* $P < 0.05$, compared with P-NGR group

P: Pearson correlation; BMI: body mass index; SBP: systolic; DBP: diastolic blood pressure; TG: triglyceride; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein; FBG: fasting blood glucose; FSI: fasting serum insulin.

**Fig. 7.** Predictive effect of serum betatrophin on GDM under the ROC

obesity. Accordingly, ethnic differences may play an important role in fat distribution, which may have led to the different results in our study.

In the present study, serum betatrophin concentration was positively correlated with FSI and HOMA-IR in the GDM group; however, there was no correlation in the P-NGR group in this regard. Erol et al. (1) found that serum betatrophin concentration increased in the GDM group, which was positively correlated with SBP, FSI, and HOMA-IR; however, no correlation was found in the P-NGR group.

In the present study, although the FBG in the GDM group was significantly higher than in the P-NGR group, there was no obvious correlation between

serum betatrophin concentration and FBG, which was consistent with the results of a study conducted by Espes et al. (19), in which it was suggested that hyperglycemia would not cause an increase in serum betatrophin concentration. In addition, there was a significant difference in HOMA-IR between the GDM and P-NGR groups in the present study, and the serum betatrophin concentration in the former was positively correlated with HOMA-IR, which supported the finding that IR may cause an increase in serum betatrophin concentration. The multiple linear regression analyses showed that HOMA-IR entered the equation in the GDM group and had an effect on the serum betatrophin concentration; this suggested that serum betatrophin in the case of GDM was correlated with insulin resistance, which verified the conclusion of Espes et al. (19) that IR may cause an increase in the secretion of serum betatrophin.

Betatrophin not only plays an important role in glucose metabolism, but also participates in lipid metabolism and has a significant influence on the regulation of TG. Research has shown that changes in the gene sequence encoding betatrophin (also known as ANGPTL8) were correlated with dyslipidemia. At present, there is no definitive conclusion regarding the correlation between serum betatrophin concentration and lipid metabolism. The results of the present study showed significant TG differences between the P-NGR and GDM groups. Correlation analysis showed that the serum betatrophin concentration

in the GDM group was positively correlated with TG. Multiple linear regression analyses indicated that TG entered the regression equation and that its concentration may have had an impact on serum betatrophin concentration. Erol et al. (1) found no significant differences in TG levels between GDM and P-NGR groups, and there was no correlation between serum betatrophin concentrations and TG in the two groups. In the present study, the positive correlation between serum betatrophin concentration and TG in the GDM group supported the findings of the above-noted studies. The inconsistency of the results in the study performed by Erol et al. (1) and those of the present study may have been due to ethnic differences among the participants. As such, the inconsistency in the results for the T2DM population requires further study.

In the current study, a correlation was observed between the serum betatrophin concentration and TC in the GDM group but not in the P-NGR group. Studies (21, 29) indicated that the serum betatrophin concentration in the T2DM population was significantly correlated with TC but that the two factors were not related in the non-diabetic group. Furthermore, among participants with insulin resistance, compared with those with normal TC levels, the serum betatrophin concentration was significantly higher in those with hypercholesterolemia. The results of the present study supported these findings. However, the present

Table III. The multiple linear regression analysis in the P-NGR group and GDM group

Variate	P-NGR			GDM		
	β	<i>t</i>	<i>p</i>	β	<i>t</i>	<i>p</i>
BMI	0.106	0.563	0.578	0.250	1.416	0.168
TG	0.224	1.219	0.233	0.523	0.523	0.003*
TC	0.038	0.202	0.841	0.126	0.607	0.549
FSI	0.176	0.946	0.352	0.117	0.530	0.600
HOMA-IR	0.188	1.014	0.319	0.518	0.533	0.005*

* $P < 0.05$, compared with P-NGR group

P: Pearson correlation; BMI: body mass index; TG: triglyceride; TC: total cholesterol; FSI: fasting serum insulin

study focused on GDM, while the above-noted studies mainly focused on the T2DM population, and there were substantial differences between the participants in those studies and the ones included in the present research. Serum betatrophin is involved in lipid metabolism; because there are differences in BMI and fat distribution among people of different races, these factors may have led to the different results of the current study.

The present study reflected a difference in the levels of LDL between the GDM and P-NGR groups, but there was no difference in the levels of HDL between the two groups. Correlation analysis found no link between serum betatrophin concentration, HDL, and LDL in the two groups. The study conducted by Erol et al. (1) also found no correlation between serum betatrophin concentration, HDL, and LDL between the same two groups. However, based on the lack of studies, the correlation between serum betatrophin concentration, HDL, and LDL in the GDM population and the normal glucose metabolism population is currently unclear and requires additional research.

Currently, there are reports on the prediction of non-alcoholic fatty liver and T2DM using serum betatrophin (30, 31). However, there are no reports concerning the prediction of GDM via serum betatrophin. The present study investigated whether serum betatrophin could predict GDM, and the results indicated that the best cut-off point for such prediction was 801.76 pg/ml, with an area under the ROC of 0.776, a sensitivity of 86%, and specificity of 76%. Kaestner (32) suggested that serum betatrophin as a new biochemical indicator may be better suitable for predicting T2DM. The results of the present study also indicated that serum betatrophin could be used as a new biochemical indicator for predicting GDM.

In summary, the results of the present study showed that serum betatrophin concentration in the GDM group was significantly higher than in the P-NGR group. In the GDM group, serum betatrophin concentration was positively correlated with BMI, TG, TC, FBG, and HOMA-IR. Furthermore, multiple linear regression analysis showed that HOMA-IR and TG were influencing factors for serum betatrophin concentration. Serum betatrophin

could potentially be used as a biochemical indicator for predicting GDM with the best cut-off point at 801.76 pg/ml. However, very few studies exist on the research topic at hand and those that are available present contradictory results. The reasons for this may be related to differences in race, age, eating habits, region, and the environment of the research participants. The correlation between serum betatrophin concentration and GDM is not yet clear, and there are contradictory findings on its relationship with clinical metabolic indicators, highlighting the need for additional studies.

The current study included the following limitations: the sample of this study was from Mudanjiang City, Heilongjiang Province, China, which may have led to limitations in terms of race, region, environment, and eating habits; the sample size of this study was relatively small, which may have led to additional limitations on the outcomes of the research.

In conclusion, serum betatrophin concentration in the GDM population was significantly increased and positively correlated with BMI, TG, TC, FSI, and HOMA-IR. Insulin resistance in the GDM population may be a mechanism for the overexpression of serum betatrophin, and serum betatrophin could potentially be used as a biochemical indicator for predicting GDM.

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Effect of miR-128-3p in regulating bleomycin-induced autophagy in lung fibroblasts and pulmonary fibrosis through the regulation of GSK3 β

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Pulmonary fibrosis (PF) may be reduced by inducing autophagy, and miR-128-3p could regulate cell autophagy through several methods, however, the mechanism is still unknown. In this experiment, whether miR-128-3p could regulate the bleomycin-induced autophagy of MRC-5 cells by regulating the PTEN/AKT/GSK3 β /mTOR signaling pathway was explored. Bleomycin (10 μ g/mL) was used to induce MRC-5 cells in vitro and form a cell model of PF. The relative proliferation rate, activity, and autophagy rate of MRC-5 cells were detected using MTT, CCK-8, and GFP-LC3 fluorescence, respectively. The expression levels of LC3-II, Beclin-1, PTEN, p-Akt, p-GSK3 β , and p-mTOR proteins in bleomycin-induced MRC-5 cells were determined via Western blotting. The expression levels of GSK3 β mRNA and miR-128-3p were determined in bleomycin-induced MRC-5 cells through real-time polymerase chain reaction. The results showed that bleomycin decreased the autophagy of MRC-5 cells and increased the activity and proliferation rate of these cells. The miR-128-3p inhibitor inhibited the expression levels of p-Akt and p-mTOR, increased the expression levels of GSK3 β , PTEN, LC3-II, and Beclin-1, and induced bleomycin-induced autophagy in MRC-5 cells. The results suggested that miR-128-3p overexpression could improve bleomycin-induced PF by increasing the bleomycin-induced autophagy of MRC-5 cells. The mechanism may be related to the decreased expression of GSK3 β gene and protein by upregulating miR-128-3p expression.

Pulmonary fibrosis (PF) is a disease causing changes in normal lung tissue structure and loss of function, resulting from excessive accumulation of fibroblasts and deposition of extracellular matrix components, such as collagen (1). It mainly involves the lung interstitium, alveoli, and/or bronchiole; it shows damages in the capillary endothelial cells and epithelial cells of acute diffuse alveoli, eventually leading to respiratory failure, with a fatality rate

of 50–70% (1, 2). PF also results in the remodeling of lung structure and progressive and irreversible damage to lung tissue. Respiratory failure is caused by pulmonary failure and air exchange interruption. Idiopathic PF (IPF) with undetermined causes and poor prognosis and secondary lesions of other diseases lead to PF, including connective tissue disease, chronic allergic pneumonia, microbial infection, chemotherapy drugs, dust, atomized environmental

Key words: MiR-128-3p; bleomycin; MRC-5 Cells; GSK3 β ; autophagy

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toxins, and radiation damage (3–5). The incidence of PF has been increasing, and the present effective clinical treatments are insufficient. The mechanism of PF also remains unclear; it may involve inflammation, oxidative stress, apoptosis, and autophagy (5). Thus, further research on the mechanism of PF could help find new therapeutic targets.

Research has shown that the decline in autophagy activity is a considerable cause of the development of PF (6). The abnormal repair of lung tissue cells, tissue structure disorder, and the abnormal accumulation of collagen fibers and proteins are common symptoms of PF, causing severe damage to pulmonary function; autophagy could effectively maintain the homeostasis of lung tissue cells, prevent the abnormal repair of lung tissue, and remove excessive aggregations to prevent lung fibrotic lesions (7). If autophagy is inhibited, foreign matters could not be removed out of the cells in time, thus, they gradually accumulate and increase and then destroy the homeostasis of the cell environment, thereby causing abnormality in lung tissue structure and inducing lung fibrosis (7). The PI3K-Akt-mTOR signal transduction pathway is a significant pathway during autophagy. According to many studies, the expression of autophagy is inevitably affected after intervening this pathway through the use of drugs (8).

MiRNA plays a considerable role in the occurrence of diseases and timing regulation of biological development, such as cell proliferation, differentiation, apoptosis, autophagy, ontogeny, body metabolism, viral infection, and tumorigenesis (9). A previous study showed that miRNA is also involved in PF (6). For instance, miR-21 could regulate the activation of lung fibroblasts, and its expression is increased in patients with IPF and in paraquat-induced PF models. TGF- β 1 could also promote the expression of miR-21 (10). MiR-101 inhibits NFATc2 signal transduction by targeting Frizzled receptor 4/6 to restrain the proliferation of WNT5a-induced lung fibroblasts, while SMAD2/3 signal transduction is inhibited by targeting TGF- β receptor 1 to prevent the activation of TGF- β -induced lung fibroblast (11). Through the targeted regulation of ZEB1/2miR-200b/c, the activities of p38 MAPK and TGF- β /smad3 signal pathways could be reduced to alleviate early lipopolysaccharide-induced PF (12).

The expression of miR-193a could be increased by inhibiting the activities of PI3K/Akt/mTOR and hedgehog signaling, while paraquat-induced PF could be improved by promoting the autophagy of lung fibrocytes (6). However, the relatively complicated mechanism of PF currently needs further research.

In U251 human glioma cell line, the transfection of miR-128-3p simulants increases the levels of phosphorylated insulin receptor substrate 1, phosphoinositide 3-kinase (PI3K), and p-AKT and promotes the apoptosis of U251 human glioma cells to decrease the proliferation of U251 cells (13). MiR-128-3p overexpression could decrease acute lung injury in sepsis rats by inhibiting the expression of induced high-mobility group box 3 (14). Recent studies have found that miR-128-3p inhibits chondrocyte autophagy and worsens knee osteoarthritis by inhibiting the expression of Atg12 (15). MiR-128-3p regulated atrial fibrosis by regulating the SP1-TGF- β 1-Smad axis (16). Therefore, miR-128-3p may be involved in the formation and development of bleomycin-induced PF via regulating autophagy. In the present study, TargetScan prediction suggested that GSK3 β may be the regulatory target of miR-128-3p. GSK3 β could activate PTEN/AKT/mTOR signaling to regulate cell autophagy (17). This study further determined whether miR-128-3p could inhibit the activity of the PTEN/AKT/mTOR pathway and improve the bleomycin-induced autophagy of lung fibroblasts to reduce bleomycin-induced lung fibrosis by increasing the expression of GSK3 β .

MATERIALS AND METHODS

MRC-5 at the logarithmic growth phase of cell culture and grouping

MRC-5 cells (Shanghai Sixin Technology Biology Co., Ltd. Shanghai, China) were cultured with 1640 culture medium containing 10% fetal bovine serum. When they grew and fused to 70%, they were cultivated at a density of 1×10^4 cells/mL per pore plate, cultured in an incubator with 5% CO₂ at 37°C, and digested with 0.25% trypsin. MRC-5 was cultured for 7 days and passed 1:2. The cells were inoculated in a 25 cm² culture vessel at a density of 1×10^5 cells/mL. Upon reaching

approximately 60% fusion, the cells were grouped after synchronously growing for 24 h. Bleomycin (American Sigma Corporation, Chicago, USA) was added in different concentrations (5, 10, 15, and 20 $\mu\text{g/mL}$), and the cells were cultured for 24 h to observe the effect of bleomycin on the activity and proliferation of MRC-5 cells. The cells were divided into several groups as follows to observe the cellular damage effect of miR-128-3p on bleomycin-induced human embryonic lung fibroblasts MRC-5: normal control, bleomycin-induced, bleomycin + miR-128-3p mimic, and bleomycin + miR-128-3p inhibitor groups. They were cultured with miR-128-3p mimic or underwent miR-128-3p inhibitor transfection for 48 h and then added with 10 $\mu\text{g/mL}$ of bleomycin solution for 24-h culture to determine various indicators.

Testing the cell activity of MRC-5 via CCK-8

ACCK-8 kit (CCK-8, Beijing Zhijie Fangyuan Technology Co., Ltd., Beijing, China) was used to determine the cell proliferation activity of MRC-5 in the different treatment groups. The MRC-5 cells were cultivated in a 96 pore-plate. When the cells fused to 70%, the medium was changed to serum-free culture medium. Bleomycin was added in different concentrations (5, 10, 15, and 20 $\mu\text{g/mL}$), and the cells were cultured for 24 h or with miR-128-3p mimic, or they underwent miR-128-3p inhibitor transfection for 48 h and then were added with 10 $\mu\text{g/mL}$ of bleomycin solution for 24 h. A microplate reader was used to measure the absorbance value ($A = 450 \text{ nm}$) at a wavelength of 450 nm, followed by the addition of 10 μL of CCK-8 solution to each pore for cell incubation for 2–4 h in the dark.

Cell proliferation assay of MRC-5

MTT colorimetry was used to detect cell proliferation and collect MRC-5 cells at the logarithmic growth phase. The cells were cultivated in a 96-pore plate at 2×10^4 cells/pore, with 150 μL for each pore. They were filled with sterile phosphate buffered saline all around and incubated for 24 h in a 37°C saturated humidity incubator with 5% CO_2 . The cells were processed using 50 μL of drugs at different concentrations, with six replicate pores for each group. Centrifugation was conducted after 48 h, the supernatant was discarded, and 20 μL of 5 mg/mL MTT solution was added to each pore to maintain incubation for 4 h. Subsequently, 100 μL of 10% SDS was

added to each pore overnight, and the absorbance value of each pore was measured at 570 nm using the microplate reader. The experiment was repeated three times. The inhibition rate of cell proliferation (%) = $(1 - A_{570 \text{ nm administration group}} / A_{570 \text{ nm blank group}}) \times 100\%$ and the half inhibitory concentration (IC_{50}) were measured.

Detection of autophagosomes by autophagy staining detection kit (MDC method)

The MRC-5 cells were cultivated in a 96 pore-plate. When the cells fused to 70%, the medium was changed to serum-free culture medium. Bleomycin was added in different concentrations (5, 10, 15, and 20 $\mu\text{g/mL}$), and the cells were cultured for 24 h or with miR-128-3p mimic GSK3 β inhibitor (CHIR-99021; American Gibco Corporation, California, USA), or they underwent miR-128-3p inhibitor transfection for 48 h and then were added with 10 $\mu\text{g/mL}$ of bleomycin solution for 24 h. Subsequently, they were gently washed twice with sterile PBS. After washing away the drugs and inhibitors, 100 μL MDC staining working solution was added to each well, and the cells were incubated at 37°C and 5% CO_2 in the dark for 15–60 min. Then, 100 μL PBS was added to each well. After washing for 2–3 times, the cells were placed under a fluorescence microscope to observe the staining of MDC. Three fields of view were randomly selected to photograph, and Image-Pro Plus 6.0 image processing software was used to analyze the integrated optical density of the stained part and calculate the MDC average fluorescence intensity. The above test was repeated three times.

Cell transfection

A moderate amount of MRC-5 cells was taken at the logarithmic growth phase and inoculated into a six-pore plate. When the cells adhered to the wall and grew to 60% overnight, the operating manual of Lipofectamine 2000 transfection reagent was followed to transfect the miR-128-3p mimic, miR-128-3p inhibitor, mimic control, and inhibitor control to the MRC-5 cells. The transfection concentration was 50 nmol/L. After 6 h of transfection, the medium was replaced with a fresh one, and culturing was continued for 24 h. The cells were then collected for RT-PCR and Western blotting.

Target gene prediction

Different target gene prediction software, including

miR-Base (<http://www.mirbase.org/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdc-berlin.de/>), were used to predict whether the target gene of miR-128-3p was GSK3 β . Wild-type (wt)-GSK3 β and mutant (mut)-GSK3 β luciferase gene vectors were constructed using conventional methods, and the two plasmids were mixed with miR-128-3p mimics, miR-128-3p inhibitor, mimic control, and inhibitor and co-transfected to the MRC-5 cells. They were counted as Wt + miR-128-3p mimics, Mut + miR-128-3p, Wt + negative control, and Mut + negative control. After the cells were cultured in a 37°C incubator with 5% CO₂ for 48 h, the dual luciferase reporter gene kit was used to detect the dual luciferase activity. Meanwhile, RT-PCR and Western blotting were used to detect the level of GSK3 β in the MRC-5 cells after the expression of miR-128-3p.

Real-time quantitative PCR

In the collected MRC-5 cells from the single-treatment group, cellular RNA was extracted using the total RNA extraction kit and reverse transcribed into cDNA by using PrimeScript™ RT reagent kit. Real-time quantitative PCR was used to detect the expression levels of GSK3 β , mTOR mRNA, and miR-128-3p. The reaction system was as follows: 10 μ L of YBR Premix Ex Taq™ II, 2 μ L of PCR primer, 2 μ L of DNA template, and 6 μ L of DEPC

water. The total reaction system was 20 μ L. Three parallel compound pores were set for each sample. The reaction system was completed on the PCR machine. TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA, USA) was used to measure the expression of miR-128-3p via 2^{- $\Delta\Delta$ Ct} method. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, reaction at 95°C for 5 s, and annealing at 60°C for 30 s, 40 cycles. The primer series are shown in Table I. U6 was used as the internal reference of miR-128-3p, while β -actin was used as the internal reference for the expression levels of GSK3 β and mTOR. After the reaction, the cycle threshold (Ct) of each gene was calculated and compared with the internal reference gene U6 to identify the expression.

Analysis of the expression of cell protein through Western blotting

Cell lysis buffer, protease inhibitor, and phosphatase inhibitor were mixed at a proportion of 8:1:1. Then, 150 μ L of RIPA cell lysis buffer was added to each pore, and the mixture was placed in an ice bath for 30-min lysis to extract the total protein from each group of cells. The mixture was then centrifuged for 15 min at 4°C, and protein purity was detected using a BCA protein quantification kit. Subsequently, 50 μ g of protein extract was separated via 10% SDS-PAGE electrophoresis, and 5% skimmed

Table I. The primer sequence for RT-PCR analysis

Gene	Primer	Product (bp)
miR-128-3p	F-5'-AGGCTCACAGTGAACCGGTC -3'	181 bp
	R-5'-CGCAGGGTCCGAGGTATTC-3'	
U6	F-5'-CGCTTCGGCAGCACATATAC-3'	204 bp
	R-5'-AAATATGGAACGCTTCACGA-3'	
mTORMRNA	F-5'-AACGCTTCACGAATTTGCGT-3'	197 bp
	R-5'-CCATTCCAGCCAGTCATCTTTG-3'	
GSK3 β mRNA	F-5'-CACAGAACCTCTTGCTGGAT-3'	181 bp
	R-5'-GGTGCCCTGTAGTACCGAGA-3'	
β -actin	F-5'-ATGGTGAAGGTCGGTGTG-3'	154 bp
	R-5'-AACTTGCCGTGGGTAGAG-3'	

milk was added for 1.5-h incubation. The corresponding TGF- β 1, Ki67, PCNA, GTCF, collagen I, LC3-II, Beclin-1, PTEN, GSK3 β , Akt, p-Akt, mTOR, and p-mTOR primary antibodies (1:1000; Abcam, England) were added at 4°C. The membrane was washed with TBST three times the next day, and then HRP-labeled goat anti-rabbit IgG was added (volume dilution ratio of 1:3000; Abcam, England) for room-temperature reaction for 1 h. Afterwards, the membrane was washed with TBST three times, each for 10 min, and ECL chemiluminescent solution was added for coloration. The band gray value was analyzed on Image Lab software, with β -actin as an internal reference.

Observation of autophagic flux under confocal microscope

MRFP-GFP-LC3 dual fluorescence-labeled autophagic adenovirus (purchased from Shanghai Hanbio Technology Co., Ltd.) was used to transfer the MRC-5 cells, which were then cultured in a confocal vessel of 35 mm. When the cell growth density reached 60%–80%, the mRFP-GFP-LC3 dual fluorescence-labeled autophagic adenovirus was added directly into the complete medium (MOI was 50) and placed in a 37°C incubator for cell culture. The medium was replaced with fresh complete medium after 2 h. The mixture was processed in accordance with each experimental group, followed by culture for 24 h. The MRC-5 cells were placed under a confocal microscope to observe the autophagic flux of the cells. Green and red fluorescence (autophagolysosome) and yellow fluorescence (autophagosome) were observed after fusion, and the intensity of autophagic flux was expressed by counting the spots with different colors. Five views were selected for each group when obtaining images, and the results were shown in a histogram after analysis.

Immunofluorescence

The cells were inoculated on 24-well plates to detect the contents of collagen I and collagen III expression in MRC-5 cells. After different pretreatments and treatments were conducted, the cells were incubated with collagen I and collagen III antibodies. Finally, fluorescence secondary antibody and DAPI staining were added to obtain images by using a fluorescence microscope.

Statistical analysis

GraphPad Prism 6 was used for statistical analysis. Experimental data were expressed by mean $\pm$ standard deviation. One-way ANOVA was used to compare groups,

and the significance between them was compared using LSD method. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Effect of bleomycin on MRC-5 cell viability and proliferation rate

The MRC-5 cells were incubated with various concentrations of bleomycin for 48 h to analyze the dose-dependent effect of bleomycin on MRC-5 cell viability and proliferation. The MRC-5 cell viability was measured using CCK-8 assay, while the proliferation rate was measured using MTT assay. As shown in Fig. 1, bleomycin increased the MRC-5 cell viability and proliferation in a concentration-dependent manner. Bleomycin concentrations of as low as 10 μ g/mL were effective in inducing the above phenotypes.

Effect of bleomycin on autophagy of MRC-5 cells

The MRC-5 cells were incubated with various concentrations of bleomycin for 48 h to analyze the dose-dependent effect of bleomycin on MRC-5 cell autophagy. The number of autophagic cells was counted via GFP-LC3 fluorescence. As shown in Fig. 2, bleomycin decreased the autophagy of MRC-5 cells in a concentration-dependent manner. Bleomycin concentrations of as low as 10 μ g/mL were effective in decreasing this autophagy.

Effect of bleomycin on LC3-II and Beclin-1 autophagic proteins in MRC-5 cells

The MRC-5 cells were incubated with various concentrations of bleomycin for 48 h to investigate the effect of bleomycin on the expression levels of LC3-II and Beclin-1 proteins in these cells. The expression levels were measured via Western blotting (Fig. 3). Bleomycin treatment decreased the expression levels of LC3-II and Beclin-1 proteins in the MRC-5 cells in a concentration-dependent manner (Fig. 3). Bleomycin concentrations of as low as 10 μ g/mL were effective in inducing these changes.

Effect of bleomycin on the PTEN/AKT/mTOR/GSK3 β signaling pathway in MRC-5 cells

The MRC-5 cells were incubated with various

concentrations of bleomycin for 48 h to investigate the effect of bleomycin on the PTEN/AKT/mTOR/GSK3 β signaling pathway. The expression levels of PTEN, GSK3 β , Akt, p-Akt, mTOR, and p-mTOR proteins were measured using Western blotting (Fig. 4). Bleomycin decreased the expression levels of PTEN and GSK3 β and increased those of p-Akt and p-mTOR in MRC-5 cells in a bleomycin concentration-dependent manner (Fig. 4). Bleomycin

concentrations of as low as 10 μ g/mL were effective in inducing these changes.

Effect of bleomycin on the expression of miR-128-3p in MRC-5 cells

The MRC-5 cells were incubated with various concentrations of bleomycin for 48 h to determine the dose-dependent effect of bleomycin on the expression of miR-128-3p, which was measured via

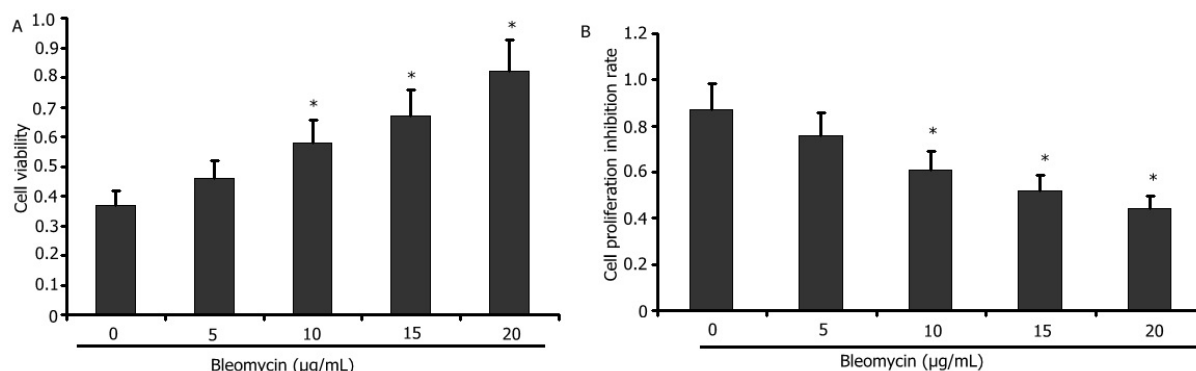


Fig. 1. Bleomycin increases MRC-5 cell viability and proliferation. MRC-5 cells were incubated with various concentrations of bleomycin for 48 h. MRC-5 cell viability was measured using CCK-8 assay, while proliferation rate was measured using MTT assay. Quantitative densitometry data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the control group.

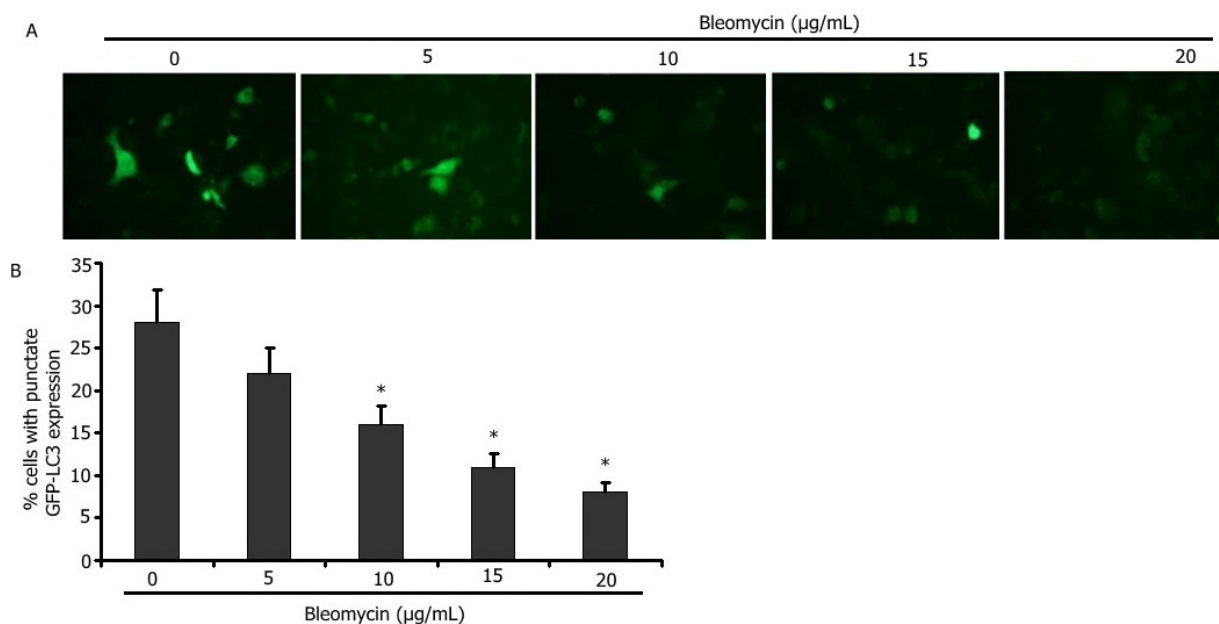


Fig. 2. Bleomycin decreases the autophagy of MRC-5 cells. MRC-5 cells were incubated with various concentrations of bleomycin for 48 h. The number of autophagy cells was measured via immunofluorescence. Quantitative densitometry data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the control group.

RT-PCR (Fig. 5), in these cells. The results showed that bleomycin increased miR-128-3p expression. Bleomycin concentrations of as low as 10 $\mu\text{g/mL}$ were effective in increasing the expression of miR-128-3p in MRC-5 cells.

MiR-128-3p mimic increases bleomycin-induced expression of miR-128-3p in MRC-5 cells

The MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin to determine whether they affected the bleomycin-induced expression of miR-128-3p. As shown in Fig. 5, the miR-128-3p mimic increased

this expression in the bleomycin-treated MRC-5 cells, whereas the miR-128-3p inhibitor further decreased it.

Effect of miR-128-3p on bleomycin-induced MRC-5 cell viability and proliferation

The MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin to determine whether miR-128-3p affected the bleomycin-induced changes in MRC-5 cell viability and proliferation rate. The MRC-5 cell viability was measured via CCK-8 assay, while the proliferation rate was measured via MTT assay

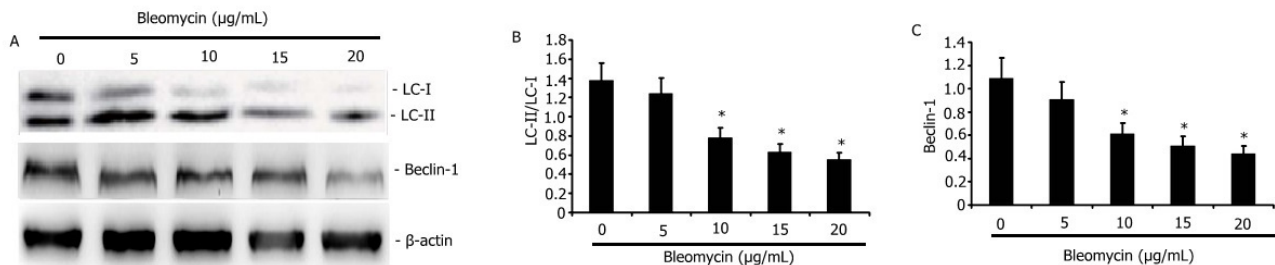


Fig. 3. Bleomycin decreases LC3-II and Beclin-1 autophagic proteins in MRC-5 cells. MRC-5 cells were incubated with various concentrations of bleomycin for 48 h. The expression levels of LC3-I, LC3-II, and Beclin-1 proteins in MRC-5 cells were measured via Western blotting. Data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the control group.

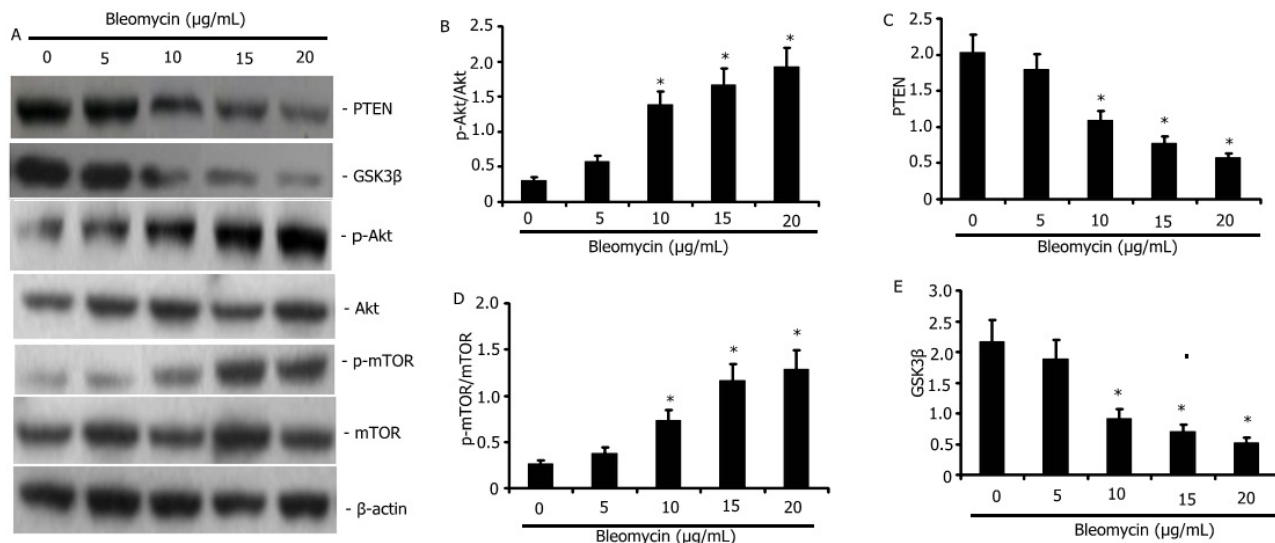


Fig. 4. Effect of bleomycin on PTEN/AKT/GSK3 β /mTOR signaling pathway in MRC-5 cells. MRC-5 cells were incubated with various concentrations of bleomycin for 48 h. The expression levels of PTEN, Akt, p-Akt, p-mTOR, GSK3 β , and p-mTOR proteins were measured via Western blotting. Data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the control group.

(Fig. 6). The miR-128-3p mimic increased MRC-5 cell viability and proliferation. Conversely, the miR-128-3p inhibitor decreased the increased MRC-5 cell viability and proliferation rate. These results suggested that the decreased expression of miR-128-3p inhibited MRC-5 cell viability and proliferation.

Effect of miR-128-3p on bleomycin-induced MRC-5 cell autophagy

The cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin to determine whether miR-128-3p affected the

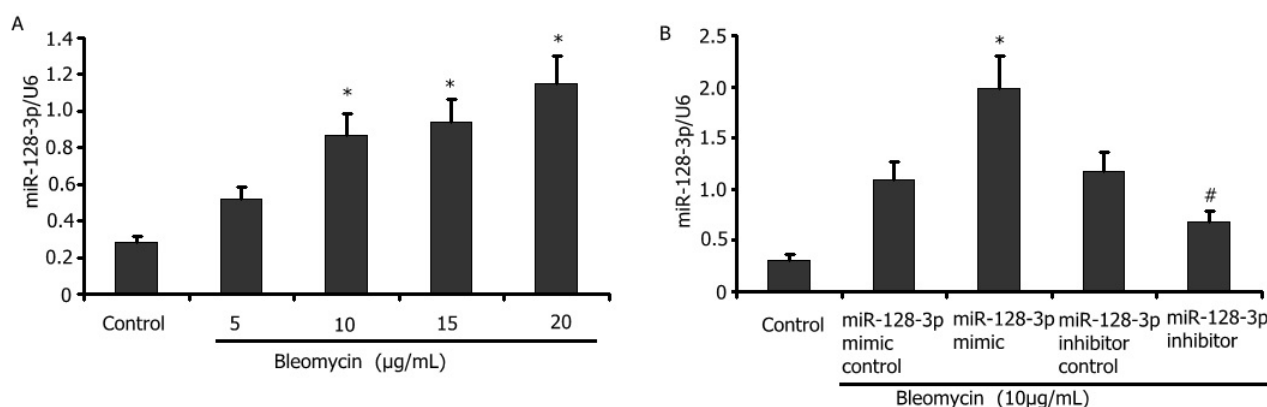


Fig. 5. Effect of miR-128-3p mimic on bleomycin-induced expression of miR-128-3p in MRC-5 cells. **A)** MRC-5 cells were incubated with various concentrations of bleomycin for 48 h. The expression of miR-128-3p in MRC-5 cells was measured via RT-PCR. **B)** MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin. The expression of miR-128-3p in MRC-5 cells was measured via RT-PCR. Data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the control group. # $P < 0.05$ vs the bleomycin-treated MRC-5 cells.

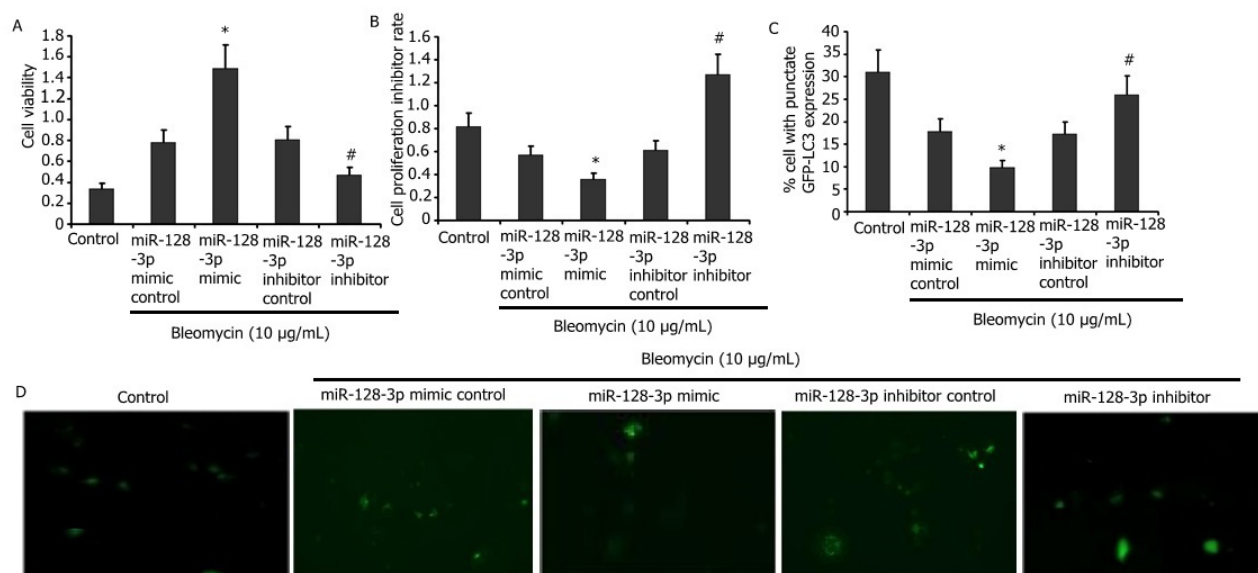


Fig. 6. Effect of miR-128-3p mimic on bleomycin-induced MRC-5 cell viability, proliferation, and autophagy. MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin. MRC-5 cell viability was measured via CCK-8 assay, while the proliferation rate was measured via MTT assay, and the number of autophagic cells was counted using GFP-LC3 fluorescence. Data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the miR-128-3p mimic control group. # $P < 0.05$ vs the miR-128-3p inhibitor control.

bleomycin-induced changes in MRC-5 cell autophagy. The number of autophagic cells was counted using GFP-LC3 fluorescence (Fig. 6). The miR-128-3p mimic attenuated the MRC-5 cell autophagy. Conversely, the miR-128-3p inhibitor increased it. These results suggested that the overexpression of miR-128-3p increased the MRC-5 cell autophagy.

Effect of miR-128-3p on LC3-II and Beclin-1 autophagic proteins in bleomycin-induced MRC-5 cells

The cells were either transfected with an miR-

128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin to determine whether miR-128-3p affected the LC3-II and Beclin-1 autophagic proteins in bleomycin-induced MRC-5 cells. LC3-II and Beclin-1 were measured via Western blotting (Fig. 7). The miR-128-3p mimic decreased the protein expression levels of LC3-II and Beclin-1 in bleomycin-induced MRC-5 cells, whereas the miR-128-3p inhibitor increased them.

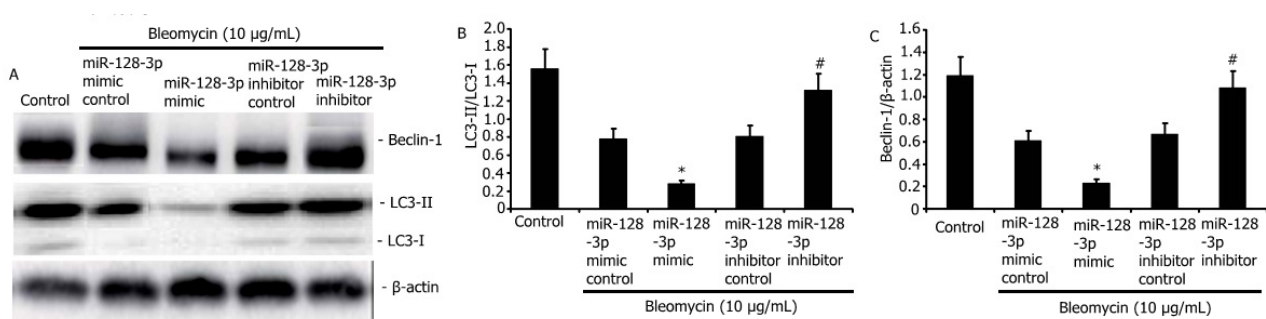


Fig. 7. Effect of miR-128-3p on LC3-II and Beclin-1 autophagic proteins in bleomycin-induced MRC-5 cells. MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin. The LC3-II and Beclin-1 autophagic proteins in bleomycin-induced MRC-5 cells were measured via Western blotting. Data from three independent experiments are presented as mean $\pm$ SD. * P < 0.05 vs the miR-128-3p mimic control group. # P < 0.05 vs the miR-128-3p inhibitor control.

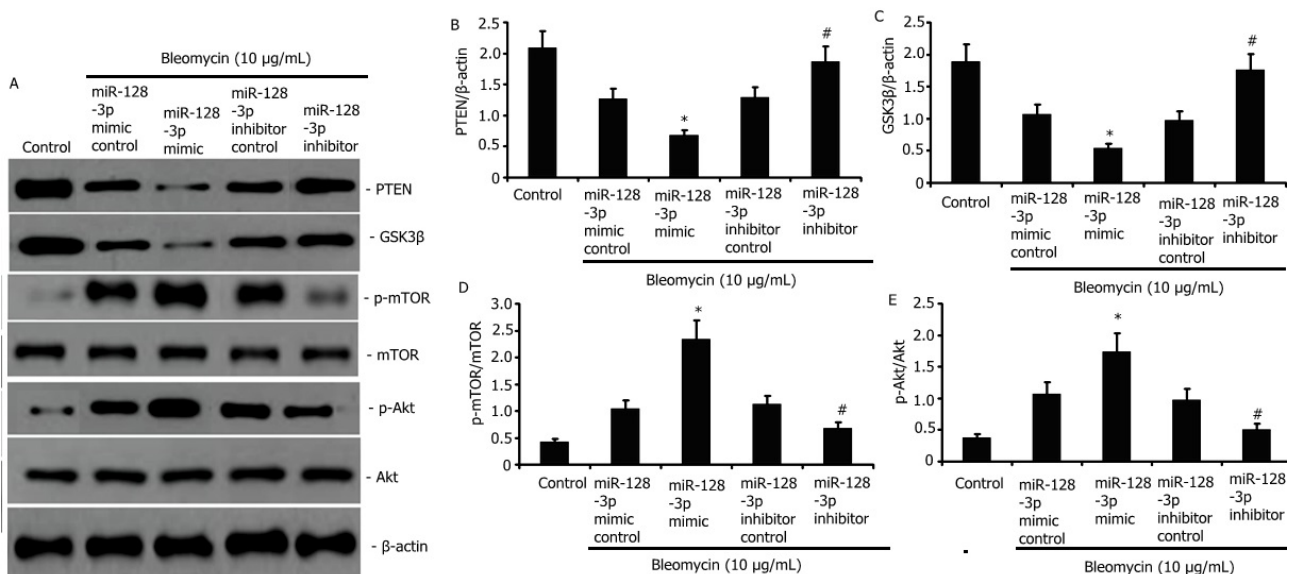


Fig. 8. Effect of miR-128-3p on PTEN/AKT/GSK3 β /mTOR signaling pathway in bleomycin-induced MRC-5 cells. MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin. The expression levels of PTEN, Akt, p-Akt, mTOR, GSK3 β , and p-mTOR proteins in the presence of 10 μ g/mL bleomycin were measured via Western blotting. Data from three independent experiments are presented as mean $\pm$ SEM. * P < 0.05 vs the miR-128-3p mimic control group. # P < 0.05 vs the miR-128-3p inhibitor control.

Effect of miR-128-3p on the PTEN/AKT/mTOR/GSK3 β signaling pathway in bleomycin-induced MRC-5 cells

The MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin to analyze the effect of miR-128-3p on the PTEN/AKT/GSK3 β /mTOR signaling pathway in bleomycin-induced MRC-5 cells. The expression levels of PTEN, Akt, p-Akt, GSK3 β , mTOR, and p-mTOR proteins in bleomycin-induced MRC-5 cells were measured via Western blotting (Fig. 8). The miR-128-3p mimic decreased the expression levels of PTEN and GSK3 β and increased those of p-Akt and p-mTOR in bleomycin-induced MRC-5 cells. Conversely, the miR-128-3p inhibitor increased the expression levels of PTEN and GSK3 β and decreased those of p-Akt and p-mTOR.

Effect of miR-128-3p on TGF- β 1, GTCF, and collagen I proteins in bleomycin-induced MRC-5 cells

The cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin to determine whether miR-128-3p affected the TGF- β 1, GTCF, and collagen I proteins in bleomycin-induced MRC-5 cells. These proteins were measured via Western blotting (Fig. 9). The miR-128-3p mimic decreased the protein expression levels of TGF- β 1, GTCF, and collagen I in the bleomycin-induced MRC-5 cells, whereas miR-128-3p inhibitor increased them.

Effect of miR-128-3p on Ki67 and PCNA protein expression in bleomycin-induced MRC-5 cells

The cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin to

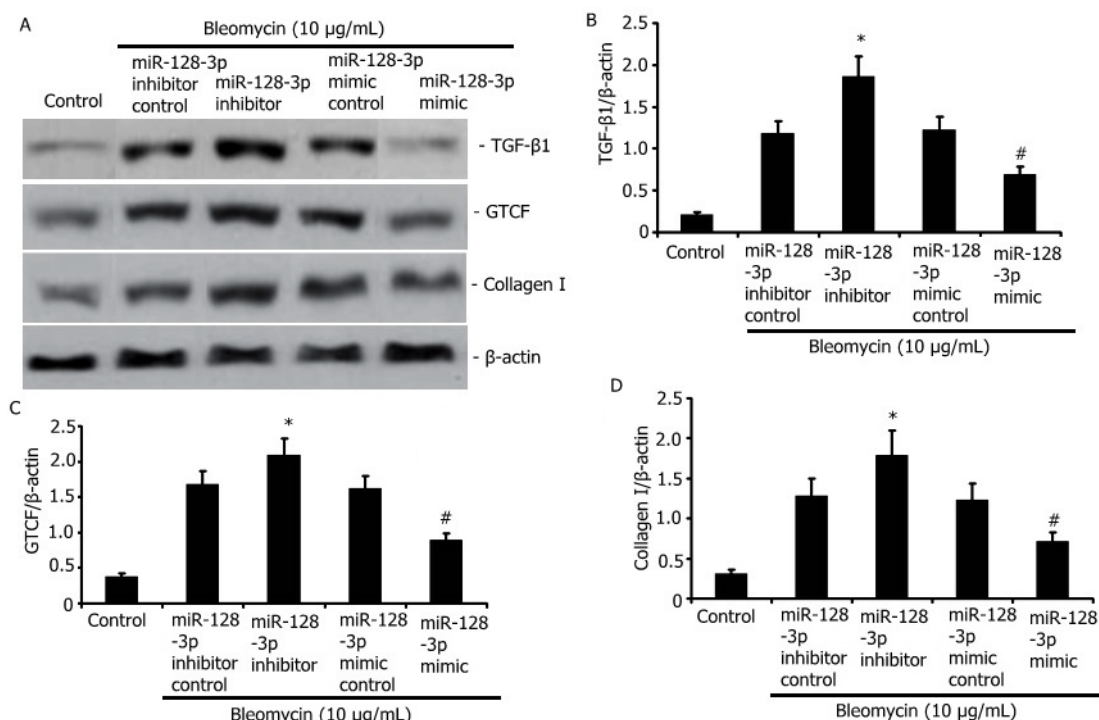


Fig. 9. Effect of miR-128-3p on TGF- β 1, GTCF, and collagen I proteins in bleomycin-induced MRC-5 cells. MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 μ g/mL bleomycin. The TGF- β 1, GTCF, and collagen I proteins in bleomycin-induced MRC-5 cells were measured via Western blotting. Data from three independent experiments are presented as mean $\pm$ SD. * P < 0.05 vs the miR-128-3p mimic control group. # P < 0.05 vs the miR-128-3p inhibitor control.

determine whether miR-128-3p affected the Ki67 and PCNA protein expression in bleomycin-induced MRC-5 cells, as measured via Western blotting. The miR-128-3p mimic decreased the protein expression levels of Ki67 and PCNA protein expression in the bleomycin-induced MRC-5 cells, whereas the miR-128-3p inhibitor increased them (Fig. 10).

Effect of miR-128-3p on bleomycin-induced collagen I and collagen III protein expression in MRC-5 cells

The cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin to determine whether miR-128-3p affected the collagen I and collagen III protein expression in bleomycin-

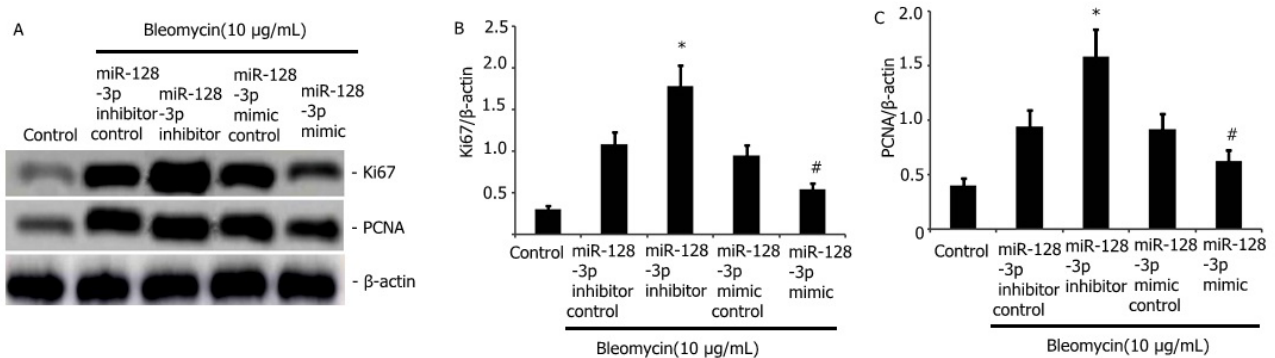


Fig. 10. Effect of miR-128-3p on Ki67 and PCNA protein expression in bleomycin-induced MRC-5 cells. MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin. The Ki67 and PCNA proteins in bleomycin-induced MRC-5 cells were measured via RT-PCR. Data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the miR-128-3p mimic control group. # $P < 0.05$ vs the miR-128-3p inhibitor control.

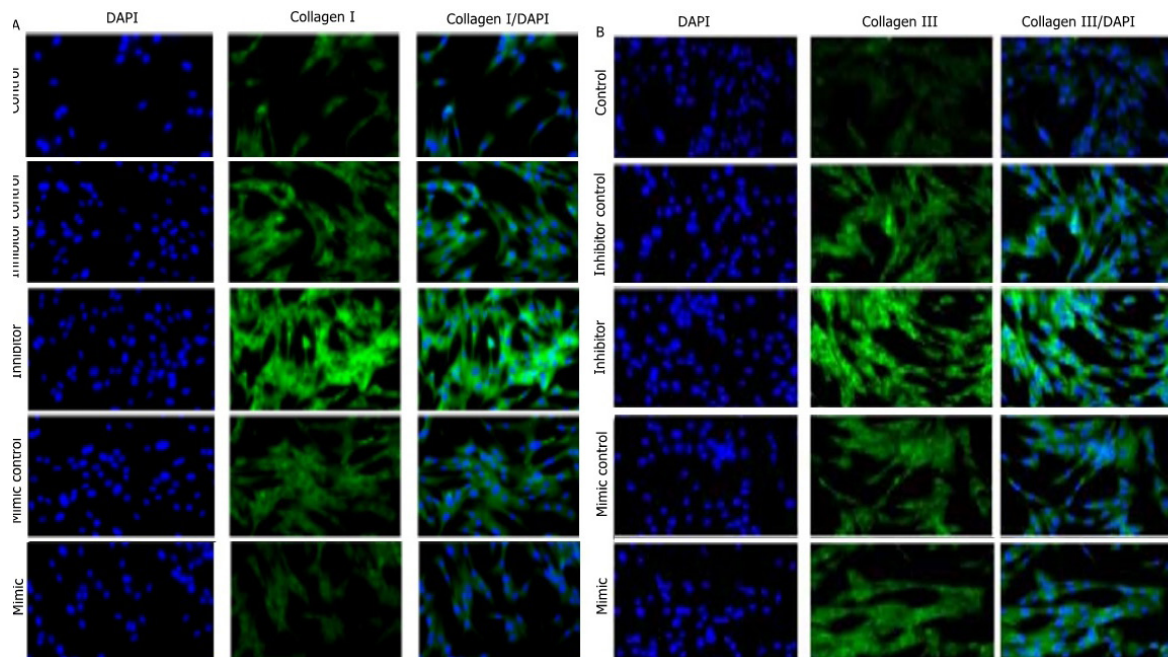


Fig. 11. Effect of miR-128-3p on bleomycin-induced collagen I and collagen III protein expression in MRC-5 cells. MRC-5 cells were either transfected with an miR-128-3p mimic or incubated with an miR-128-3p inhibitor for 48 h in the presence of 10 $\mu\text{g/mL}$ bleomycin. The expression of collagen I and collagen III proteins in MRC-5 cells were measure by immunofluorescence. Data from three independent experiments are presented as mean $\pm$ SD. * $P < 0.05$ vs the miR-128-3p mimic control group. # $P < 0.05$ vs the miR-128-3p inhibitor control.

induced MRC-5 cells. The collagen I and collagen III proteins were measured via immunofluorescence. The miR-128-3p mimic decreased the expression levels of these proteins in the bleomycin-induced MRC-5 cells, whereas the miR-128-3p inhibitor increased them (Fig. 11).

Role of GSK3 β in miR-128-3p-inhibited autophagy in bleomycin-induced MRC-5 cells

The cells were either transfected with an miR-128-3p inhibitor or/and GSK3 β inhibitor (CHIR-99021) incubated for 48 h in the presence of 10 μ g/mL bleomycin to determine whether miR-128-3p affected the LC3-II and Beclin-1 protein expression and the formation of autophagosomes in bleomycin-induced MRC-5 cells. The LC3-II and Beclin-1 protein expression levels were measured via Western blotting, while the formation of autophagosomes was measured via MDC. The miR-128-3p inhibitor increased the protein expression levels of LC3-II and Beclin-1 and the formation of autophagosomes, whereas CHIR-99021 decreased them in the bleomycin-induced MRC-5 cells. CHIR-99021 also

inhibited the miR-128-3p inhibitor-induced protein expression levels of LC3-II and Beclin-1 and the formation of autophagosomes in the bleomycin-induced MRC-5 cells (Fig. 12).

GSK3 β is a direct target of miR-128-3p

This study showed that GSK3 β is an important regulator of bleomycin-induced MRC-5 cell autophagy. A well-known database was used to predict the likely targets of miR-128-3p and determine how miR-128-3p influenced this autophagy. Analysis revealed that the GSK3 β mRNA and protein levels extensively decreased after the overexpression of miR-128-3p (Fig. 13 A, B, D, E). Wt-GSK3 β and mut- GSK3 β were constructed for testing in dual luciferase reporter assay to further validate the interaction between miR-128-3p and GSK3 β (Fig. 13 C, F). As hypothesized, miR-128-3p was bound to wt-GSK3 β but not to mut-GSK3 β (Fig. 13D).

Correlation between GSK3 β and miR-128-3p in bleomycin-induced MRC-5 cells

The results showed that the mRNA expression

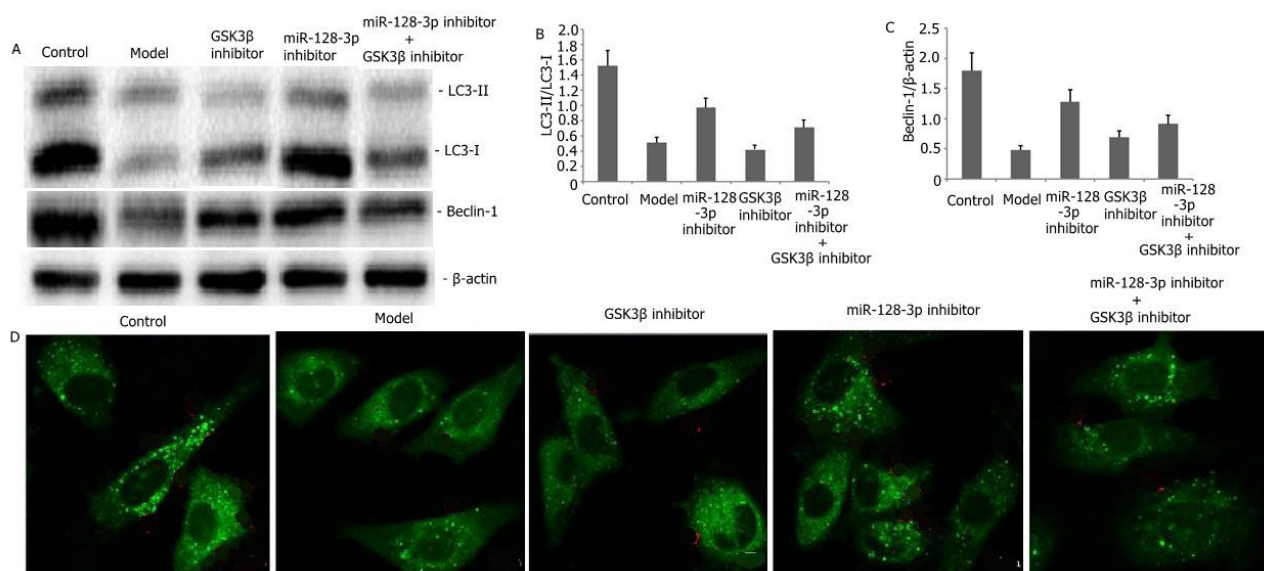


Fig. 12. Role of GSK3 β in miR-128-3p-inhibited autophagy in bleomycin-induced MRC-5 cells. Cells were either transfected with an miR-128-3p inhibitor or/and GSK3 β inhibitor (CHIR-99021) incubated for 48 h in the presence of 10 μ g/mL bleomycin to determine whether miR-128-3p affected LC3-II and Beclin-1 protein expression and the formation of autophagosomes in bleomycin-induced MRC-5 cells. The LC3-II and Beclin-1 protein expression levels were measured via Western blotting, while the formation of autophagosomes was measured via MDC. Data from three independent experiments are presented as mean $\pm$ SD. * P < 0.05 vs the miR-128-3p mimic control group. # P < 0.05 vs the miR-128-3p inhibitor control.

of GSK3 β decreased in bleomycin-induced MRC-5 cells transfected with an miR-128-3p overexpression vector compared with changes in the empty vector control ($P < 0.05$, Fig. 8). The expression of GSK3 β increased in bleomycin-induced MRC-5 cells via miR-128-3p downregulation (Fig. 14). The correlation between GSK3 β and miR-128-3p in bleomycin-induced MRC-5 cells was further analyzed. A negative correlation was found between GSK3 β protein and miR-128-3p and between GSK3 β mRNA and miR-128-3p in bleomycin-induced MRC-5 cells (Fig. 14 A, B).

DISCUSSION

PF is a lung damage caused by many factors, with complicated pathological mechanism. Different pathogenic factors initiate inflammation and immune reaction, which is the interaction of cell factors and inflammatory mediators, and various cells, such as vascular endothelial cells, alveolar epithelial cells, fibroblasts, and macrophages. Bleomycin is an alkaline glycopeptide anti-cancer antibiotic, and PF is one of the serious adverse reactions of this drug.

Animal experiments showed that the histopathological changes in bleomycin-induced PF quite resemble those in human PF; thus, it is widely used as a model for studying PF (18, 19). In the present study, a bleomycin-induced PF model was used.

Changes in the autophagy level in fibrotic diseases of various tissues were observed, along with different fibrotic changes in tissues with different autophagy levels, confirming the involvement of autophagy in the formation of various types of tissue

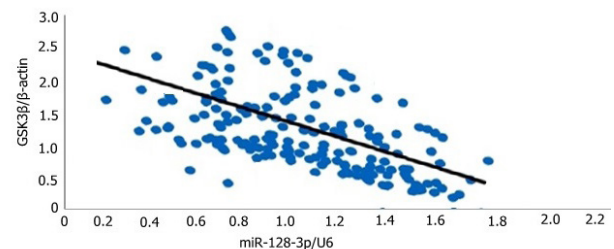


Fig. 14. Correlation between GSK3 β and miR-128-3p in bleomycin-induced MRC-5 cells. Linear correlation analysis was used to evaluate the relationship between the protein expression levels of GSK3 β and miR-128-3p. A negative correlation existed between GSK3 β protein and miR-128-3p in bleomycin-induced MRC-5 cells ($r = -0.427$, $P < 0.05$).

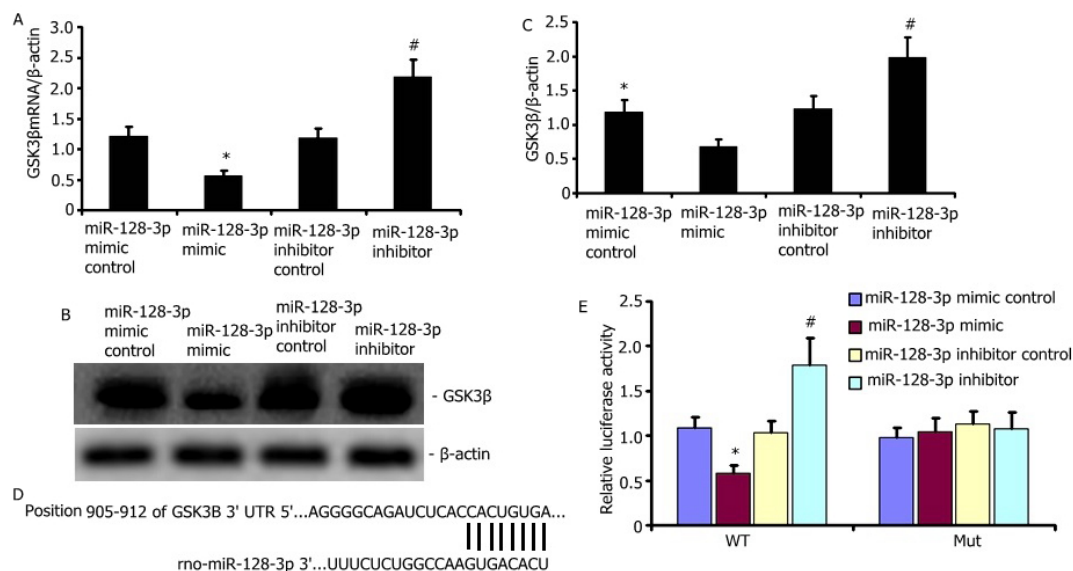


Fig. 13. GSK3 β is a downstream target of miR-128-3p. **A)** The mRNA levels of GSK3 β in bleomycin-induced MRC-5 cells decreased after the overexpression of miR-128-3p ($P < 0.05$). **B, C)** GSK3 β was decreased in bleomycin-induced MRC-5 cells through the overexpression of miR-128-3p relative to that of an empty vector control ($P < 0.05$). GSK3 β was significantly increased in cells via miR-128-3p downregulation. **D)** miR-128-3p was bound to the 3'-UTR regions of GSK3 β . Binding was interrupted in mut-GSK3 β . **E)** Dual luciferase reporter assay indicated that the miR-128-3p mimic was bound to the 3'-UTR region of wt-GSK3 β but not to that of mut-GSK3 β ($P < 0.05$).

fibrosis. Changes in the autophagy level were also observed in the lung fibrosis model. The autophagy level of lung tissue in this model was evidently inhibited compared with that in the control group. The degree of lung fibrosis was reduced through partial restoration of autophagy in lung tissue (20, 21). The present study also showed that when human embryonic lung fibroblasts MRC-5 cells were treated with bleomycin, the autophagy of these cells obviously decreased, whereas the cell activity and the expression levels of TGF- β 1, GTCF, and collagen I proteins increased.

Beclin-1 is the principal positive autophagy-controlling gene directly involved in the regulation of autophagic activity (22). LC3 is a key gene involved in the formation of autophagosomes, and its expression is closely linked with autophagic activity; it is a marker molecule of autophagic activity (23). The present study showed that the expression levels of bleomycin-induced Beclin-1 and LC3 in MRC-5 cells decreased, suggesting that bleomycin inhibited the autophagy of MRC-5 cells. Ki67 and PCNA are important indicators for evaluating cell proliferation. In this study, miR-128-3p mimics reduced the expression levels of Ki67 and PCNA mRNA proteins in MRC-5 cells induced by bleomycin, suggesting that miR-128-3p mimic promoted the autophagy of MRC-5 cells and reduced the proliferation of MRC-5 cells induced by bleomycin.

In the PTEN/AKT/GSK3 β /mTOR regulatory pathway, contrary to the function of PI3K, PTEN, which is a kind of PIP3 phosphatase, converts PIP3 to PIP2 through dephosphorylation to reduce AKT activation and regulate autophagy, which is a negative regulatory factor of the PI3K/AKT pathway (17). Activated AKT activates GSK3 β through phosphorylation. The targeted regulation of mTOR expression could be realized through GSK3 β . MTOR is the target of multiple signal pathways, and it has biological effects in the negative regulation of autophagic activities, including autophagic and antiapoptotic pathways (17). The lung fibroblasts in IPF showed continuous activation of the mTOR pathway, which has an inhibitory effect on autophagy. The autophagy level significantly decreased due to the activation of the pathway, thereby promoting

the differentiation of myofibroblasts and eventually resulting in the formation of lung fibrosis (24). In the present study, the expression levels of mTOR and AKT were increased and those of PTEN and GSK3 β were decreased by bleomycin treatment. This finding showed that bleomycin inhibited the autophagy of MRC-5 cells.

MiRNA has a crucial role in the formation and development of PF. MiR-21 is one of the most investigated miRNAs at present. Yamada et al. (25) found that lung epithelial cells could also undergo epithelial-mesenchymal transition (EMT) in addition to the myofibroblasts undergoing PF. The intracellular miR-21 is upregulated during the process. Lung epithelial cells reduces the abnormally increased expression of vimentin and α -smooth muscle actin while producing EMT by inhibiting the function of miR-21. In the fibrotic lung tissue of rats induced by bleomycin, the expression of miR-26a showed downregulation compared with that in normal lung tissue (25). In-vivo and in-vitro experiments could prove that miR-26a is downregulated by the TGF- β 1-mediated phosphorylation of Smad3, and a new positive feedback loop exists between miR-26a and p-Smad3 (26). Overexpressed miR-503 inhibited silica-induced PF by reducing the severity and distribution of internal PF and inhibiting EMT (27). In the present study, increased expression of miR-128-3p and aggregated expression levels of pro-fibrotic TGF- β 1, GTCF, and collagen I proteins in PF were found after treatment with bleomycin. Further research found that the expression levels of pro-fibrotic TGF- β 1, GTCF, and collagen I proteins significantly decreased after the administration of miR-128-3p inhibitor to reduce the expression of miR-128-3p.

MiRNA also plays a role in regulating autophagy. A previous study showed that the damage level of pancreatic autophagy of experimental pancreatitis mice could be lowered, and the prognosis could be improved by inhibiting the expression of miR-155 (28). MiR-26 enhances chemosensitivity and promotes the apoptosis of hepatoma carcinoma cells by inhibiting autophagy (29). Excessive cardiac autophagy is inhibited by increasing the activity of the PPAR γ /NF- κ B signaling pathway,

thereby restraining the expression of miR-128 to prevent pathological cardiac hypertrophy and dysfunction (30). Overexpression of single miR-128 enhances the apoptotic death of glioma cells through the activation of caspase-3/9, degradation from poly (ADP ribose) polymerase, reactive oxygen generation, loss of mitochondrial membrane potential, and formation of unprotected autophagy (31). The present study showed that bleomycin could contribute to the increase in the expression levels of miR-128-3p, mTOR, and AKT; the decrease in the expression levels of PTEN, GSK3 β , Beclin-1, and LC3; and the autophagy of MRC-5 cells. Bleomycin could also promote an increase in the expression levels of TGF- β 1, GTCF, and collagen I proteins, which aggravated PF. After miR-128-3p inhibitor was administered, the expression levels of mTOR and AKT was inhibited. In addition, the expression levels of PTEN, GSK3 β , Beclin-1, and LC3 increased, while the autophagy of MRC-5 cells and the expression levels of TGF- β 1, GTCF, and collagen I decreased, thereby improving bleomycin-induced PF. These findings indicated that miR-128-3p could regulate bleomycin-induced PF by regulating the PTEN/AKT/GSK3 β /mTOR signal pathway.

MiR-128 could regulate the apoptosis of glioma cells by regulating the PTEN/AKT/mTOR pathway (31). In the present study, miR-128-3p and GSK3 β could regulate the autophagy of bleomycin-induced MRC-5 cells. TargetScan prediction was used to further confirm the mutual regulatory relationship between miR-128-3p and GSK3 β , and the results indicated that GSK3 β may be the regulatory target of miR-128-3p. This study confirmed that GSK3 β is the regulatory target of miR-128-3p through luciferase reporter assay. In addition, miR-128-3p regulated the expression of GSK3 β via targeting method to further regulate the PTEN/AKT/mTOR signal pathway, thereby regulating bleomycin-induced MRC-5 autophagy and lung fibrosis.

In conclusion, this experiment proved that miR-128-3p plays a significant role in the formation and development of bleomycin-induced PF. Through targeted regulation of the expression of GSK3 β to reduce the expression of miR-128-3p, the activity of the PTEN/AKT/mTOR signal pathway was reduced

and the expression levels of autophagy proteins Beclin-1 and LC3 were promoted to enhance the autophagy of lung fibrotic cells and improve bleomycin-induced lung fibrosis.

This study has some limitations. MiR-128-3p may be involved in inflammation, apoptosis, and oxidative stress in bleomycin-induced PF, but the mechanism remains unknown and further study is needed. In addition, no further research was performed on the MIC and ID50 in the MRC-5 treatment with bleomycin in this study.

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Clinical indicative roles of vitamins A, D and E in preterm and term neonates with respiratory distress syndrome

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Neonatal respiratory distress syndrome (NRDS) is a leading cause of neonatal mortality, and commonly occurs in preterm neonates. Vitamins are closely related with lung development. The purpose of this study was to evaluate the clinical predictive value of umbilical cord serum vitamins A, D and E for NRDS onset in both preterm and term infants. A total of 150 NRDS neonates, including 114 preterm and 36 term cases, were included in this study. The levels of vitamins A, D and E in the umbilical cord serum of NRDS neonates were measured using electrochemiluminescence method. The predictive value of vitamins was evaluated using logistics regression analysis and receiver operating characteristic curves. Vitamins A, D and E levels were significantly reduced in NRDS neonates and served as risk factors for the onset of NRDS with certain predictive accuracy, which manifested by the area under the curve (AUC) value (0.885 for vitamins A, 0.875 for vitamins D, 0.720 for vitamins E). Additionally, in the 114 preterm cases, decreased vitamins A, D and E were also independently associated with NRDS occurrence. For term NRDS neonates, only vitamins A and D were determined as two risk factors for disease onset. Overall, vitamins A, D and E levels were lower in NRDS compared with normal control neonates, and vitamins A and D played as predictive indicators for NRDS onset in both preterm and term neonates, while relationship between vitamins E and NRDS was only found in preterm neonates.

Neonatal respiratory distress syndrome (NRDS) is a leading cause of mortality in neonates, especially in preterm infants (1). Most of NRDS cases are found in preterm newborns, and small gestational age and low birth weight have been determined as two risk factors of NRDS (2). The lack of pulmonary surfactant (PS) is one of the major causes of NRDS, leading to the progressive atrophy of alveoli (3). NRDS generally occurs in 6 to 12 h after birth, manifested as dyspnea, grunting, shortness of breath and even acute hypoxic respiratory failure, and the disease presents progressive aggravation within 24 h (4). Even if the NRDS neonate survives, there will be some sequelae (5). Therefore, early prediction of

NRDS is of great importance for timely treatment.

Vitamins are one of the indispensable nutrients for the human body (6). Vitamin deficiency is common in newborns, especially in preterm cases, owing to the limited prenatal intake of nutrients (7). Among all the types of vitamins, vitamins A, D and E have been found to be related with the development of NRDS (8-10). Vitamin A is essential to maintain body vision, gene expression, reproduction, embryonic development and immune function, and vitamin A deficiency can lead to function disorders in lung and respiratory tract (11). Vitamin D also contains 12-hydroxy vitamins D [25(OH)D], and acts as an important regulator

Key words: Neonatal respiratory distress syndrome; vitamins; pulmonary surfactant; prediction; receiver operating characteristic curve; birth asphyxia

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in lung development (12). Vitamin E has strong antioxidant functions, which could help to relieve the oxidative injury caused by NRDS (13). In addition, vitamins have been demonstrated to affect the synthesis of PS, thereby participating in the development and progression of NRDS (14, 15). Although the important roles of vitamins A, D and E have been documented in NRDS, whether the deficiency of vitamins has any clinical indication remains unclear.

To understand the clinical value of level changes of vitamins in NRDS, this study investigated the levels of vitamins A, D and E in umbilical cord serum of 150 neonates with NRDS and evaluated the relationship of the vitamin levels with the occurrence of NRDS. Given the high morbidity of NRDS in preterm newborns, this study further divided the subjects into preterm and term cases and examined the clinical prediction of vitamins for NRDS in preterm and term neonates, respectively. The analysis results may provide evidence for vitamins A, D and E as early indicators for the development of NRDS.

MATERIALS AND METHODS

Study population and sample collection

Umbilical cord blood was collected from 300 neonates, who were born in Weifang People's Hospital from June 2015 to June 2020. The 300 newborns included 150 NRDS and 150 normal controls, and informed consent was obtained from the guardians of the participants. The diagnosis of NRDS was determined based on clinical symptoms and chest X-ray, i.e. respiratory rate more than 60/min, dyspnea, grunting, cyanosis, shortness of breath, intercostal, subcostal or suprasternal retraction, and bronchial congestion, reticular shadow or white lung found via X-ray.

The newborns in the control group had no symptoms related to NRDS, and were matched with NRDS cases at gestational age, sex, birth weight and delivery mode. None of the participants had congenital disease, malformation, heart failure, severe infection, anemia, pulmonary hemorrhage or intracranial hemorrhage. In addition, according to gestational age, the included 150 NRDS infants were grouped into preterm ($n = 114$) and term ($n = 36$) groups, and the normal controls included 112 preterm

and 38 term infants. The gestational age of preterm infants was < 37 weeks, and that of term infants was ≥ 37 weeks. Umbilical cord blood was collected immediately after the umbilical cord cut off from parent end, and serum was isolated from the blood sample using centrifugation ($1000 \times g$, 10 min, 4°C). The experimental procedures of this study were conducted in accordance with the principles of the Declaration of Helsinki, and were approved by the Ethics Committee of Weifang People's Hospital.

Clinical characteristics

Demographic and clinical features, including gestational age, sex, birth weight, delivery mode, Apgar score, birth asphyxia, maternal gestational diabetes mellitus (GDM) and prenatal hormone use, were collected from the infants of both NRDS and control group. The Apgar scores at 1 and 5 min after delivery were recorded for analysis. Birth asphyxia was defined as that the newborns failed to initiate and maintain adequate breathing within 1st and 5th minutes of birth, with an Apgar score of less than 7 (16). Maternal GDM was diagnosed based on the results of fasting blood glucose examination and oral glucose tolerance test. The use of prenatal hormones meant whether the mothers of newborns used glucocorticoids before delivery.

Measurement of vitamins A, D and E

The levels of vitamins A, D and E in umbilical cord serum samples were detected by electrochemiluminescence assay using the LK3000VI vitamins detector (LANBIAO, Tianjin, China) following the manufacturer's protocols. Each sample was detected for at least 3 times.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad version 7.0 software (GraphPad Software Inc., USA). The data were expressed as mean $\pm$ SD or frequency (percentage). The comparisons between two groups were assessed using student's t-test or Chi-square test as appropriate. Logistic regression analysis was applied to examine the predictive value of vitamins A, D, and E for the onset of NRDS. Receiver operator characteristic curve (ROC) was plotted to confirm the prediction accuracy and cutoff value of vitamins A, D and E. A P value of less than 0.05 was considered significant.

RESULTS

Clinical characteristics of the study population

For NRDS neonates, the gestational age was 33.2 ± 4.3 weeks (range of 27 – 42 weeks), birth weight was 2124.4 ± 678.1 g (range of 1052 – 3755 g), and 106 male and 44 female infants were included. There were no differences between neonates in NRDS and normal control groups at gestational age, sex and birth weight (all $P > 0.05$) (Table I).

NRDS infants included 66 caesarean cases, and normal controls contained 53 cases by caesarean

delivery, and no difference was found between the two groups at delivery methods ($P = 0.125$). There were 87 cases in NRDS group and 92 cases in normal control group received hormone before birth ($P = 0.556$). Based on gestational age, NRDS group had 114 preterm infants, and normal control group contained 112 preterm newborns, showing no significant difference ($P = 0.789$). In addition, the Apgar score of NRDS group was significantly lower than that of normal control group at both 1 min and 5 min ($P < 0.001$). More birth asphyxia and maternal GDM cases were observed in NRDS group compared with normal control group ($P < 0.05$).

Table I. *Clinical characteristics of the study population*

Variables	Controls (n = 150)	NRDS (n = 150)	<i>P</i> value
Gestational age (weeks)	33.4 ± 4.5	33.2 ± 4.3	0.686
Sex			0.088
Female	58 (38.7%)	44 (29.3%)	
Male	92 (61.3%)	106 (70.7%)	
Birth weight (g)	2135.2 ± 763.5	2124.4 ± 678.1	0.897
Delivery method			0.125
Vaginal	97 (64.7%)	84 (56.0%)	
Caesarean	53 (35.3%)	66 (44.0%)	
Apgar score			
1 min	7.1 ± 1.3	5.4 ± 1.7	< 0.001
5 min	8.4 ± 1.2	6.8 ± 1.4	< 0.001
Birth asphyxia	57 (38.0%)	94 (62.7%)	< 0.001
Maternal GDM	12 (8.0%)	28 (18.7%)	0.007
Prenatal hormone use	92 (61.3%)	87 (58.0%)	0.556
Preterm cases	112 (74.7%)	114 (76.0%)	0.789

NRDS: neonatal respiratory distress syndrome; **GDM:** gestational diabetes mellitus.

Differential levels of vitamins A, D and E in newborns with NRDS

By analyzing the umbilical cord serum for vitamins A, D and E, we found that NRDS newborns had significantly lower vitamin A (0.22 ± 0.08 mg/L vs. 0.37 ± 0.09), vitamin D (18.83 ± 8.21 ng/mL vs. 41.80 ± 17.71) and vitamin E (6.67 ± 2.66 mg/L vs. 9.37 ± 3.71) compared with the infants in the control group (all $P < 0.001$) (Fig. 1).

Prediction of vitamins A, D and E for NRDS occurrence

To evaluate the predictive value of vitamins A, D and E for the onset of NRDS in the included neonates, the 3 types of vitamins and other clinical variables that might affect the development of NRDS were included in a logistic regression analysis. The univariate analysis results showed that Apgar score at both 1 and 5 min, birth asphyxia, maternal GDM, vitamins A, D and E were associated with NRDS (all $P < 0.05$) (Table II). The further multivariate analysis results revealed that birth asphyxia (OR = 2.143, 95% CI = 1.532 - 2.781, $P = 0.016$), vitamins A (OR = 2.217, 95% CI = 1.509 - 3.084, $P = 0.023$), and vitamins D (OR = 2.355, 95% CI = 1.744 - 3.103, $P = 0.009$) were independent risk factors for the occurrence of NRDS. Furthermore, ROCs based on vitamins A, D and E were plotted for NRDS neonates (Fig. 2).

The results indicated that the 3 types of vitamins had certain predictive accuracy for NRDS onset, with the area under the curve (AUC) of 0.885 for vitamin

A (cutoff value: 0.295 mg/L, sensitivity: 83.3%, specificity: 78.0%), 0.875 for vitamin D (cutoff value: 30.16 ng/mL, sensitivity: 90.00%, specificity: 73.33%), and 0.720 for vitamin E (cutoff value: 8.04 mg/L, sensitivity: 73.33%, specificity: 62.67%).

Clinical value of vitamins A, D and E in preterm neonates with NRDS

The 150 NRDS patients contained 114 preterm and 36 term neonates, and the 150 in the control group included 112 preterm and 38 term neonates. This study evaluated the predictive performance of vitamins A, D and E in preterm and term neonates, respectively. In preterm neonates, serum levels of vitamins A, D and E remained lower in NRDS cases compared with those in normal controls (all $P < 0.001$), (Fig 3A-C).

The logistic regression results found the potential relationship between NRDS and Apgar score, birth asphyxia, maternal GDM, vitamins A, D and E (all $P < 0.05$) (Table III), and that birth asphyxia (OR=1.732, 95%CI=1.278-2.240, $P=0.039$), vitamin A (OR=2.550, 95%CI=1.876-3.446, $P=0.008$), vitamin D (OR=2.693, 95%CI=1.756-3.742, $P=0.007$), and vitamin E (OR=1.387, 95%CI=1.106-1.636, $P=0.046$) were independently associated with the occurrence of NRDS in preterm neonates. The ROCs showed that the AUCs of vitamins A, D and E were 0.940, 0.902 and 0.763, respectively, with the following results of cutoff value (sensitivity, specificity): 0.275 mg/L (96.49%, 81.25%) for vitamin A; 24.15 ng/mL (92.98%, 81.25%) for

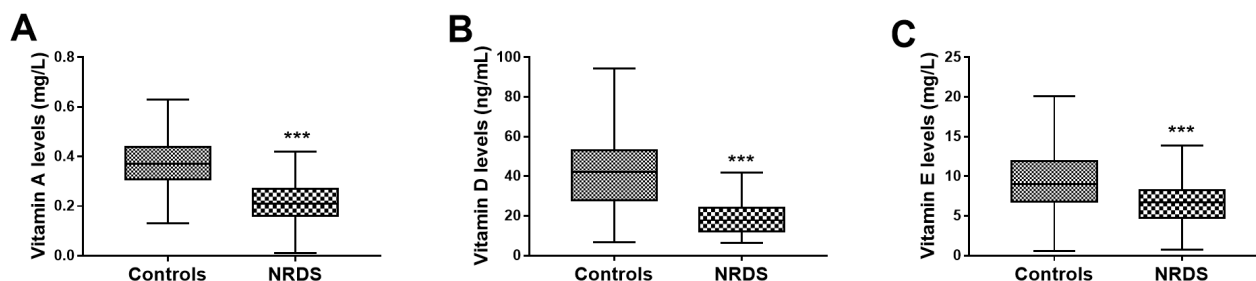


Fig 1. Umbilical cord serum vitamins A, D and E in neonates with NRDS. A. NRDS neonates had lower levels of vitamins A than normal controls. B. Vitamin D levels were lower in NRDS neonates than normal controls. C. The levels of vitamin E were lower in NRDS neonates compared with that of normal controls. *** $P < 0.001$ vs. controls.

Table II. Logistic analysis of risk factors for NRDS neonates.

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Gestational age	1.477 (0.785-2.216)	0.312	-	-
Sex	1.324 (0.657-1.993)	0.483	-	-
Birth weight	1.465 (0.815-1.752)	0.292	-	-
Delivery method	1.537 (0.952-2.136)	0.137	-	-
Apgar score-1 min	1.596 (1.210-2.096)	0.028*	1.472 (0.984-2.097)	0.066
Apgar score-5 min	1.484 (1.192-1.853)	0.034*	1.391 (0.931-1.867)	0.073
Birth asphyxia	2.252 (1.760-2.895)	0.009*	2.143 (1.532-2.781)	0.016*
Maternal GDM	1.769 (1.217-2.361)	0.029*	1.566 (0.993-2.225)	0.056
Prenatal hormone use	1.181 (0.837-1.539)	0.268	-	-
Vitamin A	2.372 (1.628-3.115)	0.006*	2.217 (1.509-3.084)	0.023*
Vitamin D	2.491 (1.832-3.156)	0.004*	2.355 (1.744-3.103)	0.009*
Vitamin E	1.686 (1.101-2.213)	0.039*	1.211 (0.996-1.612)	0.055

NRDS: neonatal respiratory distress syndrome; **GDM:** gestational diabetes mellitus; * $P < 0.05$.

vitamin D; 7.74 mg/L (86.84%, 55.36%) for vitamin E (Fig. 3D-F).

Predictive performance of vitamins A, D and E in term neonates with NRDS

For the term neonates of this study, lower vitamins A and D were also observed in NRDS cases when compared to control group (all $P < 0.001$), (Fig. 4A, B). However, no significant differences were observed in vitamins E between the two groups ($P > 0.05$), (Fig. 4C). The subsequent logistic analysis results revealed that Apgar score, birth asphyxia,

maternal GDM, vitamins A and D were related with NRDS (all $P < 0.05$), (Table IV), and that Apgar score at 5 min (OR = 1.653, 95% CI = 1.316 – 1.962, $P = 0.026$), birth asphyxia (OR = 2.175, 95% CI = 1.641 - 2.768, $P = 0.011$), maternal GDM (OR = 1.573, 95% CI = 1.017 - 2.120, $P = 0.043$), vitamins A (OR = 2.109, 95% CI = 1.463 – 2.986, $P = 0.028$) and D (OR = 2.652, 95% CI = 2.042 – 3.464, $P = 0.005$) were independent risk factors for the onset of NRDS in term neonates.

According to the ROC analysis (Fig. 4D, E), we found that vitamins A and D showed certain

Table III. Logistic analysis of risk factors for preterm neonates with NRDS.

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	<i>P</i> value	OR (95% CI)	<i>P</i> value
Gestational age	1.684 (0.816-2.579)	0.107	-	-
Sex	1.406 (0.674-2.202)	0.354	-	-
Birth weight	1.379 (0.918-1.832)	0.098	-	-
Delivery method	1.297 (0.741-1.933)	0.234	-	-
Apgar score-1 min	1.402 (1.091-1.849)	0.034*	1.340 (0.989-1.769)	0.058
Apgar score-5 min	1.798 (1.209-2.313)	0.024*	1.672 (0.966-2.476)	0.066
Birth asphyxia	1.841 (1.321-2.354)	0.013*	1.732 (1.278-2.240)	0.039*
Maternal GDM	1.261 (1.047-1.549)	0.018*	1.153 (0.992-1.448)	0.057
Prenatal hormone use	1.255 (0.895-1.724)	0.135	-	-
Vitamin A	2.572 (1.928-3.289)	0.004*	2.550 (1.876-3.446)	0.008*
Vitamin D	2.794 (1.891-3.718)	0.001*	2.693 (1.756-3.742)	0.007*
Vitamin E	1.775 (1.242-2.308)	0.038*	1.387 (1.106-1.636)	0.046*

NRDS: neonatal respiratory distress syndrome; **GDM:** gestational diabetes mellitus; * $P < 0.05$.

predictive accuracy (AUC: 0.876, cutoff value: 0.375 mg/L, sensitivity: 88.89%, specificity: 81.58% for vitamin A; AUC: 0.893, cutoff value: 40.19 ng/mL, sensitivity: 97.22%, specificity: 84.21% for vitamin D). Although vitamin E had no significant relationship with NRDS in term neonates, a ROC for vitamin E was still performed (Fig. 4F), which showed that the AUC was only 0.685.

DISCUSSION

Considering the important role of vitamins in NRDS, this study evaluated the clinical predictive value of umbilical cord serum vitamins A, D and E for the onset of NRDS. The analysis results found that NRDS neonates had significantly reduced vitamins A, D and E compared with normal controls,

and the decreased vitamins were independent risk factors to indicate the occurrence of NRDS with certain predictive accuracy. In addition, according to the gestational age at birth, the NRDS cases were

divided into preterm and term groups, and vitamins A and D levels were also lower in NRDS cases in both preterm and term cases, while reduced vitamin E was only presented in preterm neonates. In preterm

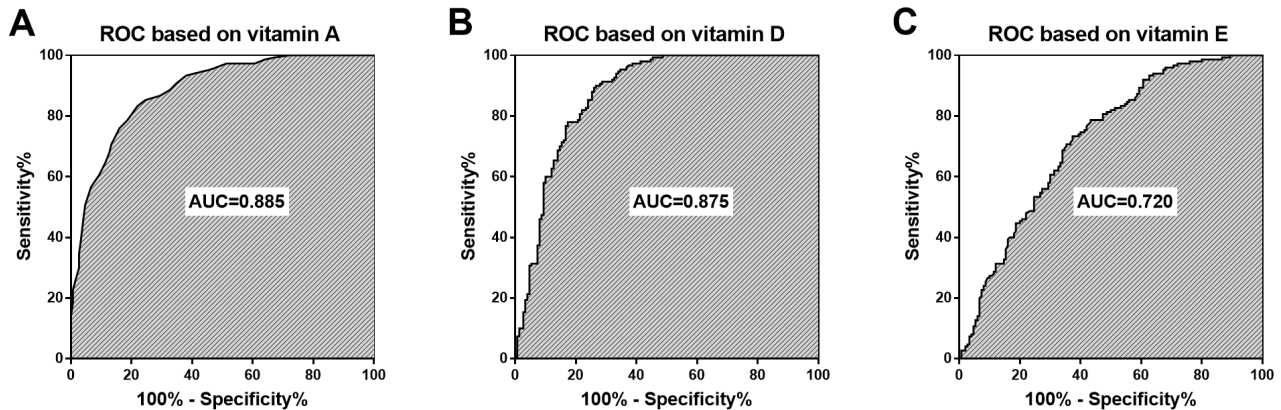


Fig 2. ROC analysis based on vitamins A, D and E for neonates with NRDS. A. ROC curve based on vitamin A levels showed that the area under the curve (AUC) was 0.885. B. ROC curve based on vitamin D had an AUC of 0.875. C. The AUC of the ROC based on vitamin E was 0.720.

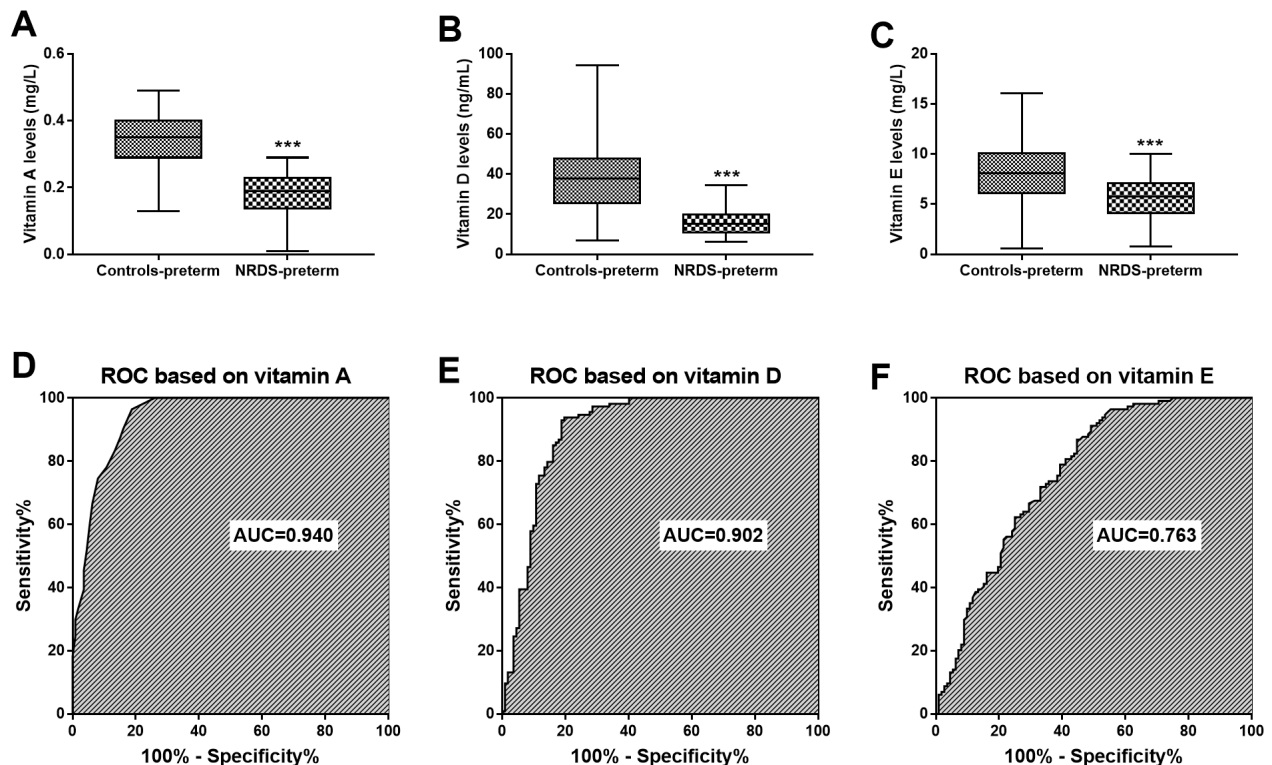


Fig 3. Expression of vitamins A, D and E and the corresponding ROC analysis in preterm neonates with NRDS. A-C. Umbilical cord serum levels of vitamins A, D and E were lower in NRDS cases compared with normal controls ($***P < 0.001$ vs. controls-preterm). D-F. ROC curves based on vitamins A, D and E, and the AUCs were 0.940, 0.902 and 0.763, respectively. AUC, area under the curve.

NRDS neonates, vitamins A, D and E could predict disease onset, while in term NRDS cases, only vitamins A and D were independently associated with disease occurrence.

Vitamin A is a type of lipid-soluble vitamins, and its active form is reticoid acid in body (17). Vitamin A has been reported to affect the development of lung, and the deficiency of vitamin A leads to diseases related to respiratory system (18). In very low birth weight infants, vitamin A supplementation could alleviate the rate of bronchopulmonary dysplasia (19). Adequate vitamin A intake can ensure lung function and reduce the morbidity of fixed airflow

limitation and incident asthma (20). In NRDS, the supplementation of vitamin A has been reported to efficiently improve the survival and oxygen requirement of neonates (21). The combination of vitamin A and PS has been proposed as an efficient therapeutic method for NRDS (8). In our study, the levels of vitamin A in the umbilical cord serum of NRDS neonates were significantly lower than that of normal controls, which proved the previously demonstrated relationship between vitamins A and NRDS (21). This study used umbilical cord blood, owing that umbilical cord is the connection between mother and fetus, which can better reflect

Table IV. Logistic analysis of risk factors for term neonates with NRDS.

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Gestational age	1.339 (0.726-1.994)	0.481	-	-
Sex	1.269 (0.773-1.856)	0.458	-	-
Birth weight	1.204 (0.802-1.638)	0.357	-	-
Delivery method	1.562 (0.953-2.230)	0.094	-	-
Apgar score-1 min	1.587 (1.286-2.009)	0.016*	1.439 (0.995-1.934)	0.052
Apgar score-5 min	1.615 (1.291-2.013)	0.013*	1.653 (1.316-1.962)	0.026*
Birth asphyxia	2.269 (1.668-2.987)	0.006*	2.175 (1.641-2.768)	0.011*
Maternal GDM	1.695 (1.327-2.034)	0.021*	1.573 (1.017-2.120)	0.043*
Prenatal hormone use	1.261 (0.824-1.712)	0.232	-	-
Vitamin A	2.184 (1.561-2.888)	0.017*	2.109 (1.463-2.986)	0.028*
Vitamin D	2.569 (1.996-3.347)	0.003*	2.652 (2.042-3.464)	0.005*
Vitamin E	1.579 (0.965-2.287)	0.072	-	-

NRDS: neonatal respiratory distress syndrome; **GDM:** gestational diabetes mellitus; * $P < 0.05$.

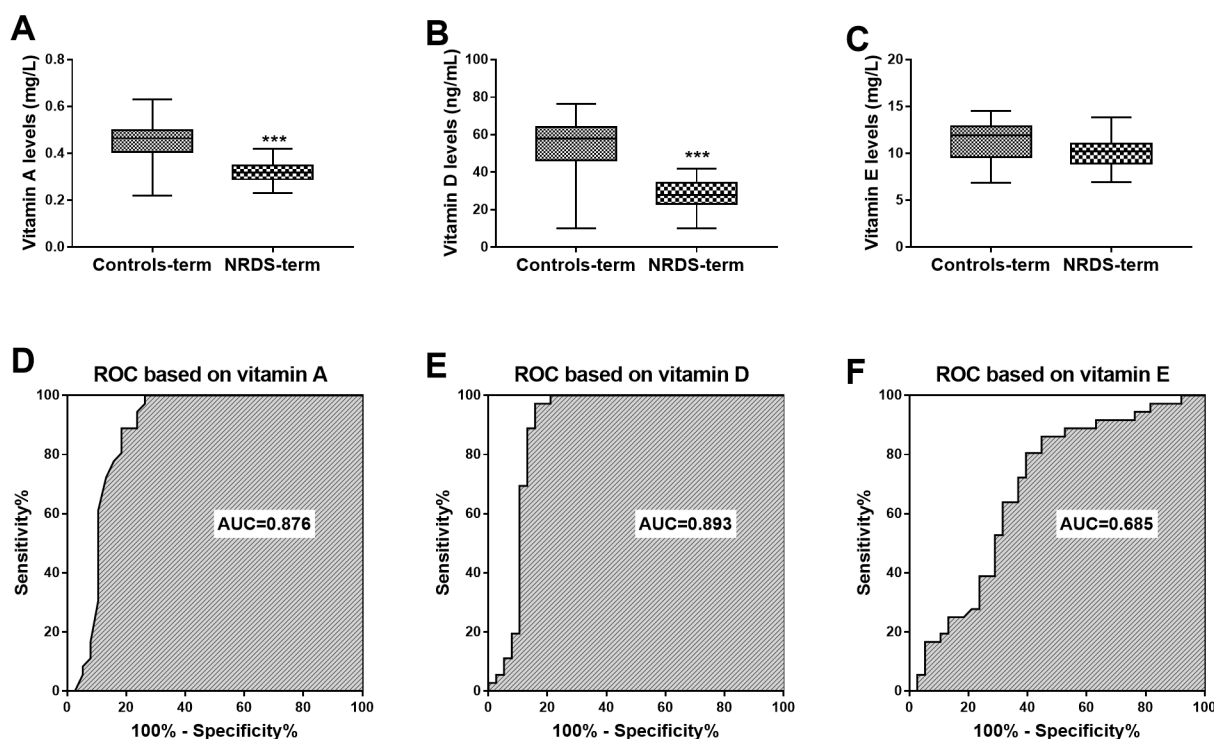


Fig 4. Expression of vitamins A, D and E and the corresponding ROC analysis in term neonates with NRDS. A and B. Umbilical cord serum levels of vitamins A and D were lower in NRDS cases compared with normal controls (*** $P < 0.001$ vs. controls-term). C. No significance difference in vitamin E was found between NRDS and normal control groups. D-F. ROC curves based on vitamins A, D and E, and the AUCs were 0.876, 0.893 and 0.685, respectively. AUC, area under the curve.

the nutrition of the mother and the neonates. More importantly, by analyzing the levels of vitamin A in NRDS and normal neonates, we demonstrated that vitamin A was independently associated with NRDS onset, indicating that vitamin A level might be a risk factor to indicate the occurrence of NRDS.

Vitamin D plays an important role in bone metabolism and neuromuscular function, and emerging evidence has highlighted its role playing in fetal development (22). Vitamin D has been reported to increase PS synthesis and secretion and enhance lung development (23). Umbilical cord vitamin D levels have been found to be associated with the risk of preterm birth, incidence of NRDS and hospitalization (24). Low levels of maternal and neonatal vitamin D were associated with the development of bronchopulmonary dysplasia in preterm infants (25). Dogan et al. has reported that vitamin D deficiency is a risk factor for NRDS in preterm neonates (26). Consistently, the umbilical

cord vitamin D in NRDS neonates in our study was also lower when compared to normal neonates, and the low vitamin D level might serve as an independent predictive indicator for NRDS with considerable accuracy.

Vitamin E is also one of important nutrients with strong anti-oxidative function, and could prevent cell oxidative damage, delay cell chromosome division, and prolong cell survival (27). Evidence showed that vitamin E deficiency promotes the sensitivity of lung to oxygen, ozone and CO_2 (28). Injection of vitamin E could significantly improve against NRDS, shorten the time of oxygen delivery, and reduce retinal damage induced by hyperoxia (29). In our study, vitamin E levels were significantly lower in preterm NRDS neonates compared with normal preterm infants and acted as an independent indicator for NRDS onset. However, the levels of vitamin E in term neonates did not show significant changes between NRDS and normal cases, and

the relationship between vitamin E and NRDS was also not found. These contradictory results between preterm and term neonates might be because of the poor nutrition condition of preterm infants. Compared to term neonates, preterm newborns have relatively low birth weight and worse development of various organs, including lung (2). The poor nutrition condition of preterm neonates makes them more sensitive to various nutrients, such as vitamins. Considering the critical role of vitamins in the alleviation of oxidative damage, vitamin E changes might be more sensitive when related with NRDS in preterm neonates. Anyway, the anti-oxidative function of vitamin E makes it an indispensable important nutrient in the treatment of NRDS.

Our analysis results provided evidence for vitamins A, D and E as indicators for the risk of NRDS and indicated that the predictive value of vitamins might only be presented in preterm infants. However, the negative results in term neonates might be due to the limited sample size. Since NRDS commonly occurs in preterm infants, this study only collected serum sample from 36 term neonates. Thus, further investigations are necessary to confirm the clinical value of vitamins using a larger study population.

In conclusion, vitamins A, D and E levels in NRDS neonates were reduced compared to normal controls, and vitamins A and D were independent predictive indicators for NRDS occurrence in both preterm and term newborns, while the association between vitamin E and NRDS was only found in preterm neonates. Our study demonstrated that vitamins A, D and E are not only important nutrients that need to be supplemented for NRDS treatment, but also play a pivotal role in the prediction of NRDS onset. Thus, the detection of vitamins levels in neonates, especially preterm cases, is of great significance for the prevention and treatment of NRDS.

ACKNOWLEDGEMENTS

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the serum extraction and examinations of vitamins. AD and XY collected the blood samples and clinical characteristics of the participants.

Ethics approval and consent to participate

The experimental procedures of this study were approved by the Ethics Committee of Weifang People's Hospital, and informed consent was obtained from the guardians of the participants.

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TREM-1 inhibition of Kupffer cells alleviates inflammation, oxidation, and apoptosis induced in rat liver by heat stroke

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To investigate the effect and probable mechanism of triggering receptor expressed on myeloid cells-1 (TREM-1) inhibition of hyperactive Kupffer cells (KCs) on acute liver injury caused by severe heat stroke (HS). A total of 32 rats were randomized into the LP17, GdCl₃, control (CON), and heat stroke (HS) groups. Serum ALT and AST activities in each group were assessed. Additionally, liver tissues were obtained to examine cellular structure, determine superoxide dismutase (SOD) concentrations, analyze apoptosis, and determine the expression levels of TREM-1, TREM-2, DAP12, TNF- α , and IL-6 in KCs, as well as Bcl-2, Bax, Cleaved-Caspase3, and Cleaved-Caspase9 in liver. Results of the study showed that compared to the CON group, the HS group had higher serum AST and ALT levels, with more serious pathological changes. Hepatocyte apoptosis and lysosome formation in KCs increased when heatstroke occurred, while SOD concentrations decreased. Moreover, expression levels of TREM-1, DAP12, TNF- α , IL-6, Bax, Cleaved-Caspase3, and Cleaved-Caspase9 increased in the HS group, while TREM-2 and Bcl-2 expression levels decreased. Pretreatment with LP17 and GdCl₃ significantly ameliorated the aforementioned indicators, relieving acute liver injury caused by heat stroke. The results of this study showed that Inhibiting TREM-1 of KCs improved severe HS-induced liver injury, which involved an underlying mechanism of regulating the imbalance of expressions in the TREMs/DAP12 pathway. Thus, inhibiting KC activities improved antioxidant consumption and alleviated apoptosis-related protein overexpression, thereby reducing HS-induced hepatocyte apoptosis.

Heat stroke (HS) is a severe heat illness, which is extremely common in daily life and work and can seriously threaten people's health. HS can be defined as a systemic inflammatory response induced by hyperthermia, leading to multiple organ dysfunction syndrome (MODS), wherein encephalopathy

dominates (1, 2). In fact, previous studies on severe HS have demonstrated that a large number of inflammatory cells infiltrate involved tissues and organs, activating and amplifying the inflammatory response (3, 4).

Of these involved organs, the liver is the most common first-acting organ for severe HS. Our pilot study

Key words: TREM-1; Kupffer cells; heat stroke; inflammation; oxidation; apoptosis

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confirmed that hyperactive Kupffer cells (KCs) induced by heat stroke played a proinflammatory role in HS-induced acute liver injury, but its molecular mechanism has yet to be elucidated (5). Notably, triggering receptor expressed on myeloid cells-1 (TREM-1) is a receptor protein mainly expressed on the surface of monocytes/macrophages and neutrophils, whose upregulated expression under heat stress is closely related to excessive cell activities and uncontrolled inflammation (6,7). It has even been shown that pathogens can stimulate KCs by upregulating TREM-1 expressions, which may contribute to hepatic inflammation and liver disease (8). Accordingly, to determine the inner relationship between abnormal TREM-1 expressions in hepatic innate macrophages and subsequent liver injury following severe HS, depressed TREM-1 expressions in rat liver macrophages using specific inhibitor LP17 and downregulated KC activities using Gadolinium trichloride (GdCl₃) were studied. Moreover, changes related to inflammation, oxidation, and apoptosis in rat liver tissues were examined, while the probable effects of LP17 and GdCl₃ were investigated.

MATERIALS AND METHODS

Animals

A total of 32 specific pathogen-free (SPF) adult male Wistar rats, weighing 180–210 g, were fed normally at an ambient temperature of 22±1°C and humidity of 50±5% for 72h before the experiment. The rats were then fasted for 12h before the experiment but had free access to water until 6 h prior. The Institutional Animal Care and Use Committee of Binhaiwan Central Hospital of Dongguan approved all procedures involving experimental animals (No.2016022).

Animal model

Rats were randomly assigned to four groups (n=8): blank control group (CON group), heat stroke group (HS group), GdCl₃ (Sigma-Aldrich, St. Louis, MO, USA) inhibitor group (GdCl₃ group), and LP17 (according to the TREM-1 sequence provided by GenBank/EMBL/DBJ, composed by Shanghai Bioengineering Technology Co. Ltd) inhibitor group (LP17 group).

Induction of HS model

The GdCl₃ group rats were injected with 10 mg/kg

GdCl₃ into the rat tail vein twice daily for 48h before HS induction, while the LP17 group rats were similarly injected with 100 mg/kg LP17 once daily (9). On the other hand, the CON group rats were placed at an ambient temperature (24 °C) for 100 mins, while rats in the other groups (HS, GdCl₃, LP17) were subjected to continuous heat stress inside a temperature- and humidity-controlled chamber (pre-set at 40±2.0°C with a relative humidity of 65%±5%). Our pilot study had confirmed that the time of HS onset in non-anesthetized rats was approximately 85–90 mins; thus, the rats in this experiment were exposed to heat stress for exactly 80 mins (5). Afterwards, the rats were removed from the incubator and allowed to passively recover at room temperature (25 ± 0.5 °C) in a new cage with food and water available ad libitum. Following this, they were anesthetized using a sodium pentobarbital (30mg/ kg), and liver tissue (right lobe) and blood samples (4 mL) acquired from the inferior vena cava were obtained for further analysis. All animals were treated based on the following guidelines - the Aggressive Guidelines 2.0 and the Guide for the Care and Use of Laboratory Animals (10,11).

Examinations of liver injury biomarkers

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were determined using commercially available kits (ALT ELISA kit, ab234579, Abcam, Cambridge, MA, USA; AST ELISA kit, ab263883, Abcam), following the manufacturer's instructions.

Histological examination of liver tissue

Liver sections were stained using hematoxylin and eosin (H&E) following standard protocol and were randomly numbered. Images were obtained using an optical microscope. Afterwards, the liver injury grading was blindly assessed by a pathologist, wherein five fields at 200× magnification were randomly selected from one liver section, and the liver injury degree was evaluated based on the Li team's injury grading score (12). The grades used were as follows: Grade 0, no pathological change; Grade 1, presence of degenerated hepatocytes with rare necrosis foci; Grade 2, small area of mild centrilobular necrosis around the central vein; Grade 3, area of mild centrilobular necrosis more severe than Grade 2; and Grade 4, centrilobular necrosis more severe than Grade 3.

Examinations of liver tissue under ultramicroscope

Liver tissues for transmission electron microscopy (TEM) were prefixed in glutaraldehyde and post-fixed in osmium tetroxide. After gradient dehydration with ethoxide and acetone, the tissues were embedded in epoxy resin, Epon812, and were examined under TEM (Hitachi H-600, Japan).

Expression of superoxide dismutase (SOD) in liver

For immunohistochemistry, liver sections were deparaffinized, rehydrated, and incubated with SOD antibody (ab51254, Abcam), following the manufacturer's instructions. Images were obtained using a light microscope, and optical densities (OD) of the positive areas were determined using Image-Pro Plus ver. 6.0 software under microscopy. The total OD value was then divided by the area of the same slice, obtaining the mean optical density (MOD).

Examinations of hepatocyte apoptosis

Using TUNEL staining, liver sections were developed with Diaminobenzidine (DAB) and were counterstained with hematoxylin, turning the apoptotic nucleus into a brown hue and making nucleus membrane lysis and apoptotic bodies evident. Five fields at 400 $\times$ magnification were randomly selected from one section, and the number of positively stained cells per 100 cells (%) was counted.

Examination of the expression of inflammatory mediators, TREMs/DAP12 pathway in KCs, and apoptosis-related proteins in liver tissue

KCs were obtained from liver tissues using collagenase IV (17104019, Gibco, USA) perfusion and was followed by Percoll (p4937, Sigma-Aldrich) density gradient centrifugation. They were then homogenized in the RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added PMSF (0.1%) protease inhibitor (Lot:P8340, Sigma-Aldrich, USA). Afterwards, cell lysates were obtained by centrifuging the homogenate at 12,000 g for 10 min at 4°C, and protein concentration was determined using the bicinchoninic acid (BCA) assay. Proteins (20 μ g) were then run on 15% SDS-PAGE gels under reducing conditions and transferred to an Immobilon-P PVDF Membrane (Millipore, Burlington, MA). After which, the membranes were blocked in 5% non-fat milk for 1h and then incubated with primary

antibodies (TREM-1 (ab214202, Abcam), TREM-2 (ARG64950, arigo, TW, China), DAP-12 (ab25098, Abcam), TNF- α (ab6671, Abcam), IL-1 β (ab9722, Abcam), Bcl-2 (ab194583, Abcam), Bax (ab32503, Abcam), cleaved-caspase3 (ab49822, Abcam), and cleaved-caspase9 (c7729, Sigma-Aldrich)) overnight at 4°C. After incubation membranes were washed 3 times with TBST, incubated with HRP conjugated secondary antibodies in 2% milk/PBST for 2 hours at RT. Following incubation, the membranes were washed with TBST and visualized using an enhanced chemiluminescent detection kit. Expression was detected by X-ray film (C500046, Sangon biotech). The Optical Density (OD) of the protein bands was determined using ImageJ software. The WB test was repeated twice for all samples.

Statistical analysis

All data were analyzed using SPSS software (version 17.0; IBM, USA), with the data presented as means $\pm$ SD. Analysis of variance (ANOVA) was used to compare the differences between groups, and pairwise comparisons were conducted using t-tests. The level of statistical significance was set at $P < 0.05$ for all analyses.

RESULTS

Elevated concentrations of liver injury biomarkers and pathologic injury score after severe HS was inhibited by LP17

AST and ALT concentration levels were examined using ELISA to assess liver injury. The results showed that severe HS could elevate AST and ALT concentration levels in the liver tissue, which could be significantly inhibited by LP17 and GdCl₃ ($p < 0.01$), with both demonstrating equivalent inhibitory effects ($p > 0.05$) (Fig. 1A). Histological liver tissue examination stained with H&E from the four groups using optical microscopy revealed that severe HS could induce central venule and sinusoid congestion (black arrow), sheet-like hepatocyte shrinkage, nucleus shrinkage (black box), and inflammatory cell infiltration around the sinusoids. Compared to the HS group, milder sinusoidal congestion was observed in the LP17 and GdCl₃ groups, with fewer inflammatory cell infiltrations,

hepatocellular ballooning, and cell shrinkage (black box) (Fig. 1B). Comparison of the pathological injury scoring showed that histopathological changes in the HS group liver tissue were more severe as compared to the LP17 and GdCl₃ group samples ($p < 0.01$), but the difference between the latter two groups was not significant ($p > 0.05$) (Fig. 1C).

LP17 modified hepatocyte apoptosis induced by severe HS and elevated activities of KCs

Observations under TEM showed obvious changes, including nuclear pyknosis, increase of heterochromatin, aggregation at the inner edge

of nuclear membrane, blurring and aggregation of endoplasmic reticulum and mitochondria. Additionally, lysosomes with high electron densities were observed in KCs after HS. The ultramicroscopic changes in the hepatocytes and KCs of the LP17 and GdCl₃ groups were milder as compared to those in the HS group (Fig. 2).

LP17 promoted synthesis of antioxidant in liver tissue.

Immunohistochemical analysis showed that, during heat stress, SOD expression in the liver tissue presented with an increasing trend, which was

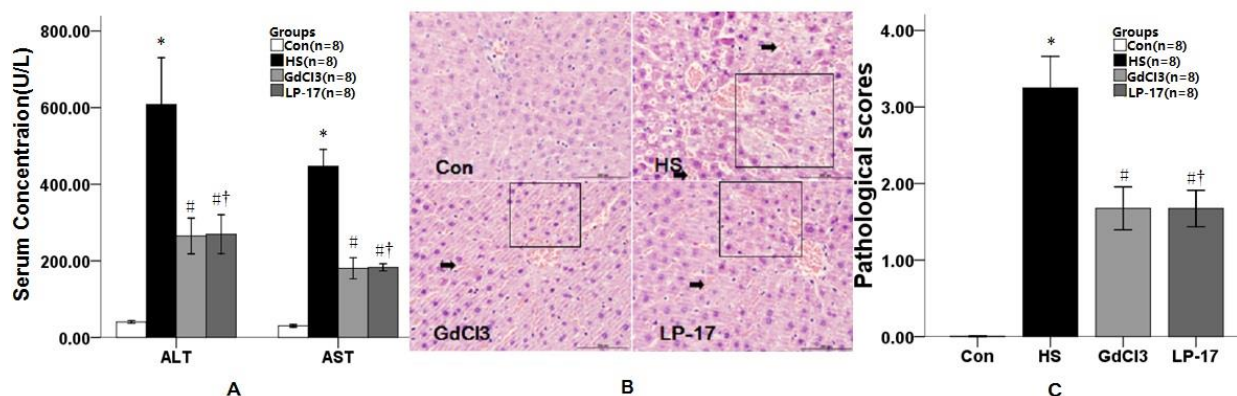


Fig. 1. The serum content of ALT and AST in the rats of each group. a): The changes in the liver by optical microscopy; b): HE staining, 200 $\times$). Pathological Scores of liver; c): * $p < 0.01$ vs Con, # $p < 0.01$ vs HS, † $p > 0.05$ vs GdCl₃.

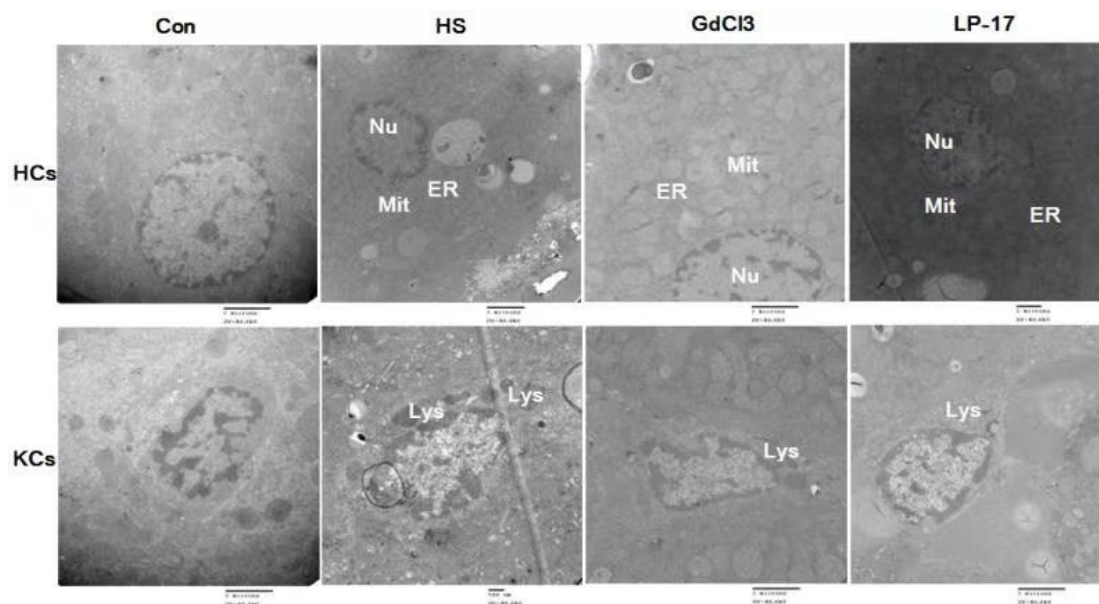


Fig. 2. Examinations of hepatocytes and Kupffer cells under ultramicroscope. HCs: Hepatic cells; KCs: Kupffer cells; Nu: Nucleus; Mit: Mitochondrion; ER: Endoplasmic reticulum; Lys: Lysosome.

mainly in the cytoplasm. The SOD levels in the HS group were the lowest ($p < 0.01$), and no significant difference was observed between the SOD levels of the LP17 and GdCl3 groups ($p > 0.05$) (Fig. 3).

LP17 ameliorated hepatocyte apoptosis induced by severe HS.

Examinations after TUNEL staining indicated that hepatocyte apoptosis increased significantly around the liver sinusoids in the HS group ($p < 0.01$), whereas hepatocyte apoptosis was milder in the LP17 and GdCl3 groups. Upon calculation of the apoptotic proportion in the count of 100 cells under microscopy, the number in the HS group was significantly higher compared to the LP17 and GdCl3 groups ($p < 0.01$),

with no difference found between the latter groups ($p > 0.05$) (Fig. 4).

Investigation of separated KCs by optical microscopy

The KCs isolated from rat livers were adherent and characterized as circles, quasi-circles, shuttles, polygons, and irregular shapes. Pseudopodia were also observed in some cells (Fig. 5)

LP modified inflammatory mediator over synthesis in KCs and abnormal protein expression in the TREMs/DAP12 pathway after severe HS

WB analysis showed that the protein expressions of TNF- α , IL-1 β , TREM-1, and DAP12 in KCs of HS group rats were significantly upregulated ($p < 0.01$), while

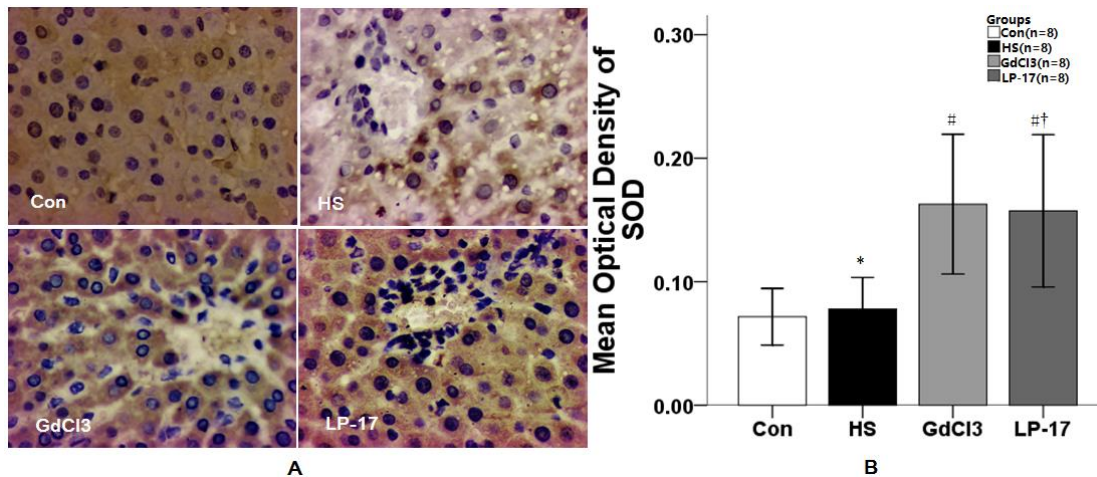


Fig. 3. SOD expression in liver under optical microscope. A): 400 $\times$) The mean optical density for SOD in liver; b): * $p < 0.01$ vs Con, # $p < 0.01$ vs HS, † $p > 0.05$ vs GdCl3.

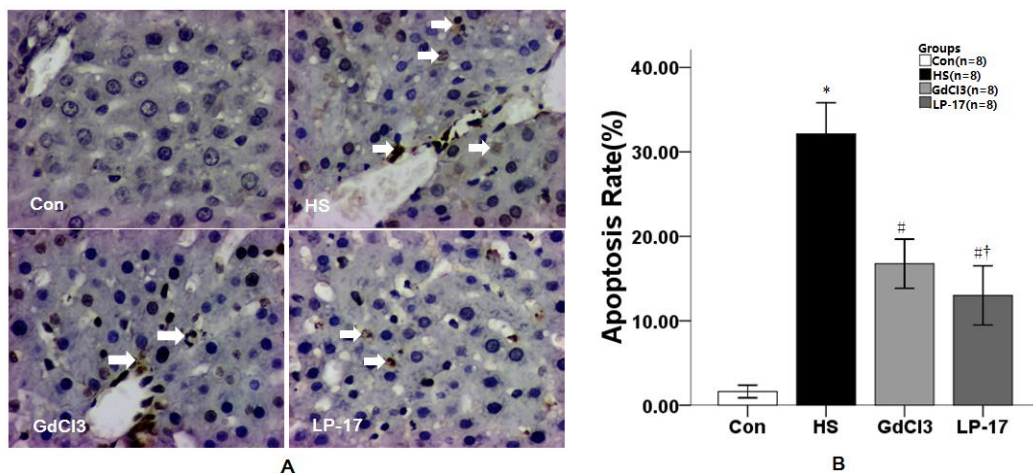


Fig. 4. The liver tissue with TUNEL staining under optical microscope. A): 400 $\times$). Apoptosis Rate in liver. b): * $p < 0.01$ vs Con, # $p < 0.01$ vs HS, † $p > 0.05$ vs GdCl3.

TREM-2 expressions decreased significantly ($p < 0.01$). In comparison, the expression levels of TNF- α , IL-1 β , TREM-1, and DAP12 in the LP17 and GdCl3 groups decreased ($p < 0.01$), whereas TREM-2 expression showed an increasing trend ($p < 0.01$) (Fig. 6).

LP17 down-regulated the expressions of apoptosis-related proteins in severe HS rat liver tissues.

The expression levels of apoptosis-related proteins in rat livers, such as Bcl-2, Bax, Cleaved-Caspase3, and Cleaved-Caspase9, were measured. Results showed that, compared to the CON group, the expression levels of Bax, Cleaved-Caspase9, and Cleaved-Caspase3 were significantly increased

in HS rats ($p < 0.01$), whereas Bcl-2 expression was significantly decreased ($p < 0.01$). Moreover, the expression levels of Bcl-2, Bax, Cleaved-Caspase9, and Cleaved-Caspase3 in the LP17 and GdCl3 groups were decreased as compared to the HS group ($p < 0.01$), with the exception of Bcl-2 (Fig. 7).

DISCUSSION

HS is a common disease during the hot season in southern China, which usually emerges during work, sports, and military training, leading to attrition or death (12). The causes of severe HS include initial thermal damage with secondary intestinal

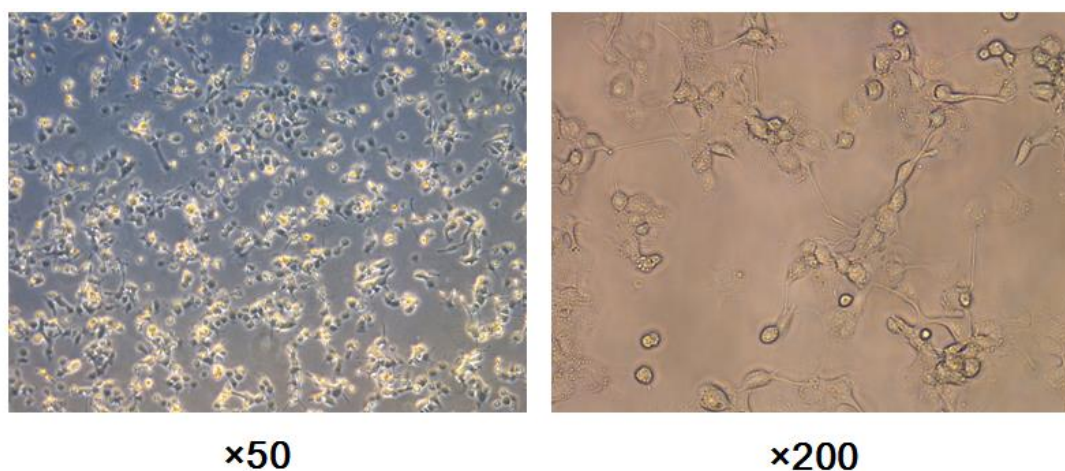


Fig. 5. The separated Kupffer cell under microscope.

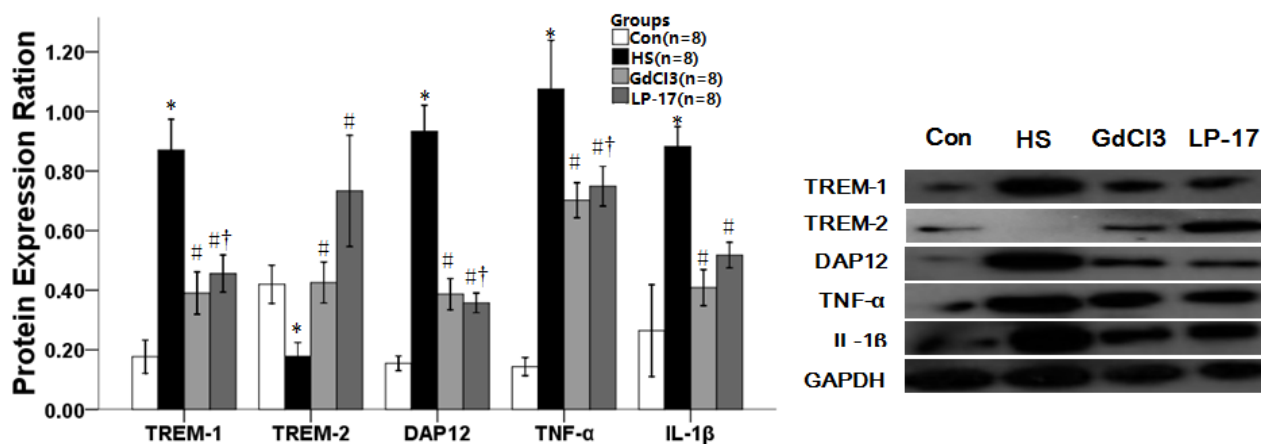


Fig. 6. The expressions of TNF- α , IL-1 β , TREM-1, TREM-2 and DAP12 in KCs. * $p < 0.01$ vs Con, # $p < 0.01$ vs HS, † $p > 0.05$ vs GdCl3.

barrier abnormalities, followed by uncontrolled inflammation and imbalanced coagulation, which are crucial pathophysiological mechanisms causing extensive tissue and organ damage. Therefore, some scholars have assumed severe HS to be a form of “heat sepsis” (14). Notably, acute liver injury or failure is more frequent in the exertional form of HS, wherein severe cases of liver damage can be irreversible and can even require liver transplantation (15, 16). Thus, determining the pathophysiology of liver injury induced by severe HS is valuable for the prevention, management, and prognosis of this condition. Interestingly, a study has documented that pyrocytosis mediated by inflammasomes plays an important role in severe HS-induced liver injury (17). Moreover, Li et al. reported that HS-induced hepatocyte exosomes promoted liver injury by activating and upregulating the NOD-like receptor signaling pathway in mice (18). As monocytes/macrophages are the core initiators and amplifiers of non-specific immune responses, Kupffer cells, which are the native liver macrophages, actively participate in the development and progression of liver damage and various liver or inflammatory diseases (19-21).

Since membrane protein receptors are extensively expressed on the surface of monocytes/macrophages, TREM-1 has been proven to be closely related to the development of inflammatory diseases and organ damage. In animal research regarding liver

injury, dramatically increased TREM-1 expression in KCs was correlated with inflammatory cytokine overproduction and inflammatory cell infiltration (22). In our study, before the HS rat model was prepared, the experimental animals were pretreated with LP17, a synthesized peptide specifically inhibiting TREM-1, and the underlying TREM-1 protective effects in KCs during severe HS-induced liver injury were investigated (23).

Liver sections of heat-stressed rats stained with H&E were presented with central venule and sinusoid congestion, hepatocyte shrinkage, nuclear shrinkage, and obvious inflammatory cell infiltration around the sinusoids, under a light microscope. These histological changes were significantly alleviated when pretreated with LP17, which inhibited TREM-1, and $GdCl_3$, which inhibited KC activities. Serum ALT and AST concentrations and blinded pathological damage scoring also helped to quantitatively evaluate liver damage severity caused by severe HS. These results suggest that LP17, similar to $GdCl_3$, had a protective effect against HS-induced liver damage. To further clarify cellular and organelle morphology changes, the ultrastructures of hepatocytes and KCs were observed under a transmission electron microscope. In fact, HS has been found to induce hepatocyte matrix edema, vacuolar degeneration, mitochondrial and endoplasmic reticulum swelling and blurring,

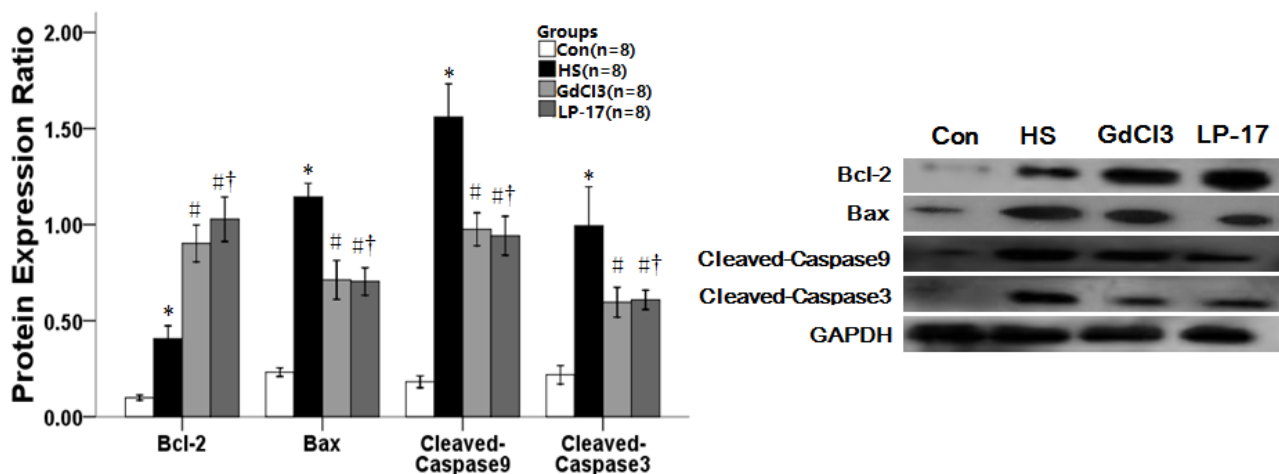


Fig. 7. The expressions of Bcl-2, Bax, Cleaved-Caspase3 and Cleaved-Caspase9 in liver: * $p < 0.01$ vs Con, # $p < 0.01$ vs HS, † $p > 0.05$ vs $GdCl_3$.

as well as pathological hepatocyte apoptosis, such as nuclear pyknosis, increase of heterochromatin, aggregation at the inner edge of nuclear membrane, blurring and aggregation of endoplasmic reticulum and mitochondria. Furthermore, increased electron-rich lysosomes in our study suggest abnormal KC activity enhancement during heat stress, which promoted the progression of severe HS-induced liver injury. Based on the histological appearances using microscopy, inhibiting TREM-1 with LP17 modified HS-induced liver injury, and this effect was equivalent in comparison to when KC activities were inhibited by GdCl₃.

To determine the pathophysiology of severe HS-induced liver injury, we measured the antioxidant levels and hepatocyte apoptosis in the liver tissue. Correspondingly, the production of antioxidants increased during the preliminary stage of oxidative stress; however, this overwhelming oxidation persistently consumed antioxidants, devastating the balance between antioxidants and oxidants and initiating tissue damage. Moreover, immunohistochemical analysis of the liver sections showed that antioxidant SOD expression was slightly increased in the hepatocyte cytoplasm during heat stress, which was more prominent in the LP17 and GdCl₃ groups than in the HS group. It has been established that SOD is a classical antioxidant that plays an important role in oxidative stress-induced liver injury (24). Therefore, we proposed that the effects of LP17 and GdCl₃ on HS-induced liver injury could be related to the inhibition of oxidative stress-induced liver injury. Furthermore, liver sections of severe HS rats stained by TUNEL showed that cell apoptosis was prominently increased, correlating with the changes in the microscopic and ultramicroscopic structures. Additionally, the proportion of apoptotic cells suggests that LP17 and GdCl₃ could improve apoptosis in the liver tissue of HS. Thus, we deduced that the protective effects of LP17 on severe HS liver were possibly related to the inhibition of oxidative stress and apoptosis.

KCs were isolated from HS rats using density gradient centrifugation to explore whether and how they are involved in the molecular mechanism of LP-17's mitigation of severe HS-induced liver damage.

In HS rats, it was found that TREM-1 and DAP-12 expressions in KCs were increased, whereas TREM-2 expression was decreased. Specifically, DAP12 is a transmembrane receptor of TREMs, and studies have confirmed that TREMs/DAP12 pathway abnormalities play an important role in the development of inflammatory liver injury that is usually characterized with upregulated TREM-1 and downregulated TREM-2, wherein TREM-1 overexpression directly led to a spurt of inflammatory activities in KCs (25, 26). Similar abnormal expression was observed in the TREMs/DAP12 pathway in our study, which was related to increased TNF- α and IL-1 β expressions in KCs. It was found that LP-17 and GdCl₃ could reduce the abnormalities of TNF- α and IL-1 β in KCs of HS rat liver, recovering homeostasis by depressing TREM-1 over-expression and improving TREM-2 under-expression. On the other hand, TREM-2 expressed in liver native macrophages inhibits classic proinflammatory signaling pathways, such as inflammatory response and TLR-4; thus, TREM-2 downregulation or absence can aggravate inflammation and oxidative damage (27). As a result, pretreatment with LP-17 can inhibit overexpression and upregulate TREM-2 expression in KCs of animals with severe HS, relieving the excessive promotion of inflammatory secretion. Additionally, to further investigate the relationship between the increased inflammatory activities of KCs and hepatocyte apoptosis after HS, WB analysis was used to determine the protein expression levels of Bcl-2, Bax, Cleaved-Caspase3, and Cleaved-Caspase9 in liver tissues. The results confirm that severe HS can lead to significant increases in the protein expression levels of Bax, Cleaved-Caspase3, and Cleaved-Caspase9 in liver tissues, whereas Bcl-2 expression levels are significantly reduced. As a classic pathway for regulating apoptosis, Bcl-2/Bax has been widely used in previous studies of liver injury, in which Bcl-2 downregulation and Bax overexpression are related to the stimulation and development of hepatocyte apoptosis, damage, and injury (28). Meanwhile, Cleaved-Caspase9 and Cleaved-Caspase3 act as the initiating and executive proteins of the downstream apoptotic pathway,

respectively, and their expression in the liver tissue of HS animals also increased significantly. In this part of the study, the LP17 upregulated Bcl-2 expression and downregulated Bax expression, while correspondingly prohibiting the production of Cleaved-Caspase3 and Cleaved-Caspase9, which was in line with other studies (29). Therefore, the mechanism of improving hepatocyte apoptosis by regulating TREM-1 in KCs was found to promote Bcl-2 expression and inhibit the expressions of Bax, Cleaved-Caspase3, and Cleaved-Caspase9.

In this study, since LP17 is an artificially synthesized peptide of monocyte/macrophage TREM-1, it cannot be completely ruled out that the protective effects on the liver after HS partially benefited from the inhibition of TREM-1 expression and activities in circulating monocytes/macrophages. The protective effects of LP17 can be considered to be potentially contributed by restraining KCs due to similarly improving liver dysfunction between LP17 and GdCl₃. Thus, a highly specific TREM-1 inhibitor in KCs is required for further study. Additionally, since the inhibition of LP17 and GdCl₃ on TREM-1 expression and activities are incomplete, TREM-1 gene knockout rats and transfection of over-expressing TREM2 plasmid were used to further investigate the effects of TREMs/DAP1 pathway abnormalities on severe HS-induced organ damage.

In conclusion, the results of our study confirmed the protective effects of inhibiting TREM-1 expression and activities in KCs against severe HS-induced liver injury. The probable mechanism involved in this condition was the regulation of the imbalanced TREMs/DAP12 pathway expressions, leading to KC activity downregulation, antioxidant synthesis promotion, amelioration of the abnormal expression of apoptosis-related proteins, and alleviation of HS-induced hepatocyte apoptosis. Moreover, the value of monocytes-macrophage TREM-1 in the pathogenesis and management of severe HS-induced liver damage was clarified.

For future studies, several unsolved problems still need to be addressed to improve the clinical application of TREM-1 inhibitors. First, the evaluation of future treatment with TREM-1 inhibitors should be carried out *in vitro* and in large animal models,

such as monkeys and pigs. Second, more clinical evidence should be collected. For the treatment of severe heatstroke, inhibiting TREM-1 expression on monocytes has broad clinical application prospects. The development and application of TREM-1 inhibitors may reduce HS-induced mortality and improve the prognosis of heatstroke patients.

ACKNOWLEDGEMENTS

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Molecular subtype classification and corresponding markers of oral squamous cell carcinoma

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This study was carried out to identify the metabolic and prognostic molecular subtypes and corresponding molecular markers of oral squamous cell carcinoma (OSCC) based on the OSCC data on The Cancer Genome Atlas (TCGA). UCSC Xena and NCBI GEO database were used to obtain RNA-seq data. The single-factor Cox regression analysis and unsupervised consensus cluster analysis were employed to screen metabolic genes and identify clusters. Survival analysis and clinicopathological analysis were used to analyze the differences of clusters. The limma package was used to obtain featured genes. Database for Annotation, Visualization and Integrated Discovery (DAVID) software was utilized to analyze the Gene Ontology Biological Processes (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of each featured gene. STRING database, Cytoscape and CytoNCA were applied to Protein-protein interaction (PPI) network construction and module analysis. GSE42743 data set was applied to genes and cluster validation. A total of 54 genes were obtained as metabolic and prognosis-related genes and 4 clusters were divided. Survival rate in cluster4 and cluster1 is significantly higher than cluster3 and cluster2, and the formation of these clusters is only related to metabolic molecules. Different numbers of featured genes were obtained in clusters and a large number of functional signaling pathways were enriched. PPI network showed a total of 338 relationship pairs and 128 genes corresponding to proteins. and then 8 hub genes were identified. The feasibilities of 6 screened molecular markers of cluster1 were successfully verified by GSE42743 data set. OSCC was divided into 4 clusters through bioinformatics analysis. There are survival differences between these four clusters. A total of 6 molecular markers were identified in cluster1 for cluster identification of OSCC.

Statistical results of the American Cancer Society show that the incidence of cancers in the oral cavity and pharynx have had an increasing trend in recent years (1-3). Oral squamous cell carcinoma (OSCC), a heterogeneous malignancy caused by the oral mucosal lining causing many deaths, is one of most common cancers in the oral cavity and pharynx (4).

The pathogenesis of OSCC is often related to lifestyle habits, including smoking, excessive drinking and betel nut chewing (5). Advanced OSCC is prone to lymph node metastasis, which is difficult to treat and has reduced survival rates (6). Therefore, it is crucial to develop a new identification strategy for OSCC.

Identification of the molecular subtype of cancer

Key words: oral squamous cell carcinoma; prognostic; metabolism; clusters

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is of great significance for the clinical analysis of various cancers and the development of targeted therapies (7, 8). Subtype identification of breast cancer may provide better treatment options for patients (9). As for adenoid cystic carcinomas, molecular subtypes are identified by proteomics methods to identify potential therapeutic targets. To date, the identification of cancer type by bioinformatics methods has been widely used in many types of cancers, including triple-negative breast cancer (11) and bladder cancer (12).

Metabolomics is widely used for cancer research. Alterations in metabolic pathways can monitor tumor occurrence (13), and affect tumor progression (14) and treatment efficacy (15). Molecular subtype differentiation of cancers based on metabolic genes is also one of the current research hotspots. Recent studies have reported metabolic genes related to prognosis through prognostic cluster identification based on metabolic genes in triple-negative breast cancer, which aids in improvement of personalized treatment based on tumor metabolism profile (11). There is no research investigating the relationship between metabolic genes of OSCC and molecular subtype identification.

The present study identifies the metabolic molecular subtypes of OSCC on the basis of The Cancer Genome Atlas (TCGA) OSCC data and determines the prognosis of metabolic molecular subtypes. Moreover, the study seeks to explain the molecular heterogeneity of OSCC, and then identify the corresponding markers for different subtypes to provide a theoretical basis for clinical treatment.

MATERIALS AND METHODS

Data source and preprocessing

For the test set, RNA-seq data, corresponding clinical information and prognostic information (OS and OS.time) of 255 tissue samples of OSCC were downloaded from UCSC Xena (<https://xenabrowser.net/datapages/>) (16). RNA-seq data used were $\log_2(\text{FPKM}+1)$ -transformed, and genes whose expression is less than 1 in more than half of the samples were filtered out. Each gene Ensemble ID was annotated according to the human gene annotation file (v22) provided by GENCODE with the corresponding gene symbol obtained.

For the validation test, original chip data of 71 oral cavity cancer tissue samples in GSE42743 data set were obtained from Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) database [Data screening conditions including: a) species: homo sapiens; b) with “oral square cell carcinoma” as the key word; c) experience type: expression profiling by array; d) the sample information contained prognostic survival information (OS and OS.time)]. R software package affy (<http://bioconductor.org/packages/release/bioc/html/affy.html>) was used for data reading and data preprocessing (17). According to the platform annotation file, the probe number and the gene symbol was matched, and the probes that did not match the gene symbol were removed. For different probes that map to the same gene, the average value of the different probes was selected as the final expression value of this gene, that is the gene expression profile. All samples were detected through the GPL570 [HG-U133_Plus_2] AAffymetrix Human Genome U133 Plus 2.0 Array platform.

Metabolic and prognosis-related gene screening and cluster identification

The metabolism-related genes were downloaded from the attachments of a previous study (18). By matching with the genes contained in the TCGA-OSCC data set, the metabolic-related gene set and the expression value of each gene in each sample of TCGA-OSCC were further obtained followed by the single-factor Cox regression analysis with the survival time (OS.time) and survival status (OS) of the samples. Then, genes with $p < 0.05$ were identified as metabolic and prognosis-related genes and were further screened for the next unsupervised consensus cluster analysis (ConsensusCluster).

Based on the gene expression matrix related to metabolism and prognosis obtained above, the R package ConsensusClusterPlus (<http://bioconductor.org/packages/release/bioc/html/ConsensusClusterPlus.html>) was used to perform a consistent cluster analysis (the set parameters were: maxK=10 (maximum cluster number to evaluate), reps=100 (number of subsamples)) on the samples of TCGA-OSCC (19). The most suitable number of clusters was selected and the samples were divided into different clusters followed by construction of expression heatmap of each metabolic-related gene in different clusters.

Survival analysis

The K-M survival curve of different clusters was constructed based on the survival time and survival status of TCGA-OSCC samples, and logRank was applied to test its significance to determine whether there were prognostic differences in each cluster.

Clinicopathological analysis

The distribution of age, gender, race, pathologic_N, pathologic_T and other clinical characteristics under different clusters were calculated, and the R package ggstatsplot (version: 0.5.0) was used for statistics, significance test and plotting, with $p < 0.05$ regarded as significantly related.

Identification of featured genes

The limma package (<http://www.bioconductor.org/packages/2.9/bioc/html/limma.html>) was used to analyze the difference between the single cluster sample and the remaining samples (20). After analysis, the corresponding p value and log fold change (FC) value of all genes were obtained. In addition, the Benjamini & Hochberg method was used for multiple test correction, and the genes with differential expression threshold p -adjust < 0.05 and $\log FC > 1$ (up-regulated by 2 times) were selected as the featured genes of this cluster. The expression heatmap of each featured gene in different clusters was plotted.

Furthermore, the commonly used enrichment analysis tool Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david-d.ncifcrf.gov/>) (21) was used to analyze the Gene Ontology (GO) biological process (BP) (22) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (23) pathway of each featured gene in different clusters. $P < 0.05$ and enrichment number more than 2 was considered as a significant enrichment result.

Protein-protein interaction (PPI) network construction and module analysis

STRING (<http://www.string-db.org/>) (24) database was applied to predict and analyze whether there was an interaction relationship between gene-encoded proteins (PPI score=0.9, highest confidence) (human species). After obtaining the PPI relationship pair, Cytoscape (<http://chianti.ucsd.edu/cytoscape-3.4.0/>) (25) was used to construct a network diagram. The parameter was set to without Weight. CytoNCA plug-in (<http://apps.cytoscape.org/apps/cytonca>) (26) was used for node analysis of Degree Centrality (DC), Betweenness Centrality (BC), and Closeness Centrality (CC). According to the ranking of the network topology properties of each node, the genes whose DC, BC, and CC ranks were all in the TOP20 were selected as the hub protein.

In the PPI network, cluster analysis was used to identify functional modules, to obtain the network topology and the relationship between network components, and finally to obtain biologically meaningful protein complexes or functional modules. Using the MCODE plug-in of Cytoscape software, the parameters were set to default (Degree Cutoff = 2, Node Score Cutoff = 0.2, K-core = 2, Max.Depth = 100), and the module with > 5 scores was selected for the following analysis. KEGG pathway enrichment analysis on the above modules was conducted using the R package clusterProfiler (version:3.8.1, <http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) and “BH” correction. After the final screening and correction, the Term with p -adjust < 0.05 was regarded as a significant enrichment result.

Molecular cluster validation

According to the method in the previous study (27), the average expression value of each metabolic-related gene was calculated and then used for consistent clustering in different clusters as the centroid of each cluster through TCGA-OSCC. Secondly, the expression values of the matched metabolic genes in each sample were screened out using GSE42743 data set; the Pearson correlation coefficient between each sample and the centroid of the TCGA-OSCC cohort was calculated through the one-to-one correspondence of genes, and the cluster with the highest correlation was assigned to the sample. In this way, the same number of clusters was obtained from GSE42743 data set, and then the K-M survival curve of the cluster was drawn by combining the survival information of the sample in GSE42743 data set to observe whether the prognosis performance was consistent with the TCGA-OSCC cohort.

RESULTS

Screening of metabolic and prognosis-related genes and identification of clusters

Firstly, 2751 metabolic-related genes were downloaded from a previous study. After matching

with the genes annotated and filtered by TCGA-OSCC, a total of 1515 metabolic-related genes were obtained. According to the method, single-factor cox regression analysis was performed based on the OS time and OS of the sample. Then, a total of 54 genes ($p < 0.05$) were obtained as metabolic and prognosis-related genes. Finally, a consistent cluster analysis was performed on the 54 metabolic and prognosis-related genes obtained above, and the samples were divided into four clusters (Fig. 1 A-C). The clinical information is listed in Table I.

In order to prove that the four clusters could distinguish the samples, we further drew the principal component analysis (PCA) chart. As shown in Fig. 1D, except that cluster2 and cluster1 were partially mixed together, different clusters were clearly separated overall, indicating that there are indeed four clusters. In addition, heatmaps were drawn to show the expression of different metabolic and prognosis-related genes in the four clusters (Fig. 1E).

Good prognosis in cluster1 and cluster 4

According to the method, the K-M survival curves of different clusters were drawn as shown in Fig. 2A. It can be seen that cluster4 and cluster1 show good prognosis, and the survival rate is significantly higher than cluster3 and cluster2.

Statistical analysis of various clinical factors under different clusters showed that only race and neoplasm_histologic_grade were closely related to the clusters (Fig. 2 B, C). Cluster3 had less G1 and higher G2-G4, which may be the reason for the poor prognosis shown in Fig. 2B. This result shows that most of the clinical factors have no significant differences among the various metabolic clusters, suggesting that the formation of these clusters is only related to metabolic molecules.

Identification of featured genes

According to the threshold set by the method, the up-regulated genes ($\log FC > 1$) of each cluster were regarded as the featured genes of the cluster. Among them, a total of 152, 109, 14, and 79 featured genes were obtained in cluster1, cluster2, cluster3, and cluster4, respectively. After analyzing the featured genes of different clusters through Venn diagrams,

the results showed that the featured genes of the four clusters basically do not overlap with each other. Only cluster3 and cluster4 have an overlapping

Table I. Clinical sample information of the TCGA-OSCC data

		Cluster 1	Cluster 2	Cluster 3	Cluster 4
Age	<65	56	34	35	33
	≥65	31	20	29	17
Gender	female	28	19	16	14
	male	59	35	48	36
Race	American Indian or Alaskan native	0	0	1	0
	Asian	1	7	0	0
	black or African American	3	3	5	5
	white	80	42	56	44
	NA	3	2	2	1
Clinical_M	M0	83	50	59	44
	M1	0	0	1	1
	NA	4	4	4	5
Clinical_N	N0	46	23	31	25
	N1	18	12	9	7
	N2	20	15	22	13
	N3	0	0	0	1
	NA	3	4	2	4
Clinical_T	T1	8	4	2	4
	T2	30	13	16	17
	T3	21	17	19	12
	T4	25	18	25	14
	NA	3	2	2	3
Clinical_stage	Stage I	5	2	1	2
	Stage II	25	10	13	10
	Stage III	17	14	11	10
	Stage IV	37	26	37	25
	NA	3	2	2	3
Neoplasm_histologic_grade	G1	13	14	3	9
	G2	49	35	49	24
	G3	23	3	10	15
	G4	1	0	1	0
	NA	1	2	1	2
Pathologic_N	N0	31	23	24	16
	N1	11	6	10	11
	N2	30	24	21	12
	N3	0	0	2	0
	NA	15	1	7	11
Pathologic_T	T1	15	6	1	3
	T2	26	14	19	16
	T3	16	17	14	9
	T4	23	16	25	14
	NA	7	1	5	8
Primary_therapy_outcome_success	CR	65	34	36	34
	PD	5	4	8	4
	PR	2	0	0	0
	SD	1	0	1	1
	NA	14	16	19	11
Tumor_stage	Stage I	9	5	0	2
	Stage II	14	6	9	6
	Stage III	13	11	14	12
	Stage IV	42	31	36	20
	NA	9	1	5	10

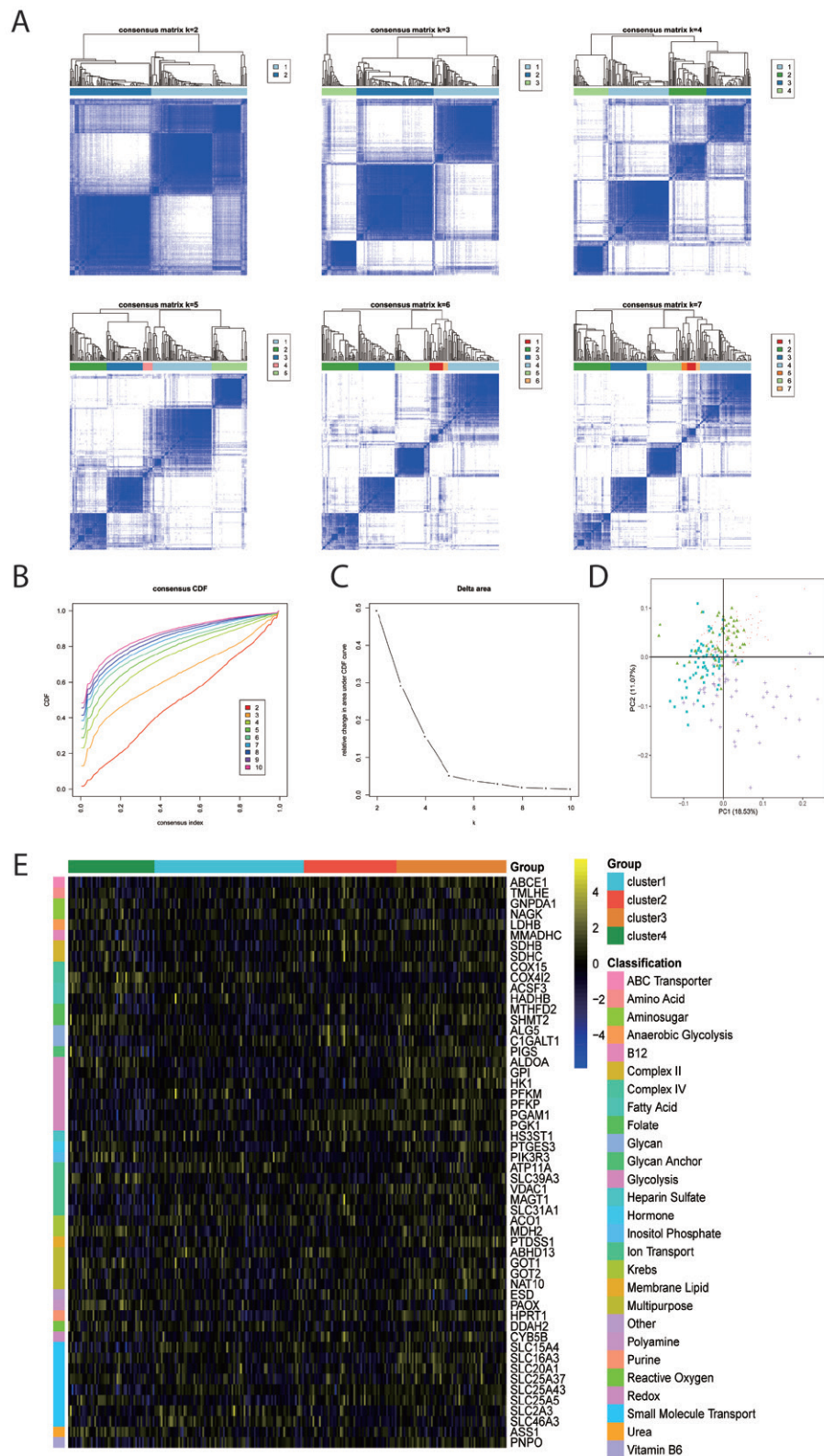


Fig. 1. Identification of OSCC clusters. **A)** Consistent cluster heatmap; **B)** Cumulative distribution function curve of $K=2-10$; **C)** The relative change in area under the CDF curve of $K=2-10$; **D)** PCA chart of 54 metabolic prognosis-related genes; **E)** Heatmap of expression of 54 metabolic and prognosis-related genes among different clusters.

gene, which indicates that the four clusters have independent expression patterns (Fig. 3A). In addition, the expression heatmap of the featured genes is shown in Fig. 3B, and it is observed that the respective featured genes were significantly highly expressed in the corresponding cluster. The results of the PCA show that the four clusters are clearly

separated, proving the accuracy of these genes as featured genes (Fig. 3C).

GO and KEGG pathway analyses of featured genes

After determining the importance of featured genes, GO_BP and KEGG pathway analyses were performed on the featured genes of each cluster. Cluster1 is

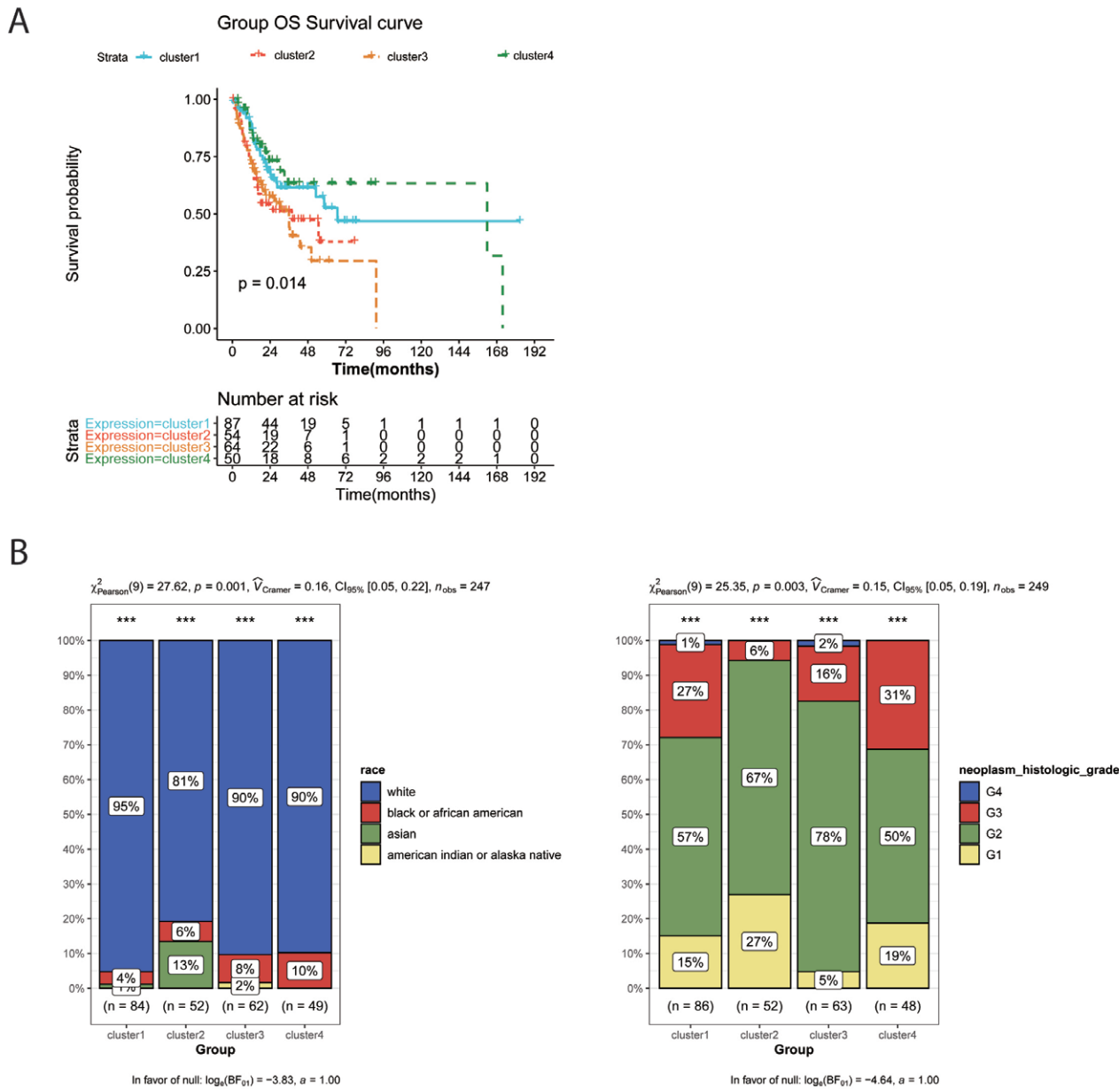
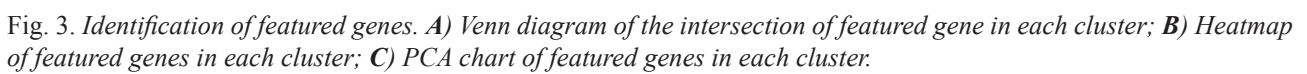


Fig. 2. Survival analysis and clinical factor analysis. *A*) K-M survival curves of 4 metabolic clusters; *B*) Distribution map of age, gender, and WHO under different clusters.



significantly enriched in cell adhesion, extracellular matrix organization and other extracellular matrix functions. The GO_BP analysis result of cluster2 shows that it is related to innate immunity and keratinization, but cluster2 cannot get the signal through KEGG enrichment. Cluster3 is mainly related to a variety of metabolic processes, and cluster4 is not only related to metabolic pathways, but also related to a variety of neurodegenerative diseases (Fig. 4 A, B).

PPI network construction and module analysis

In order to analyze the interaction relationship between gene-encoded proteins, the PPI network was constructed on the featured genes of the above clusters (Fig. 5A). It contains a total of 338 relationship pairs and 128 genes corresponding to proteins. It can be seen that featured genes from different clusters are more likely to have a close interaction relationship. In order to reveal this

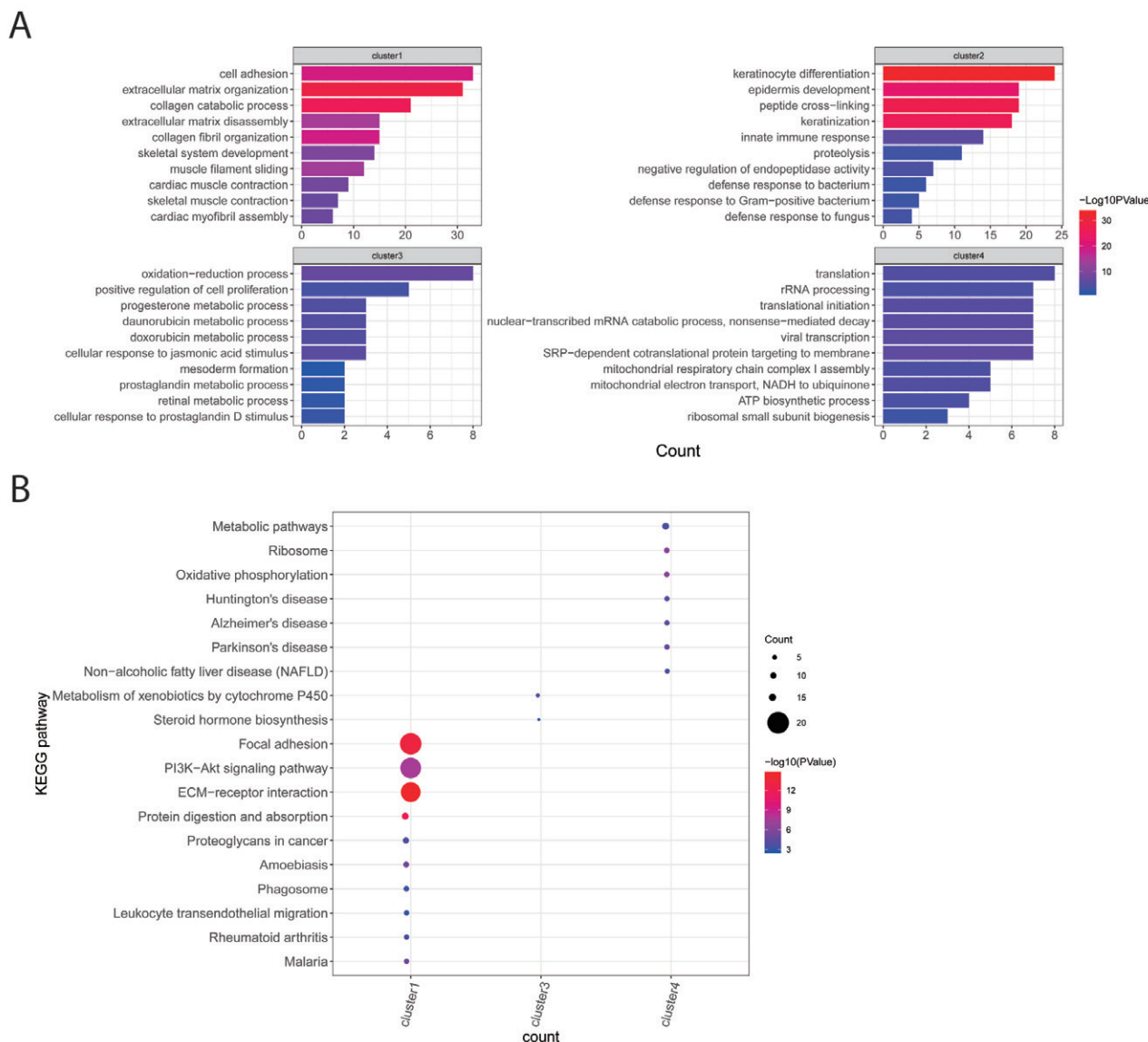


Fig. 4. GO and KEGG pathway analyses of featured genes. **A)** GO_BP bar graph of the enrichment of featured genes of each cluster (the horizontal axis represents the number of enriched genes, the vertical axis represents the enriched items, and the color from blue to red represents the decrease of *p* value); **B)** Bubble chart of KEGG pathway enriched by featured genes of each cluster (the horizontal axis represents the cluster, the vertical axis represents the name of the KEGG pathway, the size of the bubble indicates the number of enriched genes, and the color from blue to red indicates the decrease in *p* value).

phenomenon, module analysis was performed in the follow-up.

Furthermore, the CytoNCA plug-in was used to analyze the node DC, BC, CC to select the genes ranked TOP20 in each index as the key genes, including a total of 8 genes [matrix metalloproteinase 9 (MMP9), thrombospondin 1 (THBS1), alpha-2-macroglobulin (A2M), collagen type XIV alpha 1 chain (COL14A1), collagen type XV alpha 1 chain (COL15A1), collagen type III alpha 1 chain (COL3A1), matrix metalloproteinase 2 (MMP2), and transforming growth factor beta 3 (TGFB3)] (Fig. 5B, Table II). These 8 genes are all from the featured genes of cluster 1.

In order to show that the corresponding proteins of the featured genes of different clusters are more likely to have a close interaction relationship, so as to perform functions in different ways, a module analysis on the PPI network was conducted (Fig. 5C). A total of 7 modules are identified, and it can be seen that featured genes in cluster1 and cluster4 are the main ones, and the same module is more inclined to featured genes from the same cluster. Furthermore, KEGG pathway enrichment analysis was performed on different modules. The TOP10 results of each module was selected, and as shown in Fig. 5D, different modules participated in different KEGG pathways.

Cluster and molecular marker verification

The GSE42743 data set was used to verify the

above four molecular clusters. Firstly, according to the method, the Pearson correlation coefficients between each sample in GSE42743 data set and the centroids of the four clusters in the TCGA-OSCC cohort was calculated, and the cluster with the largest correlation coefficient was assigned to the sample. In the data set, there are 44 samples for cluster1 and 25 samples for cluster3, and only 1 sample for cluster2 and cluster4, respectively, so cluster2 and cluster4 samples were eliminated for subsequent verification, and only the differential expression was verified of key genes in cluster1 and cluster3.

Based on the clinical survival information (OS and OS.time) of the samples, the K-M survival curve was plotted (Fig. 6A), which showed that although there is no significant difference, the prognosis of cluster1 is better than cluster3 within 4 years, which is in line with our previous findings.

Furthermore, the expression level of the 8 hub genes obtained by the above PPI analysis were compared in the two clusters. The results showed that except for MMP9 and THBS1, the remaining six genes A2M, COL14A1, COL15A1, COL3A1, MMP2, and TGFB3 were all highly expressed in cluster1 (Fig. 6B). These results indicate that these genes can indeed be used as the featured genes of cluster1.

DISCUSSION

It is difficult to diagnose and treat OSCC (28,

Table II. The Degree Centrality (DC), Betweenness Centrality (BC), and Closeness Centrality (CC) of 8 hub genes

Gene	Degree Centrality	Betweenness Centrality	Closeness Centrality
COL3A1	17	270.71832	0.015657749
COL15A1	16	453.59137	0.015673207
COL14A1	15	316.98853	0.01566934
A2M	13	349.04132	0.015673207
MMP2	12	366.45023	0.015675142
THBS1	12	407.34665	0.01565003
TGFB3	11	337.75162	0.01565003
MMP9	11	846.174	0.015694514

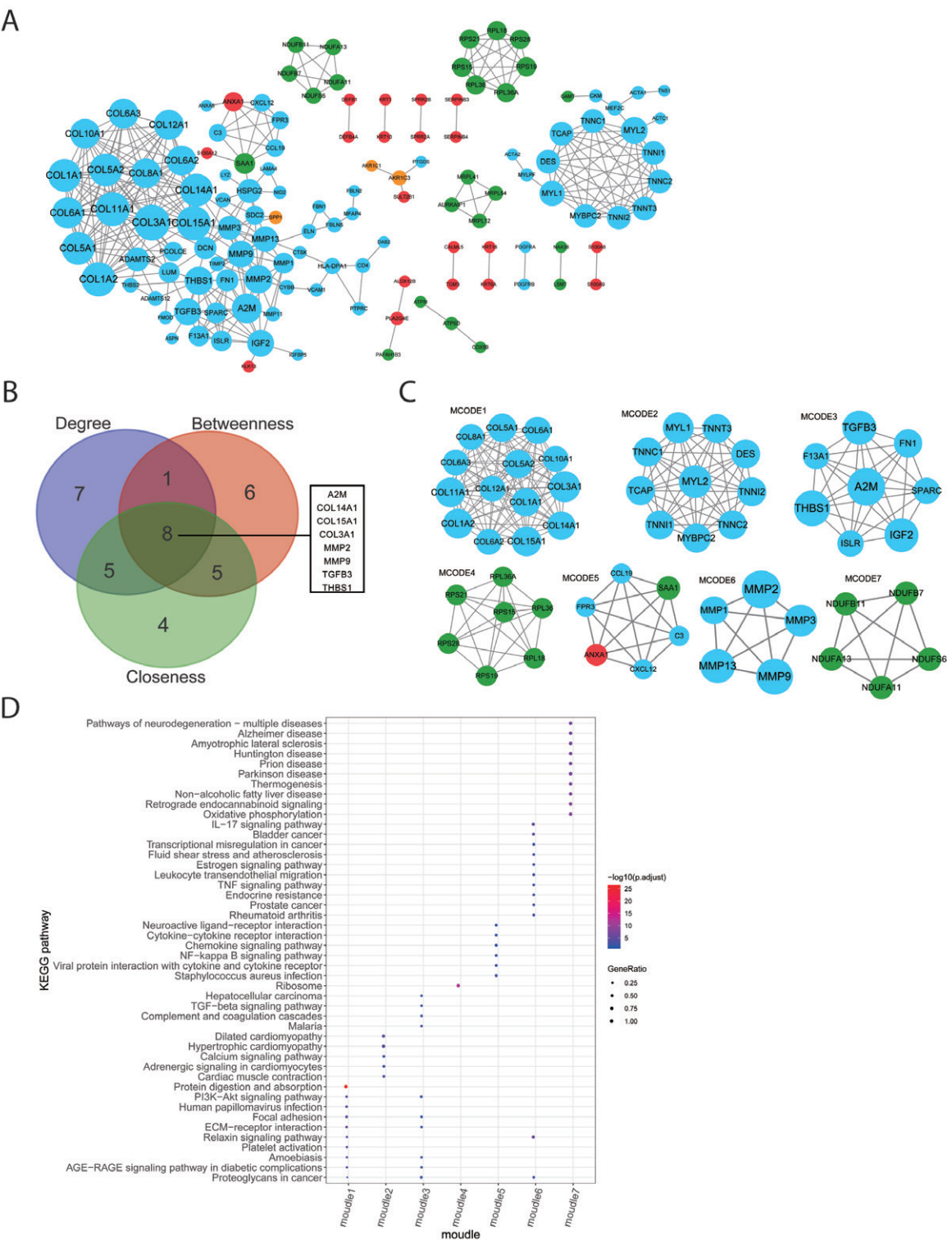


Fig. 5. PPI network construction and module analysis. **A)** PPI network diagram constructed by featured genes (different colors represent featured genes of different clusters, blue: cluster1; red: cluster2; yellow: cluster3; green: cluster4, node size indicates the size of the connection); **B)** Venn diagram of the intersection of DC, BC, and CC of TOP20; **C)** Modules with score ≥ 5 recognized by PPI network; **D)** Bubble chart of KEGG pathway enriched by different PPI modules (the horizontal axis represents the module, the vertical axis represents the name of the KEGG pathway, the size of the bubble represents the ratio of the number of enriched genes to the number of modules, and the color from blue to red represents the reduction of p value).

29). To date, few studies have investigated molecular subtype identification of OSCC (30), especially based on metabolic-related genes. In this study, we analyzed OSCC-related RNAseq data to obtain gene expression profiles and clinicopathological characteristics of a large number of OSCC samples. Fifty-four survival-

related genes were obtained and the samples were classified into 4 different metabolic clusters. We found significant differences in survival rates of patients with different metabolic clusters, but no difference regarding other clinicopathological characteristics of patients with different metabolic clusters, suggesting

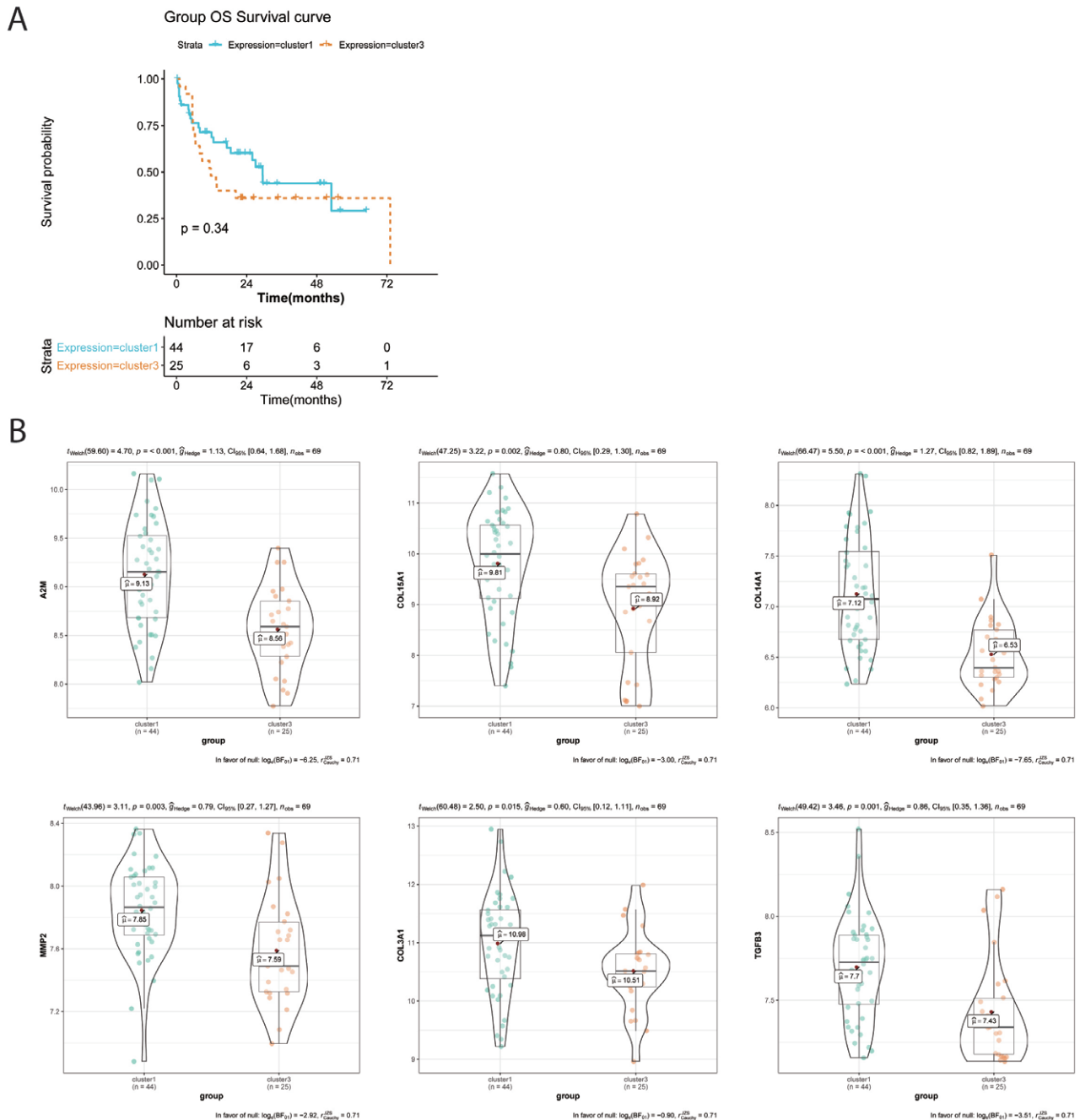


Fig. 6. Cluster and molecular marker verification in GSE42743. **A)** K-M survival curve for GSE42743 data set; **B)** Violin plot of hub gene expression in GSE42743 sample.

that the formation of these clusters was only related to metabolic molecules. We further screened the featured genes of 4 clusters and performed GO-BP and KEGG pathway analyses. The PPI network and module analyses identified 8 key genes, which were indeed verified to serve as characteristic genes of cluster1 in the GSE42743 dataset.

Metabolic pathways are closely related to the development of OSCC (31). For example, a research related to glycolysis pathways suggested that inhibiting the N-linked glycosylation of Axl and Met contributes to the anti-cancer effect of 2DG in OSCC cells (32). Also, a research related to amino acid metabolism pathways revealed that the promoted growth of OSCC cells may rely on the catabolism of branched chain amino acids (BCAAs) (33). Our data obtained different functions and signal pathways from different clusters through enrichment. Among them, the cluster1 was suggested to be related to the metabolic processes, such as protein digestion and absorption. In addition, this cluster is also closely correlated with cell adhesion, extracellular matrix organization and other extracellular matrix functions. Extracellular matrix (ECM) is a key part of the tumor microenvironment, which not only provides support for cancer cells, but also regulates their metabolism (34). Particularly, the loss of ECM attachment leads to metabolic stress, reduced nutrient absorption by cancer cells, reduced adenosine triphosphate (ATP) production and increased reactive oxygen species (ROS) levels (34). Another cluster4 with good survival rate is enriched in mitochondrial respiratory chain complex I assembly, mitochondrial electron transport and other metabolic signals, which may be one of the reasons why it regulates OSCC. These results demonstrate the accuracy of the approach to clustering by metabolic and prognosis-related genes.

According to the results of the PPI network, 8 key genes were obtained. Among them, COL14A1, COL15A1 and COL3A1 are from the collagen family. COL14A1 has been reported to be frequently methylated in a variety of cancers (35, 36). COL14A1 methylation is associated with poor prognosis in kidney cancer (37). COL15A1 and COL3A1 are closely related to ovarian cancer, and both have been reported to be highly expressed in drug-resistant

ovarian cancer cells (38, 39). In contrast to collagen, MMP2 and MMP9, members of the MMP family, mainly function to degrade ECM components. Both MMP2 and MMP9 can accelerate the metastasis of cancer cells (40). It is reported that BRD4 can promote the metastasis of OSCC by regulating the expression of MMP2 (41). Targeting MMP2 by monoclonal antibodies can impede the malignant behaviors of OSCC cells (42). Similarly to MMP2, MMP9 is also considered to be an oncogene in OSCC. Wnt family member 7A (WNT7A) activates β -catenin/MMP9 pathway to boost OSCC cell migration (43). The results of the module analysis suggested a closer interaction between characteristic genes. In previous studies, COL15A1 and COL3A1 exhibit the same expression patterns in ovarian cancer (39), which is similar to the relationship between MMP2 and MMP9 (44), indicating their possible synergistic function. In addition, THBS1 has been reported to facilitate OSCC cell migration and invasion by elevating the expression of MMP9 and MMP11 (45), which is consistent with our conclusion. These results suggest that the characteristic genes of different subtypes are more likely to interact closely with each other, thus driving their functions in different ways.

Our research also has limitations. In the verification process using the dataset GSE42743, we found that the prognosis of cluster1 is better than cluster3 within 4 years, which is in line with our previous findings, but the impact on long-term survival is reduced. This may depend on the metabolism-related genes used in the classification process of cluster, including both promotion and suppression genes, which have a certain impact on the classification. In addition, we also lack the verification through *in vivo* and *in vitro* experiments. All these issues are our future research directions.

In general, we used bioinformatics methods to identify the subtypes of OSCC based on metabolic genes and screened the corresponding markers. Our results provide a certain direction for the diagnosis and treatment of different subtypes of OSCC.

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Clinical efficacy and adverse reactions of dexamethasone combined with ademetonine in the treatment of intrahepatic cholestasis of pregnancy

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The purpose of the study was to investigate the clinical efficacy and adverse reactions of dexamethasone combined with ademetonine in the treatment of intrahepatic cholestasis of pregnancy (ICP). Ninety-four patients with ICP admitted to our hospital from February 2018 to February 2019 were selected and were randomly divided into control group (n=47) and experimental group (n=47). Among them, the patients in the control group were treated with routine ademetonine for therapeutic intervention, while the patients in the experimental group were treated with dexamethasone combined with ademetonine. After that, the clinical efficacy, and adverse reactions of these two treatment methods were compared between the two groups. The pruritus scores of the patients in the two groups after treatment were significantly lower than those before treatment, and the scores in the experimental group were significantly lower than those in the control group. The levels of liver function indexes such as AST, ALT and TBA in both groups after treatment were significantly lower than those before treatment, and the levels in the experimental group were significantly lower than those in the control group. The levels of proinflammatory cytokines such as IL-12 and TNF- α in both groups after treatment were lower than those before treatment, and those levels after treatment in the experimental group were significantly lower than those in the control group, with statistically significant differences (all $P < 0.05$). There were no adverse reactions such as nausea, vomiting, abdominal pains, etc. occurring in the two groups during the treatment. Patients' electrocardiogram and blood and urine routine examination results were normal and medication safety was ensured. Dexamethasone combined with ademetonine can effectively relieve pruritus, reduce the levels of proinflammatory cytokines and improve liver function indexes, with no obvious adverse reactions in the treatment, which is worthy of application and promotion in the clinical practice.

Intrahepatic cholestasis of pregnancy is a common disorder in the second and third trimesters of pregnancy, and initially almost all patients have mild symptoms of pruritus generally manifested in the day and severe ones in the night. The affected sites of pruritus are mostly on the palms and soles but can also spread to the limbs and face (1). Pregnant patients' symptoms can spontaneously regress

with the termination of pregnancy, but this disease remains harmful to the fetus, thus the pregnancy of ICP patients is classified as a high-risk pregnancy (2). At present, conservative drug control is clinically the top priority for treatment, and ademetonine can effectively inhibit cholestasis, hence it has become the routine drug for ICP treatment, effectively relieving pruritus, reducing bile acid excretion and decreasing

Key words: intrahepatic cholestasis of pregnancy; dexamethasone; ademetonine; adverse reactions

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the occurrence of adverse pregnancy outcomes (3). Ademetonine can play a part in transmethylation to reduce the physiological activity of catechol estrogens, to improve the bile secretion disorder caused by estrogen and progestin. Additionally, ademetonine can be converted into endogenous antidotes, such as taurine, glutathione, cysteine, which have the effect of oxidation resistance and detoxification, effectively reducing hepatocellular injuries (4). Dexamethasone for anti-inflammation and immunosuppression is a common drug for pregnant women, as it can inhibit the accumulation of inflammatory cells, reduce the synthesis and release of inflammatory mediators with phagocytosis, and restrain the release of lysosomal enzymes (5). On this basis, the present study analyzed the clinical efficacy of dexamethasone combined with ademetonine for ICP treatment.

MATERIALS AND METHODS

General information

Ninety-four patients with ICP who were admitted to Lianshi People's Hospital of Nanxun District from February 2018 to February 2019 were selected as the study

subjects and were randomly divided into a control group ($n=47$) and an experimental group ($n=47$). There were 27 primiparas and 20 multiparas in the control group, aged 20-38 years (average 28.6 ± 3.2 years) and the average gestation of (32 ± 6) weeks, while there were 28 primiparas and 19 multiparas in the experimental group, aged from 21 to 38 years, with the average age of (27.4 ± 3.1) years and the average gestation of (31 ± 6) weeks. There were no significantly statistical differences in the general information of age, gestational weeks, etc. between the two groups, which were comparable ($P > 0.05$).

Patients were included in the study according to the clinical diagnostic criteria for ICP in the Guidelines for the Management of Intrahepatic Cholestasis of Pregnancy; patients had singleton pregnancy and complete clinical medical records. Patients with the following were excluded: other organic diseases in brain, heart, kidneys or liver, abnormal liver function caused by viral hepatitis or other, fetal abnormalities due to other causes; cognitive impairment, or refused to cooperate with the study. This study was approved by the Hospital Ethics Committee, and the patients were informed of the purpose and process of this study, received the treatment protocols, and signed the informed consent.

Treatment methods in the control group were 1 g of

Table I. Comparison of the liver function indexes between the two groups ($\pm s$, $n=47$)

Group	Experimental group ($n=47$)	Control group ($n=47$)	t	P
AST before treatment (U/L)	131.70 $\pm$ 20.5	130.40 $\pm$ 21.60		
AST after treatment (U/L)	52.40 $\pm$ 11.20	70.70 $\pm$ 11.80	7.87	<0.01
t	23.76	16.98		
P	<0.01	<0.01		
ALT before treatment (U/L)	164.50 $\pm$ 22.40	163.10 $\pm$ 22.90		
ALT after treatment (U/L)	35.60 $\pm$ 7.90	57.80 $\pm$ 9.70	12.42	<0.01
t	38.99	29.64		
P	<0.01	<0.01		
TBA before treatment ($\mu\text{mol/L}$)	25.10 $\pm$ 6.00	25.70 $\pm$ 5.80		
TBA after treatment ($\mu\text{mol/L}$)	10.10 $\pm$ 1.40	15.90 $\pm$ 2.60	13.7489	<0.01
t	17.04	10.79		
P	<0.01	<0.01		

ademetionine (specification: 500 mg×10 tablets; Hospira S.P.A, Italy; Import drug registration certificate number: H20070016) was mixed with 500 ml of 5% glucose injection, and then, the intravenous drip of this injection was carried out once a day, with 12-14 days as a course. In addition, the patients took 500 mg of ademetionine in the morning and evening, respectively (6-8).

Treatment methods in the experimental group were based on the treatment of the control group, dexamethasone (Specification: 0.75 mg/tablet; Manufacturer: Tianjin Tianyao Pharmaceutical Co., Ltd.; State Food and Drug administration approval number: H20033553) was added to the injection, and 600 mg of injection was administered intramuscularly every 12 h by instillation for one course.

Observation indexes

With reference to the pruritus score evaluation criteria developed by Ribalta, 4 points represented the continuous pruritus with no changes between day and night; 3 points represented intermittent pruritus with changes of symptoms; 2 points represented intermittent pruritus with no changes of symptoms; 1 point represented occasional pruritus; 0 point represented no pruritus. All patients' pruritus scores before treatment and at 1 week after

treatment were recorded and compared for analysis.

For liver function indexes in both groups, venous blood samples before treatment and at 1 week after treatment were taken from the patients to detect their liver function indexes including AST, ALT and TBA. AST and ALT were detected by the velocity method, and TBA was detected by enzyme colorimetry.

For detection of proinflammatory cytokines in both groups, interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α) were detected by enzyme-linked immunosorbent assay, and the contents of proinflammatory cytokines of patients in the two groups were compared and analyzed.

Adverse reactions, such as nausea, vomiting, fever, abdominal pains, dizziness, of the patients during the medication period were recorded, and the electrocardiogram and blood and urine routine examinations were conducted before and after treatment.

Statistical analysis

The selected data processing software for this study was SPSS20.0, and GraphPad Prism 7 (Graphpad Software, San Diego, USA) was used to draw the pictures of the data. Measurement data were carried out by *t*-test and enumeration data were tested by χ^2 test and normality test. The differences had statistical significance when $P < 0.05$.

Table II. Comparison of the proinflammatory cytokines between the two groups ($\pm s$, ng/L)

Group	Experimental group (n=47)	Control group (n=47)	t	P
IL-12 before treatment	44.50±3.30	43.20±3.40		
IL-12 after treatment	11.70±1.20	18.50±1.90	21.18	<0.01
t	65.39	44.390		
P	<0.01	<0.01		
TNF- α before treatment	50.7±6.20	51.50±6.10		
TNF- α after treatment	22.4±2.20	33.70±3.30	19.94	<0.01
t	30.11	17.97		

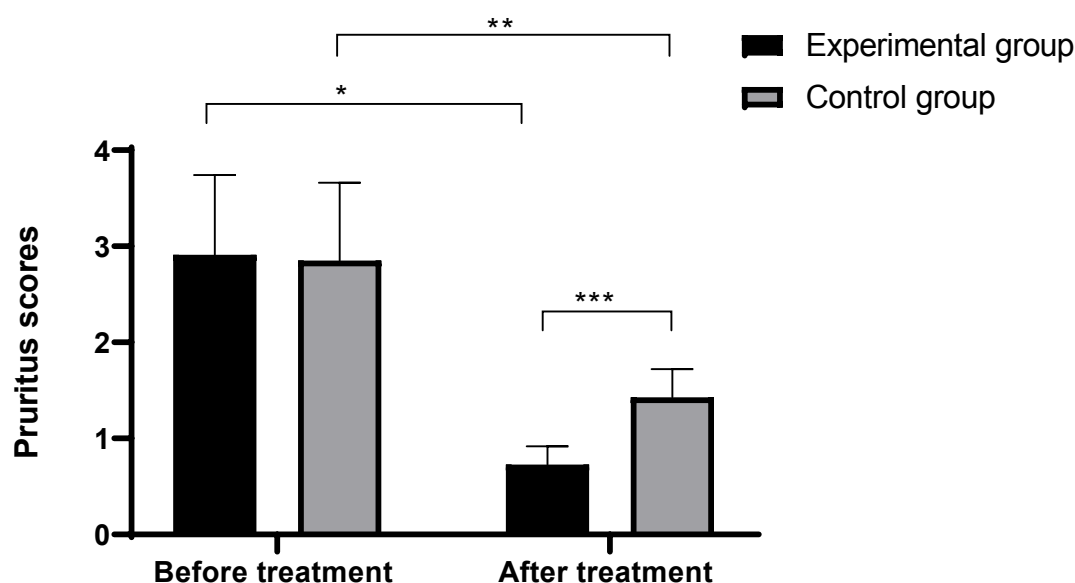


Fig. 1. Comparison of the pruritus scores between the two groups. The abscissa indicates before and after treatment, while the ordinate indicates pruritus scores (points). The pruritus scores of the patients in the experimental group before and after treatment were (2.91 ± 0.83) and (0.73 ± 0.19) . The pruritus scores of the patients in the control group before and after treatment were (2.85 ± 0.81) and (1.43 ± 0.29) . * indicates that there were significant differences in the pruritus scores in the experimental group before and after treatment ($t=17.92$, $P<0.01$). ** indicates that there were significant differences in the pruritus scores in the control group before and after treatment ($t=11.55$, $P<0.01$). *** indicates that there were significant differences in the pruritus scores between the two groups after treatment ($t=14.13$, $P<0.01$).

RESULTS

Comparisons between the two groups

After treatment, the pruritus scores of the patients in both groups were significantly lower than those before treatment, and the scores in the experimental group were significantly lower than those in the control group (all $P < 0.05$), (Fig. 1).

The levels of liver function indexes such as AST, ALT and TBA after treatment in both groups were significantly lower than those before treatment, and the levels in the experimental group were significantly lower than those in the control group, with statistically significant differences ($P < 0.001$) (Table I).

After treatment, the levels of proinflammatory cytokines (IL-12, TNF- α) reduced significantly in both groups, and after treatment, the levels in the experimental group were significantly lower than those in the control group (all $P < 0.001$), (Table II).

No adverse reactions, such as nausea, vomiting and abdominal pains, occurred in either group during treatment, and patients' electrocardiogram and blood and urine routine examination results were normal, with no significant differences between the two groups.

DISCUSSION

During pregnancy, women have difficulty in excreting cholic acid because of abnormally elevated estrogen and progesterone, which leads to an increased amount of protein synthesis in the liver and aggravates the liver burden, resulting in cholestasis (9-10). Dexamethasone has remarkable anti-inflammatory, antianaphylaxis and immunosuppressive properties, and few side effects, therefore is suitable for pregnant women (11). This study highlighted the clinical application value of dexamethasone on ICP.

Results of the present study showed that the

pruritus scores, the scores of AST, ALT and TBA and the levels of IL-12 and TNF- α of the patients in the two groups after treatment were significantly lower than those before treatment, and the scores in the experimental group were significantly lower than those in the control group. There were no adverse reactions, such as nausea, vomiting, or abdominal pains, occurring in the two groups during the treatment. Patients' electrocardiogram and blood and urine routine examination results were normal and medication safety was ensured. All these results indicate that dexamethasone combined with ademetonine has better synergistic effect to relieve cholestasis, inhibit symptoms of pruritus and improve liver function as well as immune function, with obvious clinical effects on ICP treatment. Our study results are in line with those of Ozkan (12) and others, who concluded that dexamethasone combined with ademetonine could optimize patients' biochemical criteria and relieve pruritus, thus improving pregnancy outcome.

In conclusion, dexamethasone combined with ademetonine can effectively relieve pruritus, reduce the levels of proinflammatory cytokine indexes and improve liver function indexes, with no obvious adverse reactions, therefore is worthy of application and promotion.

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Umbilical cord blood mesenchymal stem cell-derived conditioned medium induces the apoptosis and autophagy of ovarian cancer SKOV3 cells

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Mesenchymal stem cells (MSCs) are known to migrate to tumor tissues and play an important role in cancer progression. However, the effects of MSCs on tumor progression remain controversial. The aim of our study was to detect the effects of human umbilical cord blood-derived MSCs (hUCB-MSCs) on the human ovarian cancer SKOV3 cell line *in vitro* and the underlying mechanisms. MSCs were isolated from blood of umbilical vein in full-term healthy deliveries. SKOV3 cells were treated with conditioned medium (CM) from hUCB-MSCs. The CCK-8 (Cell Counting Kit) assay was used to determine the effect on proliferation. AnnexinV-FITC/PI staining was used to detect the cell cycle and apoptosis. Caspase-Glo[®]3/7 assay kit was used to detect the expression of caspase3/7 protein. Autophagy levels were determined based on expression of autophagic marker LC3, StubRFP-SensGFP-LC3 analysis, and electron microscopy. hUCB-MSCs are characteristically similar to other sources of MSCs, which are inconsistent with previous reports and exhibit multipotential differentiation capability (adipocytes and osteoblasts). The CCK-8 result revealed that hUCB-MSCs-CM inhibited the proliferation than those observed in the control group. Analysis of the cell cycle indicated that hUCB-MSCs-CM significantly increased the percentage of SKOV3 cells in the G2/M phase compared with the Ctrl group. Flow cytometry analysis and Caspase-Glo 3/7 Assay suggested that hUCB-MSCs-CM promoted apoptosis. hUCB-MSCs-CM increased LC3-II levels as well as autophagosome number in SKOV3 cells. These results indicate that hUCB-MSCs significantly inhibited the proliferation of SKOV3 cells *in vitro*, which is possibly due to blocking its cell cycle at G2/M phase and inducing SKOV3 cell death through synergistic apoptosis and autophagy. This theoretically supports hUCB-MSCs as an effective anticancer adjuvant in the treatment of ovarian cancer.

Ovarian cancer is one of the most common malignant tumors and the most lethal gynecological malignancy (1). The national cancer institute statistics show that the annual new morbidity achieves 11.9

per 100,000 women-years and the mortality achieves 7.5 per 100,000 women-years although the incidence and mortality of ovarian cancer has dropped (2, 3). The response to traditional chemotherapy is poor

Key words: umbilical cord blood mesenchymal stem cells; apoptosis; autophagy; cell cycle; ovarian cancer

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in recurrent ovarian cancer patients because of the absence of specific symptoms and the lack of early detection markers. The five-year survival rate is only about 30%. Cell lines are valuable tools for the molecular mechanisms of neoplasia and for developing potential therapeutic strategies (4). In our present study, the SKOV3 human ovarian cancer cell line was used and served as a reliable model of ovarian cancer disease progression research.

MSCs are becoming an important source for cellular therapy. hUCB-MSCs have been used in the treatment of refractory diseases, such as cerebrospinal diseases, heart diseases, diabetes, femoral head necrosis and lower limb vascular occlusion (5-7). Mesenchymal stem cells (MSCs), also called adult multipotent stem cells, with high self-renewal and differentiation capacities can be derived and propagated from a variety of sources, including bone marrow, adipose tissue, placenta, umbilical cord or umbilical cord blood (8). In addition, MSCs are known as potential therapeutic agents with unique immunological properties that allow them to survive in a xenogeneic environment and they exert a powerful potential for repairing injured tissues in cancer and regenerative medicine (9). The cytokines secreted by MSCs were used in the treatment of clinical diseases (10). More and more evidence has demonstrated that MSCs serve an essential role in tumor development and progression (11, 12).

Apoptosis and autophagy are both major modes of cell death, but whether hUCB-MSCs can influence the apoptosis and autophagy in SKOV3 cells is still unknown. In our present study, hUCB-MSCs, which have lower immunogenicity and less moral or ethical restrictions, were utilized to investigate their biological characteristics and effect on proliferation, cell cycle, apoptosis and autophagy of SKOV3 cells. In summary, the results indicated that hUCB-MSCs exhibit an anticancer effect. However, it remains unclear whether hUCB-MSCs are safe in cancer clinical therapy.

MATERIALS AND METHODS

Human epithelial ovarian carcinoma cell line SKOV3 (adenocarcinoma) was purchased from the Type Culture

Collection of the Chinese Academy of Sciences (Shanghai, China). Primary hUCB-MSCs were isolated from healthy full-term deliveries and their biological characteristics were identified. All donors provided written informed consent, and the present study was approved by the Ethics Committee of Zhengzhou Central Hospital Affiliated to Zhengzhou University (Zhengzhou, China). Mouse anti-human McAb CD45-FITC, CD34-FITC, CD11b-FITC, CD19-FITC, CD29-FITC, CD73-PE, CD105-APC-A750, CD90-APC and HLA-DR-FITC were purchased from BioLegend. Unless otherwise stated, other chemicals used in the present study were purchased from Gibco Life Technologies (Waltham, MA, USA).

Isolation and identification of hUCB-MSCs from human umbilical cord blood

Umbilical vein samples were harvested from 12 healthy full-term female deliveries who underwent cesarean section. The umbilical vein samples were collected by aseptic blood bag and the MSCs were successfully isolated from 3 samples. Ficoll gradient was used to eliminate non-MSC umbilical cord blood cells. The samples were centrifuged at 500×g for 20 min and all the isolated cells were plated in 75-mm petri dishes containing Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 100 U/mL penicillin, 100µg/mL streptomycin and 10% fetal bovine serum (FBS) at a concentration of 2×10^6 cells/mL. The medium was replaced every 3 days after the cells had been incubated at 37°C in a humidified atmosphere of 5% carbon dioxide, and non-adhering cells were removed. After expansion to 80~90% confluence, the cells were harvested by 0.25% trypsinization and subcultured for further experiments. Inverted microscope was used to analyze the phenotypes of hUCB-MSCs. Markers of hUCB-MSCs were analyzed by flow cytometry and differentiation potential assays. At the end of differentiation, cells were stained with Alizarin Red S and Oil Red O.

Preparation of hUCB-MSC-derived conditioned medium

The hUCB-MSCs were seeded at a cell density of 1.5×10^4 cells/cm². They were cultured in MSC medium and were maintained for 1 day. The attached cells were washed three times with PBS, and the medium was replaced with advanced DMEM/F12, supplemented with 100 /mL penicillin, 100µg/mL streptomycin and 10% fetal bovine serum (FBS). The cells were then maintained

for an additional 24 h. The medium was collected and centrifuged at 300g for 5 min, sterile-filtered through a 0.2µm membrane and stored at -20°C.

CCK-8 assay

SKOV3 cells were seeded in 96-well plates at a density of 6×10^3 cells/well. After attachment, the cells were treated with hUCB-MSC-derived conditioned medium in the CM-treated group and treated with RPMI-1640 medium in the control group. The effect of hUCB-MSC-derived conditioned medium on proliferation of SKOV3 cells was assessed via CCK-8 assay according to the manufacturer's protocol after culture of 1, 2, 3, 4 and 5 days, respectively. Briefly, cells were obtained and incubated with 10 µL of CCK-8 reagent at a final concentration of 5 g/L at 37°C for 4 h. Wells without cells containing culture medium were used as blank controls. The optical density (OD) value was measured at a wavelength of 450 nm. Each assay was performed in triplicate.

Flow cytometric assay for cell cycle and apoptosis

SKOV3 cells were seeded in 6-well plates at a density of 5×10^5 cells/well at 37°C for 24 h in an incubator. Then the culture medium was renewed with 200 µL CM per well in the CM treated group and renewed with the same volume of RPMI-1640 medium in the control group. The cells were harvested after cultured 1, 3, and 5 days, respectively. AnnexinV-FITC/PI kit was used according to the manufacturer's protocol and the cells were analyzed by flow cytometry. Each experiment was repeated three times and Flow jo7.6 analysis program software was used to analyze the results.

Caspase-Glo[®]3/7 assay for caspase3/7 protein

SKOV3 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. The cells were treated with CM and treated with RPMI-1640 medium in control group. Cells were obtained after culture of 1, 3 and 5 days, respectively, followed by incubation with 100 µL of Caspase-Glo[®]3/7 reagent for 3 h. Wells without cells were used as blank controls and wells without Caspase-Glo[®]3/7 reagent were used as negative controls. The fluorescence values of each group were measured by ELISA detector at the wavelength of 405 nm. This experiment was repeated three times.

StubRFP-SensGFP-LC3 transfection for autophagosomes

SKOV3 cells were transfected with lentivirus vector containing stubRFP-SensGFP-LC3 gene, and the StubRFP-SensGFP-LC3-mut lentiviral vector was used as negative group. Then puromycin (2µg/mL) was used for stable screening. Transfected SKOV3 cells were treated with conditioned medium or RPMI-1640 complete medium for 1, 3, and 5 days, respectively, and then fixed with 4% paraformaldehyde for 20 min. SensGFP is sensitive to pH change owing to the fusion of autophagosomes and lysosomes, whereas stubRFP is stable. The expression of autophagosomes in the cells was monitored under fluorescence microscope (Olympus BX51, Japan) and the yellow spots which appeared after the fusion of red fluorescence and green fluorescence were autophagosomes. This experiment was repeated three times.

Transmission electron microscope for ultrastructure

SKOV3 cells were treated with conditioned medium or RPMI-1640 complete medium for 1, 3, and 5 days, respectively. The cells were harvested and fixed with 2.5% glutaraldehyde overnight after centrifugation, then post-fixed with 1% osmium tetroxide at 4°C for 2 h, then stained with 2% uranyl acetate and dehydrated through an acetone. Then the cells were embedded in Epon-812 for semi-thin sectioning, and they were stained with toluidine blue. Ultrathin sections were prepared based on light microscopic observations. Cellular ultra-structures were visualized under a transmission electron microscope (TEM) (Hitachi, Japan). This experiment was repeated three times.

Western blot analysis for LC3-II protein

SKOV3 cells were cultured with conditioned medium in the CM treated group and cultured with RPMI-1640 complete medium in the control group for 1, 3, and 5 days, respectively. Total protein from cultured cells was extracted using a radioimmunoprecipitation assay (RIPA) buffer containing proteinase inhibitors and quantified using a Bradford assay according to the manufacturer's protocol. Proteins (30 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies: anti-LC3-II antibody (1:1000 dilution), followed by secondary HRP-conjugated antibodies (1:5,000 dilution) for 1 h at room

temperature. Enhanced chemiluminescence (ECL) was used to visualize proteins on the membrane. The protein bands were quantified by using Quantity One v4.62 software. Each experiment was repeated at least 3 times separately.

Statistical analysis

All data are expressed as the mean $\pm$ standard deviation ($n \geq 3$). The statistical significance of differences between two groups was analyzed using a two-tailed Student's *t*-test

via SPSS statistical software package, version 16.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Characteristics of hUCB-MSCs

hUCB-MSCs were successfully isolated from umbilical cord blood of healthy full-term deliveries (Fig. 1A). The cells were positive for CD11b, CD29, CD73, CD90, CD105 but negative for CD34, CD45, CD11b, CD19, HLA-DR (Fig. 1D). From day 4, the cells entered the logarithmic growth stage and the growth peak was observed on day 6 (Fig. 1E). Oil droplets could be observed by oil red O staining after 21 days of adipogenic differentiation (Fig. 1B) and red calcium deposits could be seen by alizarin red S staining after 21 days of osteogenic differentiation (Fig. 1C).

Proliferation of SKOV3 cells

To investigate whether hUCB-MSCs-CM inhibit the proliferation of SKOV3 cells, SKOV3 cells were treated with conditioned medium derived from hUCB-MSCs for 1, 2, 3, 4 and 5 days, respectively. The proliferation of cells was then assessed by CCK-8 assay. The OD value of the CM treated group was higher than that of the control group. The cells exhibited a time-survival curve and the OD values on days 2, 3, 4 and 5 were statistically significant between the two groups (Fig. 2). These findings suggested that hUCB-MSCs-CM inhibit the proliferation of SKOV3 cells.

Cell cycle and apoptosis of SKOV3 cells

To further determine whether hUCB-MSCs-CM induce the change of cell cycle and apoptosis of SKOV3 cells, SKOV3 cells were treated with conditioned medium derived from hUCB-MSCs. Flow cytometric analysis demonstrated that the co-culture group SKOV3 cells revealed significantly higher apoptosis rate compared with the control group cells (Fig. 3A, Table I). The difference of apoptosis rate between the two groups was statistically significant on day 5. In addition, the proportion of SKOV3 cells in the phase of G2/M in the CM-treated group was higher than that of the control group (Fig. 3B, Table II).

To further explore the molecular mechanisms

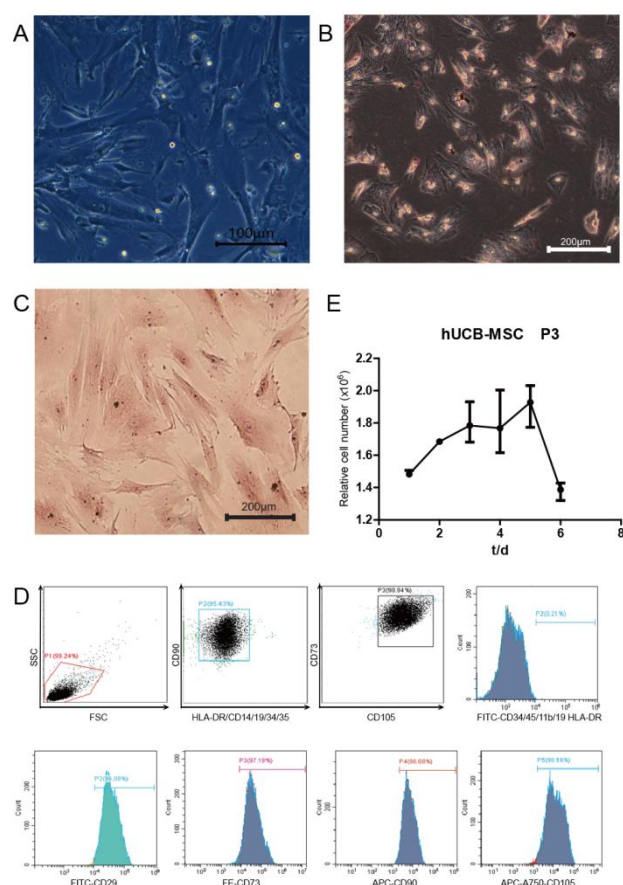


Fig. 1. Morphology, immunophenotype, adipocyte and osteoblast differentiation of hUCB-MSCs. A) Representative micrographs depict morphology of hUCB-MSCs (Scale bars indicate 100 μm). B) hUCB-MSCs were induced in adipogenic medium, and adipogenic differentiation was indicated by oil red O staining. C) hUCB-MSCs were induced in osteogenic medium, and osteoblast differentiation was indicated by Von Kossa staining for calcium phosphate. D) Mean fluorescence intensity (MFI) of CD34, CD45, CD11b, CD19, HLA-DR, CD73, CD90 and CD105 was determined on hUCB-MSCs by flow cytometric analysis. E) Cell growth curve of P3 hUCB-MSCs

underlying the hUCB-MSC-mediated apoptosis of SKOV3 cells, caspase-3/7 was evaluated by Caspase-Glo®3/7 assay kit. The expression levels of caspase-3/7 in the co-culture group SKOV3 cells were significantly increased compared with the control group cells (Fig.3C). These findings suggest that hUCB-MSCs-CM induce apoptosis in SKOV3

Table I. Cell cycle of ovarian cancer SKOV3 cells after being treated with hUCB-MSCs-CM(%)

Group	1 d G2/M	3 d G2/M	5 d G2/M
Control	12.53±1.26	8.95±1.11	3.50±0.76
CM-Treated	15.46±1.16	12.7±1.82	8.36±0.92
P	<0.0	<0.0	<0.01

^a vs Control, *P<0.05, **P<0.01

Table II. The apoptosis of ovarian cancer SKOV3 cells after being treated with hUCB-MSCs-CM(%)

Group	1 d	3 d	5 d
Control	1.89±0.046	7.79±0.83	8.39±1.44
CM-Treated	1.83±0.12	8.86±0.84	13.69±0.72
P	>0.05	>0.05	<0.01

^a vs Control, *P <0.05, **P<0.01

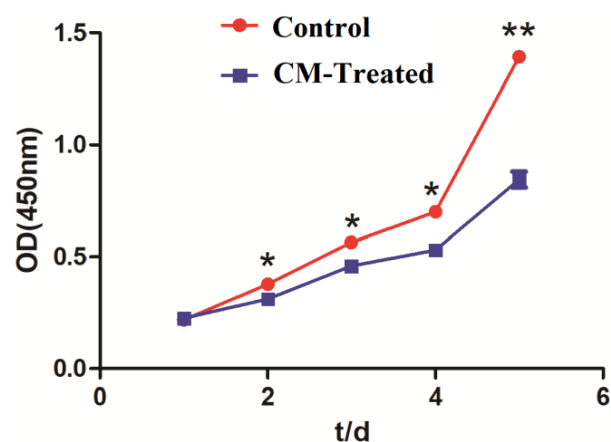


Fig. 2. Proliferation curve of ovarian cancer SKOV3 cells under conditioned medium of umbilical cord blood mesenchymal stem cells

cells by inhibiting the cell cycle in the phase of G2/M and promoting the activity of caspase-3/7 protein.

Effects of hUCB-MSCs-CM on autophagy in ovarian cancer SKOV3 cells

StubRFP-sensGFP-LC3 is widely used to monitor accumulation of the cytoplasmic LC3-positive puncta and autophagosomes. We established SKOV3 cells stably expressing stubRFP-sensGFP-LC3 by using a lentiviral vector and assessed the autophagic flux. Enhanced autophagosome-lysosome fusion in SKOV3 cells was observed by a laser scanning confocal microscope. The number of yellow dots (LC3-positive puncta) on day 5 was higher than that on days 1 and 3 (Fig. 4A), suggesting accumulation of autophagosomes in SKOV3 cells. Besides, the results of TEM showed that the number of autophagosomes and autolysosomes with double membranes appeared more in SKOV3 cells after treatment with conditioned medium on day 5, which was significantly more than that on days 1 and 3 (P<0.05) (Fig. 4B). Consistent with the results described above, hUCB-MSCs-CM significantly enhanced the expression of LC3-II protein and the corresponding LC3-II/GAPDH optical density in SKOV3 cells after being treated with conditioned medium derived from hUCB-MSCs on day 5 (Fig. 4 C, D).

DISCUSSION

hUCB-MSCs are cells with high self-renewal and multi-differentiation potential in umbilical cord blood. Each 100 mL umbilical cord blood only contains 1000~5000 hUCB-MSCs. Although the number of hUCB-MSCs is very small, they may be a very therapeutically valuable cell subset (8), but their specific role on tumor cells is still controversial. Previous research has shown that hUC-MSCs can induce cell death of breast cancer by secreting interferon- β . Our study shows that hUCB-MSCs isolated from the umbilical vein of normal full-term deliveries and the conditioned medium of hUCB-MSCs was capable of inhibiting SKOV3 cell survival.

Caspase protein is an aspartate-specific cysteine protease, and its overexpression can cause apoptosis, in which caspase 3/6/7 plays an executive role (13).

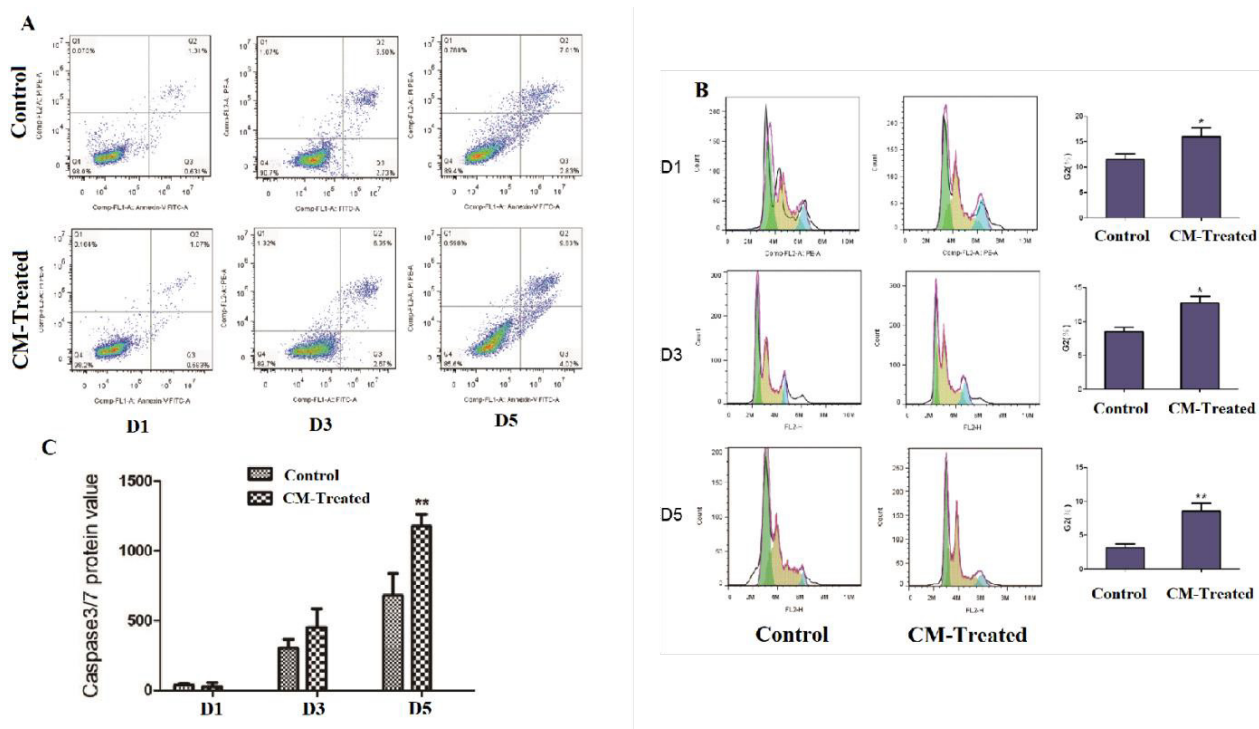


Fig.3. *hUCB-MSCs blocks its cell cycle at G2/M phase and induces the apoptosis of SKOV3 cells. A)The apoptosis of ovarian cancer SKOV3 cells after being treated with hUCB-MSCs-CM. B)Cell cycle of ovarian cancer SKOV3 cells after being treated with hUCB-MSCs-CM. C)hUCB-MSCs-CM can promote the expression of caspase3/7 protein in ovarian cancer SKOV3 cells*

Studies have shown that umbilical cord mesenchymal stem cells inhibit the expression of PI3K, Akt and Bcl-2 in ovarian cancer SKOV3 cells and promote the mRNA expression of caspase-3 (14). It was identified that indirectly co-culture with hUCB-MSCs significantly induced apoptosis and blocked cell cycle at the phase of G2/M accompanied by an increased level of caspase-3/7 protein in SKOV3 cells. Similarly, a previous study reported that umbilical cord-derived MSC (UC-MSC) fusion with ovarian cancer cells displayed normal MSC-like properties with reduced proliferative capacity in conjunction with a loss of tumorigenic potential. Research has also indicated that UC-MSCs could inhibit the proliferation and induce the apoptosis of ovarian cancer cells (15). These findings are consistent with the results of our study.

As a mechanism of tumor inhibition, autophagy is usually monitored by measuring the levels of autophagy-related proteins, such as LC3-II protein. Autophagy has been involved in various anticancer

therapeutic studies including clinical application (16). Moreover, other studies confirm that autophagy and apoptosis may cooperate to play an anti-tumor effect on tumor cells (17). The present study identified that hUCB-MSCs significantly promote the accumulation of autophagosomes in SKOV3 cells. The stimulatory role of hUCB-MSCs in autophagy was demonstrated by western analysis of LC3-II, mRFP-GFP-LC3 assay and TEM.

Therefore, we inferred that hUCB-MSCs can significantly inhibit the proliferation of ovarian cancer SKOV3 cells *in vitro*, which may be induced by blocking the cell cycle at the G2/M phase, synergizing the effect of apoptosis and autophagy to induce SKOV3 cell death. This study provides some insights into the process and mechanism of anti-tumor effects of hUCB-MSCs, suggesting that hUCB-MSCs may serve as an effective anti-cancer adjuvant for ovarian cancer. However, this study is an *in vitro* experiment, and the exact conclusion needs

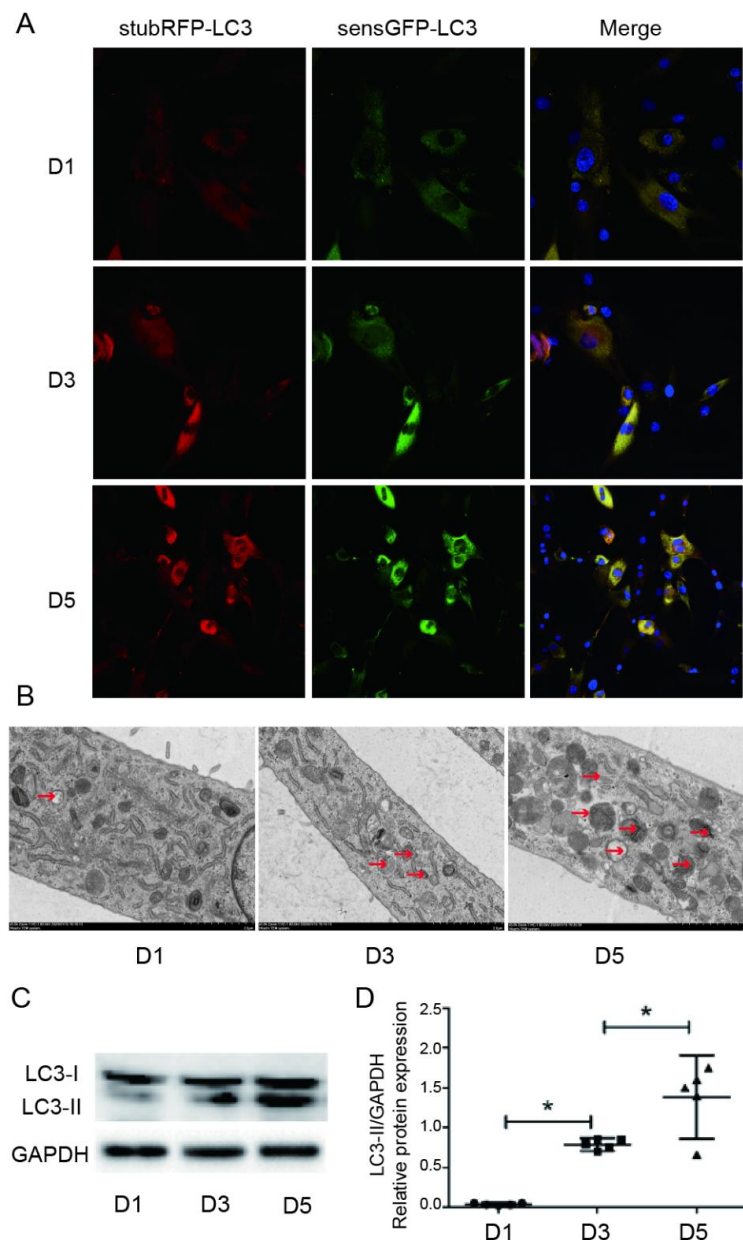


Fig. 4. Effects of hUCB-MSCs on autophagy in ovarian cancer SKOV3 cells. Representative images are shown. **A)** stubRFP-sensGFP-LC3 assay was used to detect the number of autophagosomes in SKOV3 cells. **B)** SKOV3 cells ultrastructure was examined by TEM. The red arrow indicates autophagosomes and autolysosomes in the cytoplasm. **C)** SKOV3 cells were treated with hUCB-MSCs-CM for 1,2,3 days, and LC3-II expression was analyzed by western blotting. **D)** The optical density of the LC3-II/GAPDH bands was analyzed. Data represent mean $\pm$ 95% confidence interval (CI), * P <0.01, n =5.

further study to explore the specific mechanism of apoptosis and autophagy in tumor cells, which will be further discussed in future studies.

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Identification and molecular mechanism study of hallmark genes associated with steroid-induced osteonecrosis of the femoral head

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Steroid-induced osteonecrosis of the femoral head (SONFH) is a severe disease. Nonetheless, the pathogenesis of SONFH is not fully understood, and it is challenging to make an early diagnosis. This study aimed to explore the hallmark genes associated with SONFH and predict potential biomarkers for SONFH. The microarray dataset GSE123568 was obtained from the Gene Expression Omnibus (GEO) database. The Weighted Gene Co-expression Network Analysis (WGCNA) and limma R-package were used to screen the intersection genes between the interest module genes and differential expression genes (DEGs). A protein-protein interaction (PPI) network, cross-talk, and receiver operating characteristic (ROC) curve were utilized to identify the optimal hallmark genes. The Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Set Variation Analysis (GSVA), and Gene Set Enrichment Analysis (GSEA) were performed to explore the potential molecular mechanism of genes. The single-sample Gene Set Enrichment Analysis (ssGSEA) and CIBERSORT algorithm were used to analyze the immune infiltration level in SONFH. There were 96 intersection genes acquired between the interest module and DEGs. Based on the PPI network, cross-talk, and ROC curve analysis, we identified 6 SONFH-related hallmark genes, including *TYROBP*, *TLR8*, *TLR2*, *LILRB2*, *HCK*, and *TREMI*, which showed heterogeneous biological functions or molecular mechanisms. The Toll-like receptor (TLR) signaling pathway was considered the primary pathway in the development of SONFH. Finally, we found that the neutrophils might be the culprit in the SONFH. Our study identified some SONFH-related hallmark genes and revealed novel insights into the biological mechanism of SONFH, which provided a theoretical foundation for further experimental study and contributed to understanding the pathogenesis of SONFH.

Steroid-induced osteonecrosis of the femoral head (SONFH) is a disease in which ischemia and necrosis of bone occur due to femoral head partial blood circulatory disorder because of long-term or mass glucocorticoid application (1, 2). In clinics, SONFH may be asymptomatic at the early stage and

difficulty diagnosed. Therefore, the femoral head of some patients has already collapsed at the diagnostic phase (3). Once the collapse of the femoral head appears, the course of the disease will be difficult to reverse (4). Although the total hip arthroplasty is currently recognized as the only reasonable way to

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treat extensive post-collapse of the femoral head, as SONFH mainly occurs in middle-aged and young patients, it is preferable to preserve the patients' joints if possible (5, 6). Besides, growing clinical evidence shows that joint-preserving techniques used in the pre-collapse stage of osteonecrosis of the femoral head have been successful (7). Therefore, there is an urgent need to determine the pathogenesis of SONFH and make an early diagnosis to delay disease progression effectively (8).

In recent years, the pathogenesis of the disease has been extensively studied, but there is still no clear consensus on the exact origin of the disease (9-11). Furthermore, there are few comprehensive bioinformatics studies on SONFH by high-throughput platforms. Biomarkers can provide potential leads for disease diagnosis and may be applied to decide the clinical practice (12). Supposing SONFH's potential diagnostic biomarkers can be identified in the early stage of the disease, or even before the condition, patients' life quality will be improved.

In the present study, we determined the key biomarkers associated with SONFH and explored the molecular mechanism of SONFH in-depth through a series of comprehensive bioinformatics analysis. Our study revealed a novel mechanism of SONFH that involved the burst of neutrophils via Toll-like receptor (TLR) signaling pathway. This new information could contribute to understanding the pathogenesis of SONFH further.

MATERIALS AND METHODS

Microarray data from the Gene Expression Omnibus (GEO) database

The dataset of human peripheral serum GSE123568, including 30 SONFH samples and 10 normal samples, was acquired from the GEO database (<https://www.ncbi.nlm.nih.gov/geo>) (13). The annotation and matrix files were downloaded from the GEO database for analysis. Then, the data were normalized and transformed to $\log_2(\text{count}+1)$ using limma R-package (14) downloaded from Bioconductor (<https://www.bioconductor.org/>) (15). The probes were matched to the corresponding gene symbols based on the annotation file in the platform. When

the same gene corresponded to multiple probes, its average expression value was calculated.

Construction of key co-expression modules

The WGCNA algorithm (16) was utilized to excavate the modules of significantly correlated genes among samples for relating modules to external sample traits. To create a scale-free network, the function `pickSoftThresholdsoft` was used to select a soft threshold, which mainly affected the independence and the mean connectivity (k) of the co-expression modules. We performed the gradient method here and ranged the soft threshold from 1 to 20. When the correlation between k and $p(k)$ reached 0.85, the optimal soft threshold was chosen, an adjacency matrix (AM) and a topological overlap matrix (TOM) were also gained. After that, we constructed a hierarchical clustering dendrogram of the 1-TOM matrix to stratify similar gene expressions into different gene co-expression modules. For any modules, as the module Eigengenes (ME) provided the most suitable explanation of the gene expression profile, we related the ME to clinical features, including SONFH or normal status in this study. According to module-trait relationships, we selected the module displaying the highest correlation.

Identification of differential expression genes (DEGs) and intersection genes

The limma R-package was employed to screen the DEGs via comparing the SONFH samples with normal samples (14). The false discovery rate (FDR) was utilized to adjust the p -value. The adjusted p -value < 0.05 and $|\log_2 \text{fold change (FC)}| \geq 1$ were considered as the cut-off criteria. Subsequently, we extracted the intersection genes between DEGs and the interest module, and presented it as a Venn diagram (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>).

Functional enrichment analysis of intersection genes

The DAVID website (<https://david.ncifcrf.gov/home.jsp>) was applied to perform the Gene Ontology (GO, including cellular component, molecular function, and biological process) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (17). The thresholds were set as p -value < 0.05 .

Construction of PPI network and identification of hallmark genes

The PPI network of intersection genes was constructed

using the STRING website (<https://string-db.org/>) (18) and then visualized by the Cytoscape version 3.7.1 (19). Whereafter, SONFH-related genes were determined via the cytohubba plugin (20) in Cytoscape. Next, the Pearson correlation coefficients (PCC) were calculated among the above genes through the Hmisc R-package (21) and then visualized using the corplot R-package (22). The p -value < 0.05 and $|PCC| > 0.6$ were regarded that two genes have a significant co-expression relationship. After that, the expression levels of the genes were visualized as box plots. The p -values were identified with asterisks. *, p -value < 0.05 ; **, p -value < 0.01 ; ***, p -value < 0.001 ; ****, p -value < 0.0001 . The receiver operating characteristic (ROC) curve analysis was performed to assess the predictive capacity of the hallmark genes for SONFH (23).

Molecular mechanisms of hallmark genes in SONFH

We downloaded the gene sets of the GO and KEGG pathways as the reference gene sets from the Molecular Signature Database (MSigDB, <https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>) (24). The hallmark genes' expression profiles were subjected to the Gene Set Variation Analysis (GSVA), a non-parametric, unsupervised method for estimating alteration of gene set enrichment through the samples of an expression dataset (25). PCC was further calculated to show the correlation between the expression level of the hallmark genes and the activity of gene sets. $|PCC| > 0.6$ and p -value < 0.05 were considered to have statistical significance. The relationships between the activity of gene sets and the expression of hallmark genes were visualized by network diagrams via the Cytoscape software.

Consensus clustering of hallmark genes

We collected the expression data of 6 hallmark genes from the dataset to perform unsupervised clustering utilizing factoextra R-package. The Euclidean distance of different samples was calculated, and then the ward. D2 method was applied to perform the hierarchical clustering (26). Ultimately, the GSE123568 samples were stratified into two clusters and visualized the expression of hallmark genes in cluster1/2 using the heatmap R-package.

Immune infiltration analysis

To calculate the proportions of 22 immune cell types for each sample, we performed the cell type identification by estimating relative subsets of RNA

transcripts (CIBERSORT, <https://cibersort.stanford.edu/>) algorithm (27). The threshold was set as p -value < 0.05 . Meanwhile, we acquired the gene sets of 24 immune cell types from the published literature (28) and then estimated the infiltration level of gene sets using the single-sample Gene Set Enrichment Analysis (ssGSEA) method (29). The immune-related gene signatures used in our research consisted of innate immunity, including natural killer cells, CD56bright NK cells, CD56dim NK cells, dendritic cells (DCs), immature DCs (iDCs), activated DCs (aDCs), plasmacytoid dendritic cells (pDCs), macrophages, mast cells, eosinophils, and neutrophils, and adaptive immunity, containing B cells, T cells, T helper cells, CD8 T cells, T central memory cells (Tcm), T effector memory (Tem), cytotoxic cells, T follicular helper (Tfh), T gamma delta (Tgd), Th1, Th2, Th17, and Treg cells. The following equation standardized the ssGSEA score for each immune cell type:

$$\text{score} = [x - \min(x)] / [\max(x) - \min(x)]$$

Gene Set Enrichment Analysis (GSEA)

To validate the correlation between the activity of KEGG pathways and the expression of hallmark genes, we separated the SONFH samples into two cohorts based on the median expression value of individual hallmark genes. Then we conducted the Gene Set Enrichment Analysis (GSEA) in the KEGG pathways gene set via the JAVA program. The algorithm of random sampling was 1,000 permutations. The significant enrichment pathway between the two cohorts was determined by utilizing the FDR < 0.25 , p -value < 0.05 , and the NES (Normalized Enrichment Score) (30).

RESULTS

Construction of key co-expression modules

We firstly screened the top 25% genes sorted by the variance of expression in the dataset. 5021 genes were screened and then subjected to create a scale-free co-expression network by the WGCNA method. $\beta = 18$ was determined as the optimal soft threshold (Fig. 1A), and the correlation between k and $p(k)$ reached 0.89 (Fig. 1B). To merge the highly familiar modules, we chose a cut line of < 0.25 and a minimum module size of 30 utilizing the dynamic hybrid tree cut method

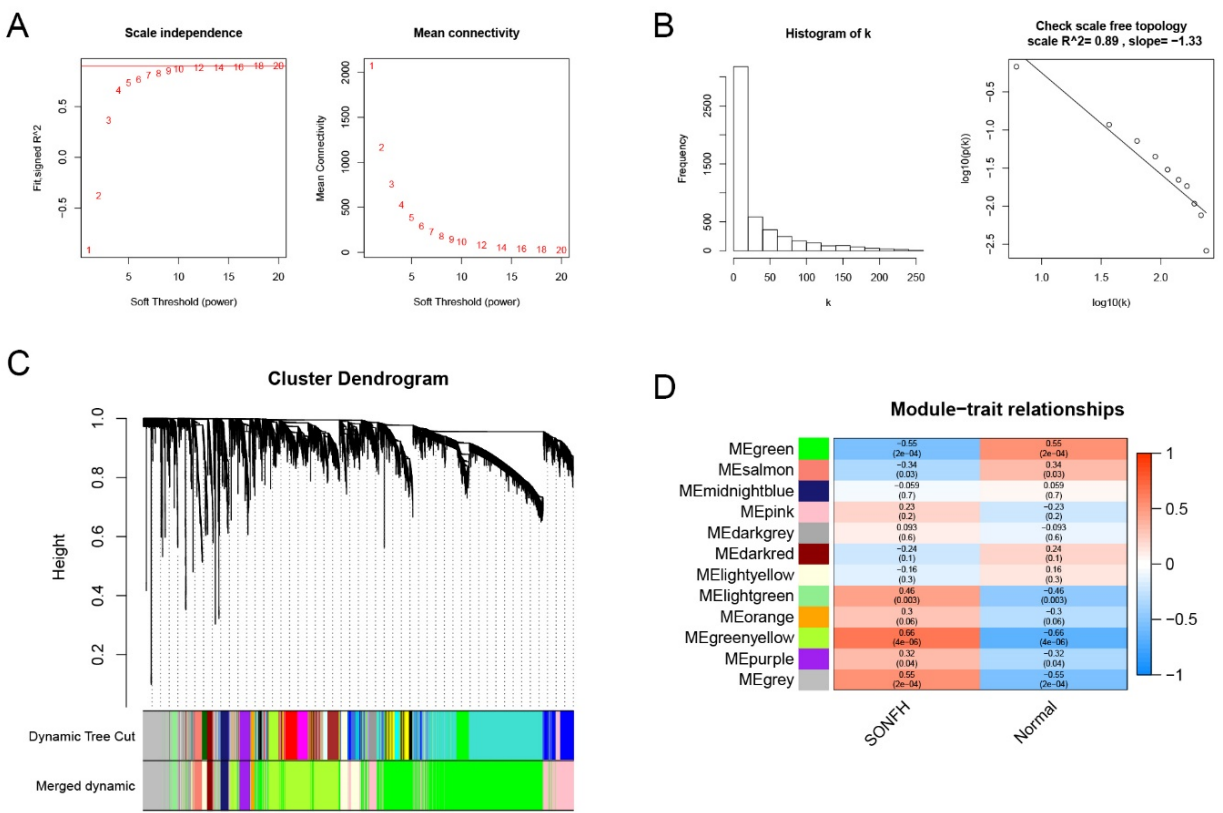


Fig. 1. Weighted gene co-expression network of GSE123568. **A)** Analysis of the scale-free fit index and the mean connectivity for soft threshold (β). **B)** K shows the logarithm in the whole network connectivity, $p(k)$ shows the corresponding frequency distribution logarithm. K is negatively related to $p(k)$. When $\beta = 18$, the correlation coefficient was 0.89, which represents scale-free topology. **C)** The cluster dendrogram is based on the 1-TOM matrix. **D)** Module-trait relationships. The heatmaps were depicted to show the correlation between module Eigengenes and clinical traits (SONFH and normal); correlation coefficients and p -values were shown in each module.

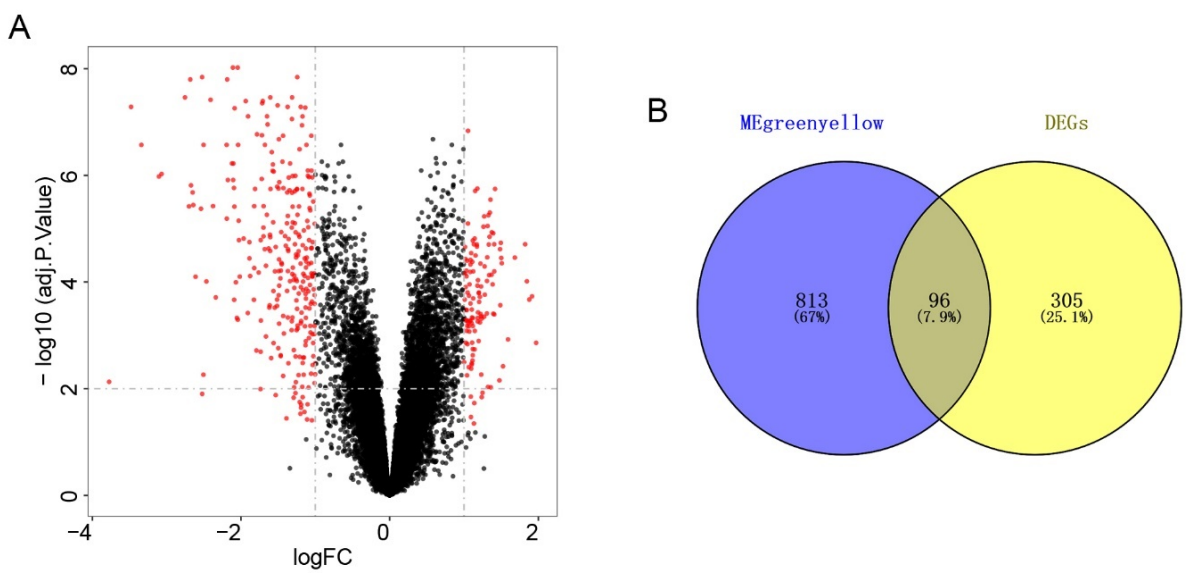


Fig. 2. **A)** DEGs were selected by volcano plot filtering (adjusted p -value < 0.05 and $|\log_2 FC| \geq 1$). **B)** Venn diagram between the interest module genes and DEGs.

(Fig. 1C). Next, the interest module was determined via the average linkage hierarchical clustering. The greenyellow module ($r = 0.66$, $p = 4e-06$) was the highest correlation with the SONFH status (Fig. 1D).

Identification of DEGs and intersection genes

A total of 401 DEGs were screened using limma

R-package, including 278 downregulated genes and 123 upregulated genes, and then visualized via volcano plot (Fig. 2A). As shown in Fig. 2B, 96 intersection genes were extracted between the interest module and DEGs.

Enrichment analysis of intersection genes

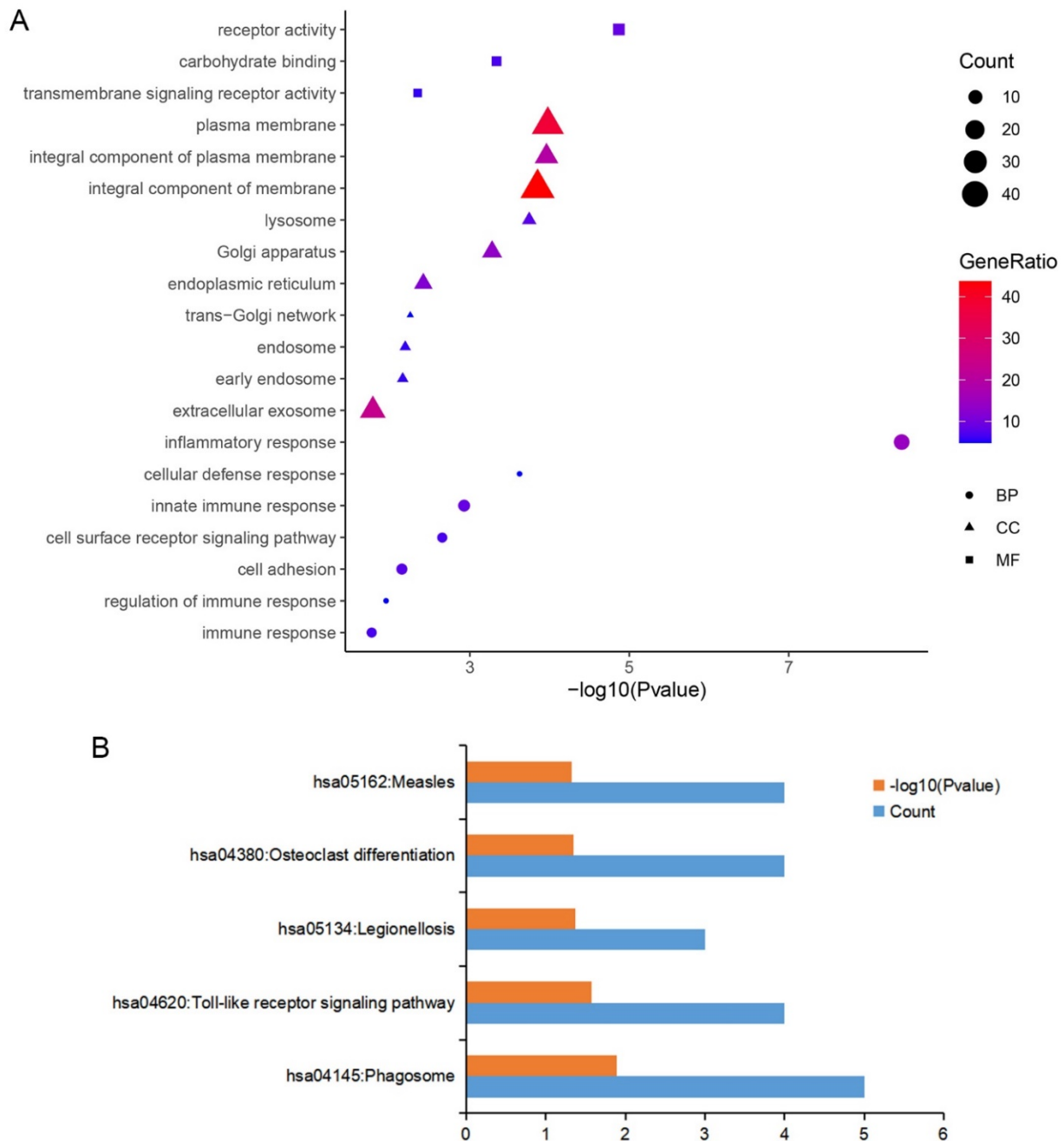


Fig. 3. The functional enrichment analysis of 96 intersection genes. **A)** Top 20 enriched GO terms of the intersection genes. **B)** Top 5 enriched KEGG pathways.

Using the DAVID website, GO analysis presented the top 20 enriched terms of the 96 intersection genes (Fig. 3A). In terms of biological process (BP), these genes significantly were enriched in some processes associated with the inflammatory response, cellular defense response, and cell surface receptor signaling pathway. Moreover, for the cellular component (CC), these genes were mainly enriched in plasma membrane, integral component of membrane, and multiple organelles, such as comprising lysosome, Golgi apparatus, endoplasmic reticulum, and extracellular exosome. As far as the molecular function (MF) enrichment analysis, these genes might perform the functions related to receptor activity and carbohydrate binding. In addition, these genes were also significantly enriched in several KEGG pathways, such as Phagosome, Toll-like receptor (TLR) signaling pathway, and Osteoclast differentiation (Fig. 3B).

Construction of PPI network

The PPI network of the intersection genes was established using the STRING website (Fig. 4A).

Based on the cytoHubba plugin, 10 SONFH-related genes were determined according to the degree of connectivity in the entire PPI network (Fig. 4B), including *TYROBP*, *TLR8*, *TLR7*, *HCK*, *CTSS*, *TLR2*, *TREM1*, *CCR2*, *LILRB2*, and *FGL2*.

Cross-talk among the SONFH-related genes and identification of hallmark genes

To explore the correlation among SONFH-related genes, we calculated the PCC based on their expression in SONFH (Fig. 5A). We noticed a significantly positive correlation among *TYROBP*, *TLR8*, *TLR2*, *LILRB2*, *HCK*, and *TREM1*. Then, we observed that the expression levels of above 6 genes were significantly upregulated in SONFH tissues compared with their corresponding normal controls (Fig. 5B). Based on the expression level of these genes, ROC curve analysis was performed by comparing the respective AUC values. As shown in Fig. 5C, the AUC values of these genes were all > 0.8, which indicated that they might have potential diagnostic value for SONFH. Thus,

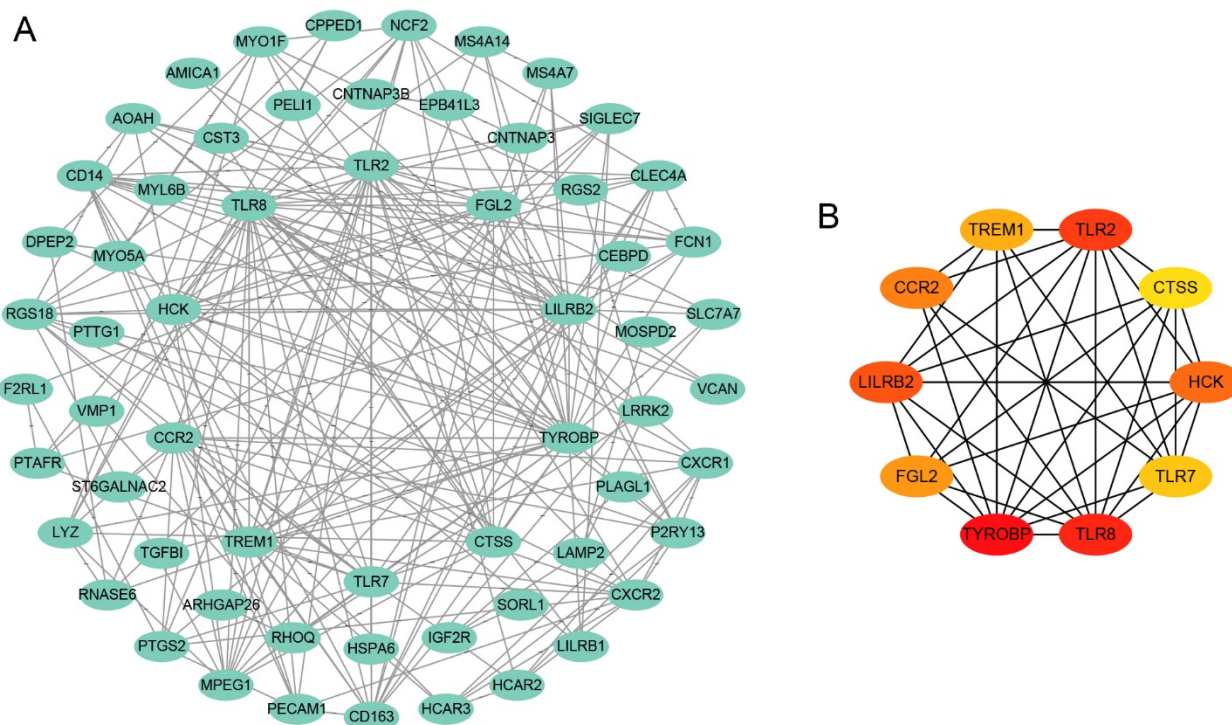


Fig. 4. A) The PPI network of 96 intersection genes. B) 10 SONFH-related genes were determined according to the degree of connectivity in the entire PPI network.

we considered that these 6 genes were SONFH-related hallmark genes.

Molecular mechanisms of hallmark genes in SONFH

To investigate the molecular mechanism of 6 hallmark genes responsible for SONFH, we calculated

the PCC between the expression of these genes and the activity score of KEGG pathways. We found that different genes were significantly correlated with varying pathways (Supplementary Table I). The expression of HCK and TREM1 showed significantly positive correlations with the activity of many KEGG

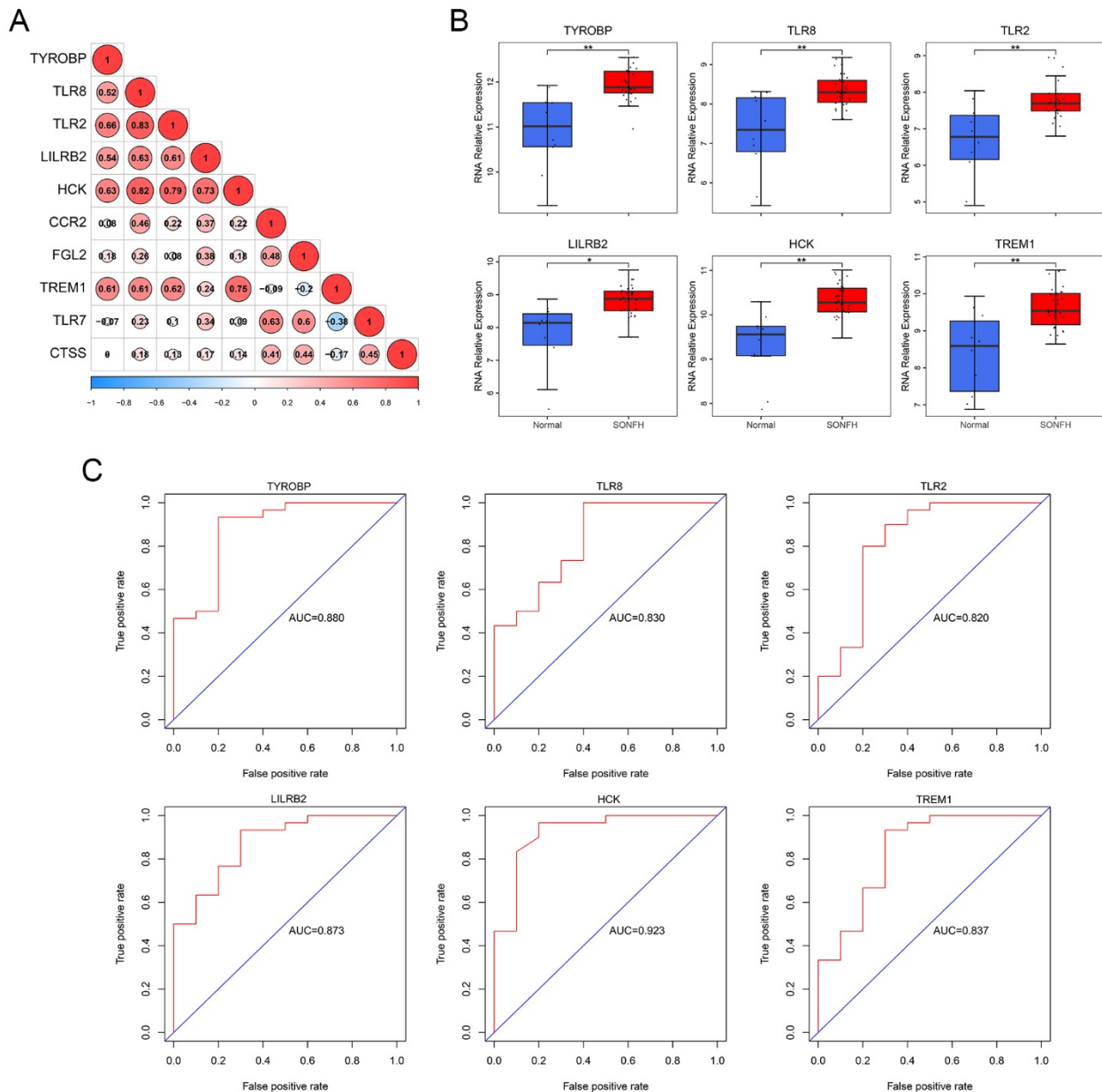


Fig. 5. A) The correlation plot shows the correlations among 10 SONFH-related genes. Red represents positive correlation, and blue represents negative correlation. The size of the point means the p-value. B) The box plots present the expression of 6 hallmark genes in SONFH and normal tissues, and t-test was performed to calculate the significance level between two samples. p-values are identified with asterisks. *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001. C) The ROC curves of hallmark genes. The ROC curves were used to show the capacity ability with true positive rate and false positive rate.

pathways (Fig. 6A). Notably, we found the expression of TYROBP, TLR8, and HCK was highly correlated with the activity of Toll-like receptor signaling pathway. Meanwhile, the hallmark genes were highly associated with the activity of multiple BPs, MFs, and CCs in SONFH (Supplementary Table I). Among these, more BPs, MFs, and CCs were highly related to *TLR8* and *HCK*, suggesting that *TLR8* and *HCK* might play crucial roles in regulating SONFH development. To further validate the potential regulatory mechanism of *TLR8* and *HCK* resulting in SONFH, we performed the gene set enrichment analysis (GSEA). Based on the expression of *TLR8* and *HCK*, we stratified the data into two groups (including high expression and low expression cohort) and subjected the data to the GSEA program. The results showed that Toll-like receptor signaling pathway was correlated with high *TLR8* and *HCK* expression cohorts (Fig. 6B), which were consistent with Fig. 6A, suggesting that the Toll-like receptor signaling pathway might be the primary mechanism to regulate the development of SONFH.

Consensus clustering of hallmark genes and distinct immune cell infiltration level of different clusters

The development of SONFH is usually regulated

with multiple genes. After that, we conducted an unsupervised clustering based on the expression of hallmark genes (Fig. 7A). A total of 30 patients with SONFH were separated into two subtypes, namely cluster1 (n = 21) and cluster2 (n = 9). We found that the expression of all hallmark genes was higher in cluster2 than that in cluster1 (Fig. 7B). To investigate the relationship between hallmark genes and immune infiltration in SONFH, we evaluated the immune infiltration level and the proportion of 22 immune cells between cluster1 and cluster2. We found the two clusters represented no significant difference in immune infiltration level and proportion. Interestingly, the proportion and immune infiltration level of Neutrophils in cluster2 were significantly higher than that of cluster1 (Fig. 7C and 7D).

DISCUSSION

In this study, to explore the key biomarkers associated with SONFH and the molecular mechanisms of SONFH, we analyzed the only human SONFH dataset from GEO database. We firstly screened 96 intersection genes through differential expression analysis and WGCNA. Then, we performed

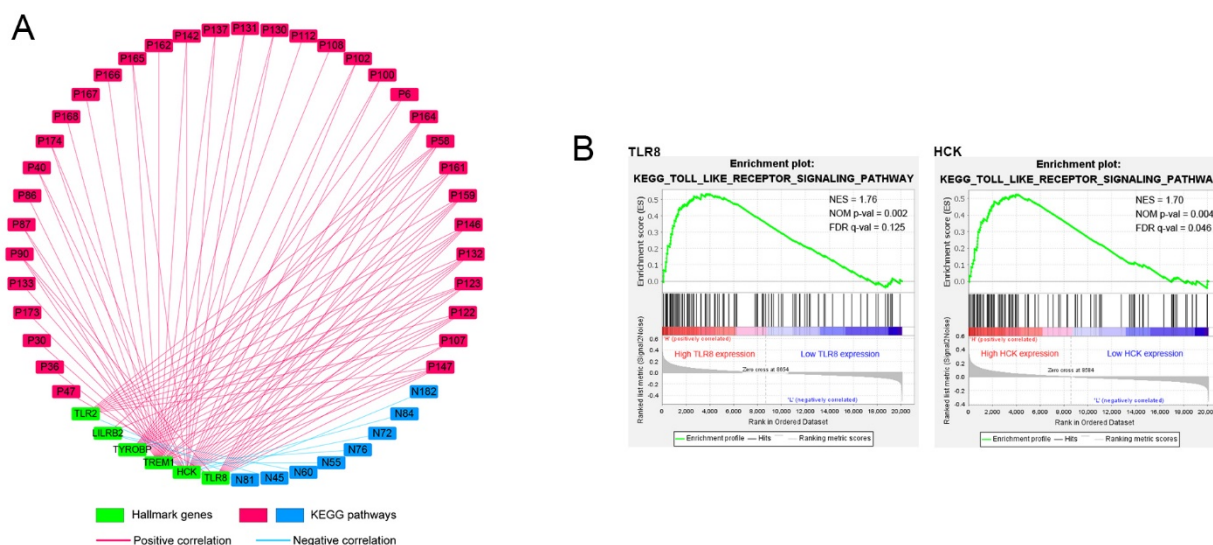


Fig. 6. *A)* The network planning shows the correlations between KEGG pathways and hallmark genes. $|PCC| > 0.6$ and p -value < 0.05 are selected. Positive correlations are shown by red; negative correlations are shown by blue; hallmark genes are represented by green. *B)* Gene set enrichment analysis based on the expression of *TLR8* and *HCK*, the Toll-like receptor signaling pathway highly enriched in high expression cohort are shown. NES, Normalized Enrichment Score; NOM p -val, Normalized p -value; FDR q -val, False Discovery Rate q -value

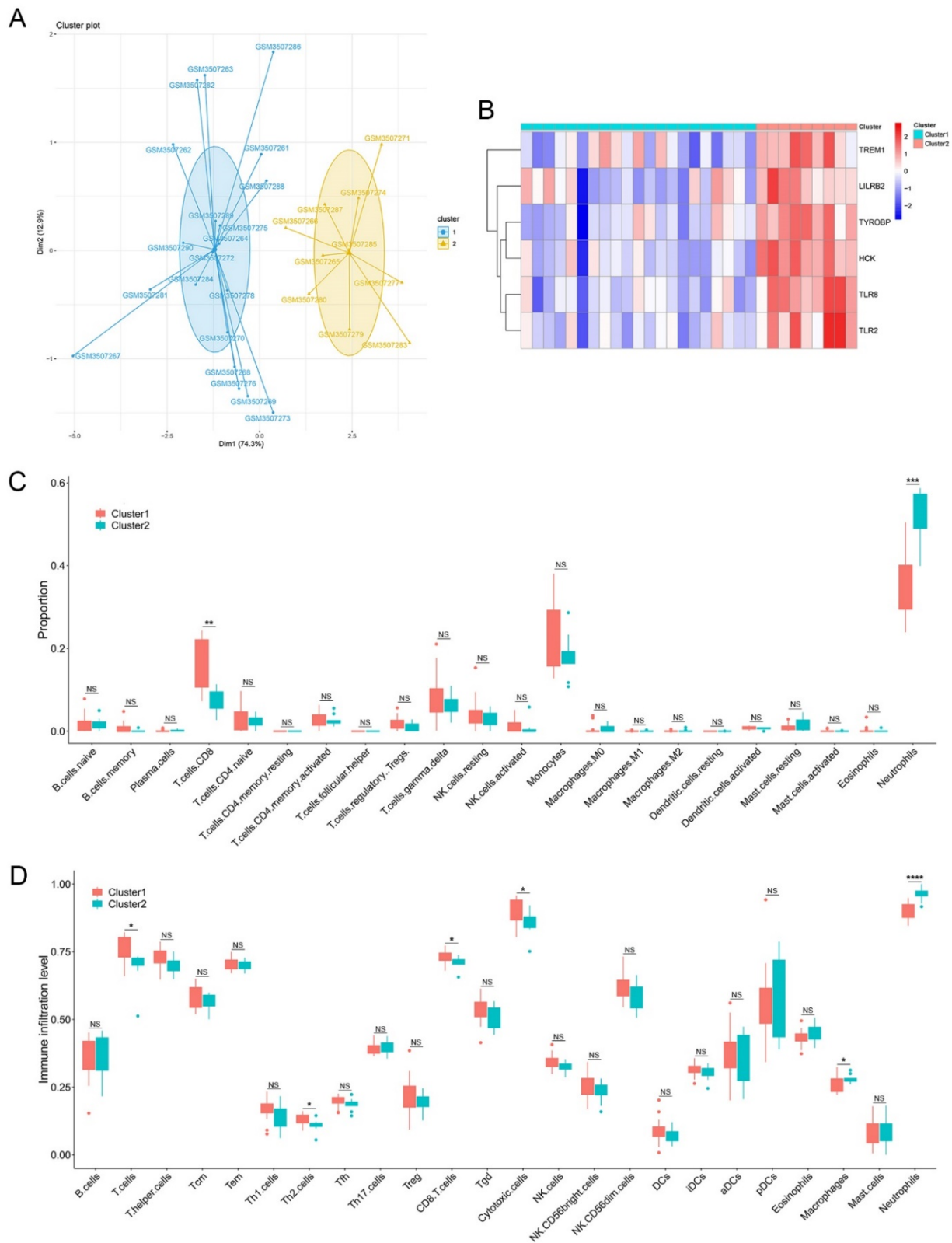


Fig. 7. *A*) The cluster plot of SONFH samples (cluster1/2). *B*) Heatmap of the two clusters (cluster1/2). *C*) Proportion of the 22 immune cells in cluster1 and cluster2. *D*) Immune infiltration levels of 24 immune cell gene signatures in cluster1 and cluster2. *, p -value < 0.05; **, p -value < 0.01; ***, p -value < 0.001.

the functional enrichment for intersection genes to ascertain significant GO terms and KEGG pathways. These genes showed heterogeneous biological functions or molecular mechanisms, indicating that SONFH development was subjected to multifaceted regulation (31). Based on the GSVA and GSEA results, we observed TLR8 and HCK were involved in Toll-like receptor signaling pathway, suggesting that might be the primary mechanism to regulate the progress of SONFH. The abnormal activation of Toll-like receptor signaling pathway activates the regulatory pathways of innate and adaptive immunity, leading to chronic inflammatory diseases (32). There is evidence that manipulation of this signaling pathway in disease has therapeutic potential, but its safety remains studied (33). Therefore, targeting the pathway might be a feasible strategy for the treatment of SONFH patients.

Identification of the biomarkers is necessary for the early diagnosis and detection of SONFH. Our research determined 6 SONFH-related hallmark genes (namely TYROBP, TLR8, TLR2, LILRB2, HCK, and TREM1) based on the PPI network, cross-talk, and ROC curve analysis. TLR8 and TLR2 belong to the family of toll-like receptors (TLRs), which are membrane-associated innate-immune sensors (34, 35). TLRs can lead to the synthesis and secretion of pro-inflammatory cytokines and costimulatory molecules through the Toll-like receptor signaling pathway, thereby promoting the inflammatory response (36, 37). Previous reports have revealed that the disorder of immune response via the TLR4 signaling pathway provokes the proliferation of osteoclasts, which plays a critical role in the pathogenesis of SONFH (38, 39). However, the exact role of TLRs in SONFH is not understood, especially TLR8 and TLR2, which remains to be explored. LILRB2 (known as ILT4, CD85d, and LIR2) is one member of leukocyte immunoglobulin-like receptor B, which modulates the immune response in the middle and late activation stages of the neutrophil lifecycle (40). HCK is one member of the Src-family of non-receptor tyrosine kinases, which can interact with steroid hormone receptors and transmit the downstream signals of the receptors (41). Besides, *HCK* and *LILRB2* seem to play specific roles in SONFH, but they still need further validation because no reports about these

genes contribute to SONFH. Moreover, *TREM1* is one isoform of the triggering receptors expressed by myeloid cells (TREMs), which signals through the use of the intracellular activating adaptor *TYROBP* (TYROsine kinase Binding Protein, also known as *DAPI2*) to induce downstream signaling (42-44). Carrasco et al. revealed that an initial priming via TLRs might be needed to allow *TREM-1* signaling (45). Additionally, accumulating evidence demonstrated that TREM-1 and TLR2 pathways could exert synergistic effects at the level of cytokine induction, as well as, *TREM-1* and *TLR8* regulated the burst of neutrophils together (46, 47).

There is an inevitable connection between the immune system and the skeletal system (48). Based on our immune infiltration analysis, we observed all hallmark genes were significantly upregulated in cluster2, as well as, the proportion and immune infiltration level of neutrophils in cluster2 were also substantially higher than that of cluster1. Furthermore, at a steady-state, the ability of the immune system to maintain the balance between amplifying and inhibiting the immune response is necessary to avoid potential autoimmunity and tissue damage while generating a successful immune defence. When the immune system is activated, the upregulation of stimulatory receptors is an essential feedback mechanism to promote pathogenic inflammatory responses and autoimmune disorders (49). Hence, the activated immune system of SONFH in cluster2 promoted the development of SONFH, which might be involved in the imbalance between the activated and suppressed immune responses. We speculate that the upregulation of hallmark genes mainly promotes the excessive formation and increased infiltration level of neutrophils in the immune system. Besides, excessive formation of neutrophils in small vessels surrounding the femoral head has been demonstrated to be closely associated with SONFH (50). However, the molecular mechanism of how neutrophils in small vessels contribute to tissue ischemia still needs to explore by future studies.

Based on the result of our comprehensive bioinformatics analysis, we hypothesize that excessive use of corticosteroids activates the immune response, TLRs initiate and amplify inflammatory signals through

Toll-like receptor signaling pathway, promote TREM1/DAP12 pathway to participate in the inflammatory response, and form molecular cross-talk, leading to the burst of neutrophils, and excessive formation of neutrophils in small vessels surrounding the femoral head further contribute to tissue ischemia, which may be the primary molecular mechanism for SONFH.

However, our study also had limitations. Due to the small amounts of samples from the GSE123568 dataset, false positive results may occur, so further experimental support is still necessary. Besides, the potential molecular mechanism of hallmark genes in SONFH should be further validated through a series of experiments, including tissue samples, cell or animal models. All in all, we expect these organized data and information will be mined, and many hypotheses will be validated by other researchers over time.

In conclusion, we offered a comprehensive bioinformatics analysis to identify the 6 SONFH-related hallmark genes and revealed new perspectives into the biological mechanism of SONFH. In particular, this study was the first to explore the molecular mechanism of SONFH through immune infiltration analysis. Our findings provided a theoretical foundation for further experimental study and establishing new roads for understanding the pathogenesis of SONFH.

Conflicts of interest

All authors declare that they have no competing interests regarding the publication of this paper.

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Availability of data and materials

The datasets analyzed during the present study are available from GEO (<https://www.ncbi.nlm.nih.gov/>

[gds/](https://www.gsea-msigdb.org/gsea/msigdb/index.jsp)) and MSigDB (<https://www.gsea-msigdb.org/gsea/msigdb/index.jsp>) database.

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Correlation between nasal inflammation assessed by nasal cytology and blood neutrophil-to-lymphocyte ratio in patients with COPD

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To the editor,

The upper and lower airways form a single anatomical and functional system, which is exposed to the same pathogens. In this regard, the coexistence of nasal inflammation and bronchial symptoms is common in Chronic Obstructive Pulmonary Disease (COPD) patients (1), and several studies have shown that the clinical manifestations of both diseases overlap in the same subjects with a combined and worse effect on quality of life (2-3). To date, the relationship between COPD and nasal inflammation has not been clearly determined (1).

Nasal cytology is easy to apply and is a useful diagnostic tool to explore nasal pathological conditions and related inflammatory patterns (4-5). To date, no studies have applied nasal cytology in the field of COPD, and it is reasonable that examination of nasal cells might be useful in the diagnosis and monitoring of patients with this condition.

Based on the above, the aim of our study is to evaluate nasal cytology in a group of COPD patients compared to a control group with similar smoking status and without COPD. Moreover, we aimed to compare in the nasal inflammation of the

aforementioned groups with blood parameters of inflammation such as neutrophils-to-lymphocyte ratio and lung function indices of obstruction.

MATERIALS AND METHODS

A total number of 57 patients participated in the current study. Forty-three patients with COPD were enrolled as well as 14 individuals as a control group.

Inclusion criteria for the COPD groups were as follows: age > 45 years and an established diagnosis of COPD; (post-bronchodilator FEV1/FVC < 0.7; personal history of chronic productive cough and/or exertional dyspnea in the presence of exposure to noxious inhaled particles) strictly following GOLD recommendations (6). Exclusion criteria were as follows: personal history of asthma or other chronic respiratory diseases; personal history of known nasal pathologies; either anatomical malformations or changes and nasal inflammatory disease; comorbidities such as cancer, severe heart failure and severe kidney failure; recent acute respiratory infection and/or COPD exacerbation (in the four weeks prior to the study); alcohol or drug abuse.

The control group consisted of smokers or former

Key words: COPD; nasal cytology; inflammation; neutrophil-to-lymphocyte ratio; upper airways

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smokers with normal lung function and no personal history of respiratory symptoms. Exclusion criteria were the same as for the COPD group. All participants were enrolled from the outpatient clinics of Respiratory Disease Department, University Hospital Policlinico, Bari, Italy, and signed informed consent according to the Declaration of Helsinki. The study was approved by the Institutional Review Board of Bari University General Hospital (protocol number: 6228).

Study design

A cross-sectional study with a two-day visit for each participant was performed. During the first day, inclusion and exclusion criteria were carefully checked. Then, all subjects underwent complete lung function with body plethysmography (Carefusion MasterScreen PFT System, Jaeger, Germany) in order to establish the diagnosis of COPD. During the second day, peripheral blood samples for complete blood count and differential white blood cell count were collected from the patients. Moreover, systemic subclinical inflammatory status was estimated with C-reactive protein (CRP) and neutrophils-to-

lymphocytes ratio (NLR). Then, nasal cytology analysis was performed on all patients (see below).

Nasal cytology

Nasal cytology was performed as previously described (4-5). The technique involves sampling, processing and microscope reading. This procedure was performed by scraping the middle part of the inferior turbinate with a Rhino-Probe® device (Arlington Scientific). The sample is immediately smeared on a glass slide, air-dried, then stained with the May-Grumwald Giemsa preparation. The stained sample is read at optical microscopy (Nikon® E600) at x 1000 in oil immersion. The minimum number of fields used for differential cell counting is 50.

Statistical analysis

Statistical analysis was conducted by using SPSS software version 23. Being a pilot study, no sample size evaluation was performed. Patients characteristics were reported as mean $\pm$ standard deviation (SD) for continuous variables and as a percentage (%) for categorical variables. We verified the normal distribution of continuous variables.

Table I. *Clinical characteristics of the study population*

Parameter	COPD	CONTROLS	<i>p</i>
Subjects (n.)	43	14	ns
Males (%)	68	57.1	ns
Age (years)*	70.8 $\pm$ 8.1	68.7 $\pm$ 5.4	ns
Smokers (%)	29.3	25.4	ns
Ex-smokers (%)	74.6	70.7	ns
Pack/Year*	43.1 $\pm$ 24.8	31.4 $\pm$ 14.7	ns
ICS users (%)	34.1	0	<0.01
BMI*	27.2 $\pm$ 5.3	28.4 $\pm$ 6.2	ns
FEV1 (% pred)*	71.4 $\pm$ 25.1	100.4 $\pm$ 29.3	<0.01
FVC (% pred)*	83.7 $\pm$ 16.8	101.5 $\pm$ 18.6	<0.01
Blood neutrophils (%)	60.4 $\pm$ 7.8	62.3 $\pm$ 6.7	ns
Blood lymphocytes (%)	26.3 $\pm$ 6.4	29.2 $\pm$ 5.6	ns
Blood eosinophils (%)	1.2 $\pm$ 0.5	1.1 $\pm$ 0.2	ns

* Values are intended as mean and standard deviation (SD)

Continuous parameters were analyzed with Student's *t*-test, whereas discontinuous variables were analyzed with *Chi*-squared test. Pearson correlation, multivariate linear and logistic regression were used to assess relations between nasal cytology and clinical parameters. A value of $p < 0.05$ was considered as significant.

RESULTS

General characteristics of the COPD population are shown in Table I. Mean age was 70.85 ± 8.01 years. In this study, 68% of patients were males and 36.6% were frequent exacerbators. Mean cigarette packs/year was 37.7 with 29.3% current smokers and 70.7% ex-smokers. The control group was comparable with COPD group with mean age of 68.7 ± 5.40 years and 57.1% of males. All these patients were current smokers (25.4%) or ex-smokers (74.6%) with mean pack/years of 31.4 ($p = \text{ns}$).

COPD patients had a mean FEV1% of 71.4. According to GOLD spirometric classification, 34.1% of patients belonged to stage I, 43.9% to stage II, 14.7% to stage III and 7.3% to stage IV. Following combined GOLD classification (6), 41.5% of patients belonged to stage A, 24.4% to stage B, 4.9% to stage C and 29.2% to stage D. Percentage of inhaled steroid users was 34.1%.

Altered nasal cytology was found in 14 (34.1%) COPD patients, distributed as follows: 8 patients with neutrophilic pattern, 1 with eosinophilic pattern, 3 with neutrophilic and eosinophilic pattern and 2 with mast cells. Furthermore, in COPD patients we found bacteria in nasal mucosa in 8 (19.5%) patients, with biofilm in 3 (6.9%) patients. In the control group 4 (28.6%) patients presented nasal cytology abnormalities. There was no difference in nasal cytology between COPD patients and the control group considering frequency of alteration and pattern of nasal inflammation ($p = \text{ns}$). Nasal inflammation of COPD patients resulted to be similar to that of the control group.

Moreover, in the COPD group, no differences in nasal cytology were reported between patients with and without inhaled corticosteroid (ICS) therapy, nor among different GOLD stages. Similarly, no correlation was found between smoking status and nasal cells in both COPD and control groups.

Interestingly, COPD patients with altered nasal inflammation showed higher neutrophil-to-lymphocyte ratio (NLR) compared to patients with normal nasal mucosa (2.77 vs 2.05, $p < 0.01$), whereas no correlation between nasal cytology and NRL was reported in the control group (2.31 vs 2.19; $p = \text{ns}$, Fig. 1). Finally, no relationship between CRP and

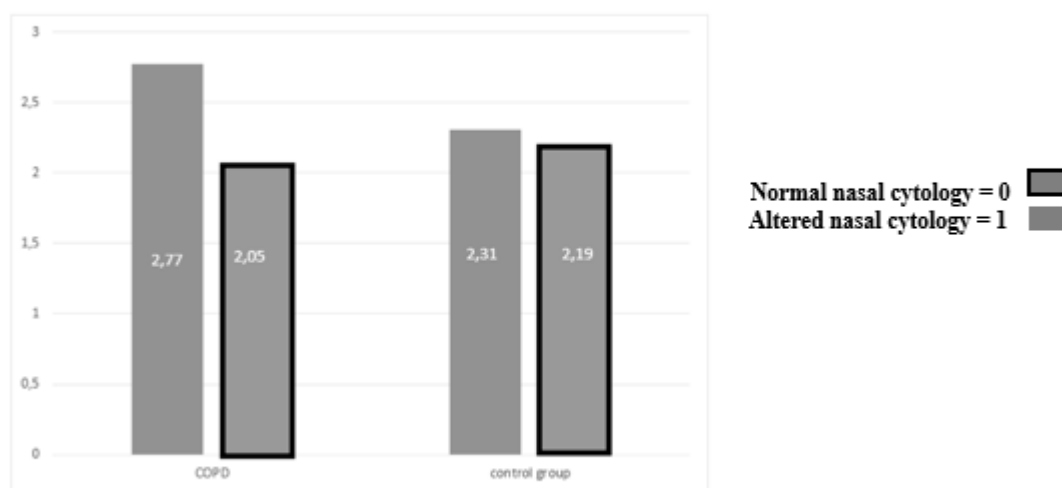


Fig. 1. Neutrophil-to-lymphocyte ratio between patients with cytological nasal abnormalities and normal nasal mucosa in COPD and control groups.

nasal cells was reported in either the COPD group or the control group ($p=ns$).

DISCUSSION

To the best of our knowledge, this is the first study that investigates nasal cytological features in COPD patients in order to define the possible role of nasal inflammation in this chronic disease. From a cytological point of view, in this study no differences emerged between COPD patients and the control group. Interestingly, we observed a correlation between nasal inflammation assessed by nasal cytology and blood neutrophil-to-lymphocyte ratio (NLR) in patients with COPD. Previous data investigating nasal inflammation and COPD are ambiguous. Hurst et al. analyzed nasal lavage with paired sputum in 47 COPD patients and 12 control patients and found higher interleukin 8 (IL-8) levels in nasal wash and sputum in COPD patients (7). This result is in line with our findings, as in our study 57% of COPD patients with nasal abnormalities presented neutrophilic pattern. Similarly, Hens et al. reported an increase of inflammatory mediators such as eotaxin, granulocyte colony stimulating factor (G-CSF), and IFN gamma in nasal secretions of COPD patients (8).

Conversely, Vachier et al. performed nasal and bronchial biopsies in smokers with and without COPD and in a non-smoking group (9), showing increased number of neutrophils and macrophages in smokers, whereas the presence of eosinophils, neutrophils and macrophages was similar in smokers with or without COPD. In this study, smoking seemed to represent the crucial factor that determined nasal histological inflammatory pattern (9). However, our data showed no correlation between nasal cells and smoking status, thus requiring further investigation specifically focused on the above.

The current study has some limitations due to the relatively limited number of patients and to the difference between nasal histological and cytological analyses. Indeed, while in the cytological analysis final samples are read at optical microscopy at $\times 1000$ in oil immersion, histological samples are examined at $\times 400$. Future studies with larger populations should

also compare the two aforementioned techniques. Moreover, we enrolled our COPD patients during a stable phase of their diseases. It would be worth to monitor these subjects during their exacerbation phase, to assess the role of nasal cytology as potential predictor tool.

The most interesting result of our study is the significant presence of nasal cell infiltration (neutrophils, eosinophils, lymphocytes, and muciparous cells) which was observed exclusively in COPD patients with elevated blood neutrophil-to-lymphocyte ratio. NLR is a reliable marker of systemic inflammation derived from routine complete blood count. In addition to its availability, rapidity and easy detection usefulness, it is important to underline its cost effectiveness that makes this parameter easy to evaluate in any medical setting. Previous studies have shown a correlation between NLR and clinical/functional parameters (FEV1, mMRC, and BODE index) in patients with COPD (10), highlighting that a high NLR may be related to a worsening of dyspnea, degree of bronchial obstruction and clinical performance. Furthermore, it was suggested that NLR could be useful in predicting the risk of in-hospital and late mortality in COPD patients (10). Notably, our data showed no correlation between nasal cells and CRP, warranting further investigation on this discrepancy.

How can we interpret our findings? According to our results, a pro-inflammatory state was evidenced in COPD patients with nasal abnormalities. However, unlike asthma that shows presence of the role of nasal inflammation in pathogenesis and clinical worsening of pulmonary disease, in COPD this link appears to be inconsistent. Even though the role of COPD as a widespread systemic disease is emerging with increasingly scientific strength, this seems to be more related to noxious effect of COPD itself on cardiovascular and metabolic systems, not directly involving nasal inflammation.

In conclusion, our data warrant further prospective studies to address confounders with larger sample sizes to better clarify the potential clinical value of both nasal inflammation and NLR in COPD. Furthermore, these studies should include patients during exacerbation phase.

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The usefulness of amelogenins in the treatment of periodontal defect on the distal surface of the lower second molar after adjacent third molar extraction: a pilot study

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To the Editor,

A periodontal defect on the second molar distal surface is the most common complication after surgical extraction of the contiguous lower third molar. Deep impaction, mesio-angular or horizontal position, and bacterial plaque are the main predisposing factors for the development of this defect which is characterized by both a variable bone loss and a worsening of probing depth and clinical attachment (1, 2). Although many techniques and biomaterials have been proposed in preventing and treat such a defect (1, 3), there are no studies regarding amelogenins (AMGs), whose use is easy and safe. Surprising results have been found, over the last 20 years, with AMGs, in the regeneration of bone, root cement and periodontal ligament, in sites severely compromised by periodontitis (4). The present study primarily aims to evaluate whether AMGs have some benefits in reducing second molar periodontal defects after adjacent third molar surgical extraction and, secondarily, to determine the possible need to improve, modify or simplify some protocol aspects for further research as well as the feasibility of recruiting patients for a larger main study.

MATERIALS AND METHODS

Study type and aims

The present pilot study was performed at the Oral

Surgery Unit of the Department of Odontostomatological and Maxillofacial Sciences of Sapienza University of Rome and was set up as a single-blind randomized controlled clinical trial (RCT) to comparatively evaluate the effect of AMGs (EMD-Emdogain® Straumann Switzerland) *versus* no regenerative treatment at 1 year from the treatment of the distal periodontal defect distal to the second molar, after extraction of the adjacent lower third molar. The study followed the principles of the Helsinki Declaration concerning research work involving the human species. The enrollment of each patient occurred only after approval of the relative consent to participate in the study. The research was approved by the local Ethics Committee with protocol no. 3522 and was only retrospectively registered on ClinicalTrials.gov (Identifier: NCT4314726) because, although set up as an RCT, it was an intra-patient study and was configured as a feasibility study, with a very limited sample size, which was imposed by the Ethics Committee.

Study sample and randomization

The study enrolled 5 periodontitis-free patients, for whom the extraction of both lower third molars was prescribed. A bone defect at the distal surface of the second molar ≥ 5 mm, had to be present on the intraoral periapical radiography, bilaterally. EMD effect was evaluated by applying it only in the test site and comparing healing results with those obtained on the contra-lateral site (control site), in which the conventional treatment was performed, and healing

Key words: amelogenin; periodontal regeneration; experimental design; wound healing; third molar surgery

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was ensured only by simple blood clotting. Randomization was performed by a person not involved in the patient recruitment, generating a sequence of codes (1=EMD; 2=no EMD) via a Microsoft Excel spreadsheet, which were individually sealed inside an envelope and casually assigned to each study participant; the contents of the latter was then viewed by an assistant only after the surgeon defined which of the two third molars would be extracted first. Likewise, the evaluation of clinical and radiographic healing parameters was performed by an operator who was not aware of how the site under examination was treated, ie whether it was the test or the control site.

Inclusion criteria

The following inclusion criteria were used for the study: patients for which the extraction of both lower third molars was indicated, aged <27, since the risk of surgical complications is higher for older patients; bilateral total (bone or osteo-mucosal) impaction of mesially or horizontally inclined third molars; bilateral bone defect ≥ 5 mm distal to the lower second molars, evaluated on pre-operative periapical radiographs performed with Rinn's film holders; periodontal probing depth (PPD) ≥ 8 mm distal to the second lower molars, in at least one of the following probing sites: buccal, disto-buccal, disto-central, disto-lingual; intra-operative integrity of both third molar buccal and lingual alveolar cortices.

The following exclusion criteria were used for the study: smoking habit; systemic disorders with serious immunologic impairment; taking cortisone or other drugs that might interfere with the healing process; allergy to penicillin; previous periodontal treatment; less than 2 mm of adherent gingiva at second molar level; second molars with a prosthetic crown; previous endodontic treatment of second molars; furcation involvement of the second molars, less than 5 mm of bone loss at the distal surface of the second molar and lack of integrity of both buccal and lingual bone cortices, intraoperatively evaluated.

Assessment parameters and detection methods at second molar level

The following parameters were detected on the second molar distal surface:

- Pre-operative PPD, at 3 and 12 months after surgery, in four different sites (buccal, disto-buccal, disto-central and disto-lingual).

- Pre- and post-operative clinical attachment loss (CAL), at 3 and 12 months after surgery, in four different sites (buccal, disto-buccal, disto-central and disto-lingual)
 - Pre- and post-operative gingival recession (REC), at 3 and 12 months after surgery, in four different sites (buccal, disto-buccal, disto-central and disto-lingual)
 - Bone level (distance between the defect bottom and the cement-enamel junction - CEJ) evaluated both clinically, at the moment surgery, and radiographically, pre-operatively - and at 3 and 12 months post-operatively, with periapical radiographs performed with parallel beam technique and Rinn's film holders
 - Post-operative complications or other adverse events
- PPD, REC and CAL distal to the second molar were evaluated each time by the same researcher with a PCP 15-UNC 15 periodontal probe (Hu-Friedy, Leimen, Germany). Two different measurement methods were used:
- standard method: four-point measurement (buccal, disto-buccal, disto-central and disto-lingual);
 - personalized method: four-point measurement (buccal, disto-buccal, disto-central and disto-lingual) using a self-curing acrylic resin customized guide, according to the 2014 study by Pippi (5) in order to overcome the clinical difficulty of CAL detection, due to the possible gingival pseudo-hypertrophy, and to obtain measurements from identical sites over time and thus a more precise and reliable comparison of CAL variations at each follow-up visit.

Radiographic evaluations were performed on periapical radiographies performed with the long cone technique, pre-operatively and after both 3 and 12 months from surgery, with the same shooting parameters and the use of an adhesive non-metallic millimeter grid, applied to the radiographic film, to measure the residual bone level as the distance from the CEJ to the most coronal bone margin, radiographically identifiable on the second molar distal surface. For this purpose, two different values were registered, corresponding to the lingual and buccal bony contours of the defect distal to the second molar, when radiographically identifiable, or two identical values if a single bone contour was identifiable.

Patient pre-surgical treatment and peri-operative oral hygiene procedures

In the days prior to surgery each patient underwent a professional oral hygiene session and received

personalized oral hygiene instructions to achieve an O'Leary plaque index of $\leq 25\%$. Immediately before surgery, the patient was asked to rinse with 0.20% chlorhexidine mouthwash for 1 min. In order to prevent post-surgical infectious complications, third molar extraction was carried out on antibiotic prophylaxis with 2 g of amoxicillin+clavulanic acid, taken 1 h before surgery and 1 g of amoxicillin+clavulanic acid every 12 h for the

following 3 days. In the post-operative period, all patients were instructed not to brush the surgical wound for at least 3 weeks after surgery, but to perform local mechanical cleaning with the help of a gauze and/or a cotton stick. From the fourth week all patients were asked to take up a gentle brushing with the roll-stroke technique (apico-coronal) with a single-toothbrush. Daily rinses with a 0.12% chlorhexidine antiseptic mouthwash, twice a day,

Table I. Overall data of the study sample (significant results are highlighted in bold).

Patients	RG		VG		SA		FS		DM		Mean $\pm$ SD (mm)		p-value
Tooth	3.7	4.7 EMD	3.7	4.7 EMD	3.7 EMD	4.7	3.7	4.7 EMD	3.7	4.7 EMD	EMD	No EMD	
<i>Measures (mm)</i>													0.04
T0 PPD											6.05 $\pm$ 2.04	7.05 $\pm$ 2.09	
<i>B</i>	8	5	7	3	3	4	7	5	7	3	11.15 $\pm$ 3.50		0.95
<i>DB</i>	8	7	7	5	7	7	2	5	7	5			
<i>DC</i>	5	6	9	5	8	9	5	7	6	5			
<i>DL</i>	10	8	9	10	6	5	9	9	10	9			
T0 CAL with the guide											11.15 $\pm$ 3.50	11.35 $\pm$ 3.37	0.95
<i>B</i>	15	11	14	7	9	5	8	12	15	14	4.10 $\pm$ 1.37		0.07
<i>DB</i>	14	13	12	10	10	11	5	10	15	14			
<i>DC</i>	10	12	12	13	10	11	6	10	15	10			
<i>DL</i>	15	11	10	12	9	10	9	13	13	13			
3-month PPD											4.10 $\pm$ 1.37	4.90 $\pm$ 1.74	0.07
<i>B</i>	6	3	3	3	4	4	3	3	3	2	11.05 $\pm$ 1.96		0.59
<i>DB</i>	4	4	7	3	3	4	6	5	3	3			
<i>DC</i>	3	6	5	3	5	7	8	7	4	4			
<i>DL</i>	7	4	4	4	4	8	5	7	4	5			
3-month CAL with the guide											11.05 $\pm$ 1.96	11.15 $\pm$ 2.03	0.59
<i>B</i>	9	8	10	10	10	13	10	10	11	12	4.70 $\pm$ 1.45		0.48
<i>DB</i>	9	10	15	16	9	11	13	13	11	12			
<i>DC</i>	9	12	15	14	10	12	13	12	12	11			
<i>DL</i>	8	9	11	11	9	12	11	10	8	13			
12-month PPD											4.70 $\pm$ 1.45	4.95 $\pm$ 1.43	0.48
<i>B</i>	4	3	3	2	4	4	3	4	3	3	8.70 $\pm$ 1.42		0.00054
<i>DB</i>	7	4	4	4	3	6	6	7	4	5			
<i>DC</i>	6	6	5	5	6	6	8	7	4	3			
<i>DL</i>	5	6	5	6	5	7	4	6	5	5			
12-month CAL with the guide											8.70 $\pm$ 1.42	10.45 $\pm$ 1.43	0.00054
<i>B</i>	10	8	10	6	10	11	10	10	12	10	5.60 $\pm$ 0.55		0.62 ¹
<i>DB</i>	8	7	10	8	8	11	12	10	12	10			
<i>DC</i>	7	7	11	10	9	12	8	8	10	9			
<i>DL</i>	11	10	11	9	7	12	11	7	10	11			
<i>T0 xr</i>	5	6	8	8	5	7	5	6	5	6	5.60 $\pm$ 0.55		0.62 ¹
<i>3-month xr</i>	4	3	4	1	2	4	4	2	3	4			
<i>12-month xr</i>	5	1	2	1	1	2	2	1	3	2			
Healing index											5.60 $\pm$ 0.55	5.40 $\pm$ 0.55	0.62 ¹

PPD: periodontal probing depth; CAL: clinical attachment level; B: buccal; DB: disto-buccal; DC: disto-central; DL: disto-lingual; T0: pre-operative measure; SD: standard deviation; EMD: Emdogain; xr : periapical radiograph; ¹: paired t-test (95% CI).

up to 4 weeks after surgery, were finally recommended for the post-operative plaque chemical control.

All surgeries were performed by a single expert operator following the same protocol with the buccal approach and the use of rotary instruments for both bone removal and tooth section.

Intra-operative treatment of second molar distal root surface

Immediately after tooth extraction, any residual deposits of sub-gingival calculus were removed from the exposed root surface of second molar, by means of manual and ultrasonic instruments. In the test side, the root surface was chemically conditioned for cleansing with Straumann® PrefGel (EDTA) for 2 min and, after careful rinsing with sterile saline solution, Straumann® Emdogain gel was applied (0.15 ml-30 mg/ml), and it was left undisturbed for about 2 min on the root surface before the access flap was sutured. The same steps were followed in the control site, using the same type of dispenser and the same execution times, but sterile saline instead of EDTA and AMGs. No other biomaterials were used in association with AMGs because they could have prevented the evaluation of the real regenerative contribution of AMGs and could have potentially exposed to a greater risk of infections, rather unusual for the enamel matrix derivatives compared to other biomaterials (4).

Surgical wound healing assessment

At suture removal, 7 days after surgery, surgical wound features were evaluated through the wound evaluation scale, giving each site a total final numerical score, based on the presence or absence of step margins, contour irregularities (wrinkling), more than 2 mm distance between surgical margins, edge inversion, and inflammation, as well as on the overall positive or non-positive cosmetic appearance (6). Any difference in the overall average numerical score, in excess or in default, between one study group compared to the other, was considered as indicative of better or worse healing, respectively.

Statistical analysis

Data with a normal distribution were presented as mean $\pm$ standard deviation. The variations of each clinical and radiographic parameter were calculated in the different recording times (baseline, 3 months and 12 months). The Wilcoxon test was used to evaluate any statistically

significant differences between the means of test and control sites' measurements, within each recording interval, and it was also used to identify, between test and control sites, any significant changes in clinical and radiographic parameters, between different recording times. A p-value <0.05 was considered statistically significant.

RESULTS

Five female patients were enrolled, with a mean age of 25 ± 1.09 , in a 13-month period, from March 2016 to April 2017. No side effects occurred during the entire follow-up period. The results of periodontal probing, with and without the guide, and those of the radiographic residual bone defect distal to the second lower molar, in the different recording times (T0/pre-operative, 3 and 12 months after surgery), are summarized in Table I. In no cases was gingival recession present, either before surgery or at the follow-up visits.

Although randomization of surgical sites revealed a slight significant difference ($p=0.04$) in PPD registered without the guide, with mean control group values about 1 mm higher than those of the test group, CAL values registered with the guide did not reveal statistically significant differences between the two groups of sites ($p=0.95$). Actually, the mean difference of CAL with the guide was about 0.2 mm, with an approximate similar standard deviation between the two groups (11.15 ± 3.50 in the EMD sites and 11.35 ± 3.37 in the control sites, Table I).

The PPD registered without the guide revealed no statistically significant differences in both the 3-month ($p=0.07$) and 12-month ($p=0.48$) follow-up visits. On the other hand, the CAL values with the guide showed a significant difference ($p<0.01$) only at the 12-month clinical follow-up visits with a mean PPD 1.75 mm lower in the test group than in the control one (mean CAL in EMD sites= 8.70 ± 1.42 , mean CAL in control sites= 10.45 ± 1.43).

Regarding the mean PPD change registered without the guide among the different recording times (Table II), no statistically significant difference was found between the pre-operative and the 3-month measures ($p=0.52$), as well as between the 3-month measures and those registered at 12 months

($p=0.48$), and between the pre-operative and the 12-month measures ($p=0.21$). After a slight initial reduction in PPD during the first post-surgical three months, both study groups reported a slight average increase in PPD between the third and twelfth month, but all study sites reported a mean PPD reduction of -1.40 ± 1.73 mm for test sites and of -2.10 ± 2.77 mm for control sites, between pre-operative and post-operative measurements after 12 months.

The mean CAL variation with the guide (Table II) found statistically significant differences only between 3- and 12-month measures ($p<0.01$) and between pre-operative and 12-month measures ($p<0.05$), but not between the pre-operative and 3-month measures ($p=0.93$). One year after surgery, in the test sites was found an average CAL reduction with the guide, corresponding to -2.45 ± 1.85 mm, unlike the control sites (-0.80 ± 3.62 mm).

As to the average CAL per site variation registered with the guide (Table III), the only statistically positive differences ($p<0.01$) were detected in disto-buccal and disto-lingual sites between 3- and 12-month measures. The average variation of CAL in disto-buccal site was -3.40 ± 2.70 mm in the test group and -1.20 ± 2.28 mm in the control group, while, in the disto-lingual site, it was -1.60 ± 1.52 mm for the test group and 1.00 ± 1.41 mm for the control group. On the contrary, as to the average variation per site in the PPD without the guide (Table III), only buccal sites, within the measures recorded pre-

operatively and those recorded at 3 or 12 months, showed a significantly greater reduction in the control group compared to the test group ($p<0.05$ and $p<0.01$, respectively). The mean PPD variation at buccal sites, between pre-operative and 12-month post-operative measures, was -0.60 ± 1.14 mm in the test group and -3.40 ± 1.34 mm in the control group, respectively. The bone defect reduction, evaluated through periapical radiographs, showed no significant differences (Table II). No statistical difference was finally found between test and control sites as to the clinical healing index, the results of which are reported in Table I.

DISCUSSION

Regarding PPD, a slightly lower, not statistically significant average reduction was found in test vs control sites. Although many studies found significant reductions in PPD at sites where membranes and/or biomaterials were applied (7-9), a considerable result variability exists and preventive techniques of second molar periodontal defect did not always show significant advantages compared to spontaneous healing (10). This variability could be related to different factors such as methods of both measurement and patient selection, initial site features, Pell & Gregory Class, quality, characteristics of soft tissues adjacent to the second molar, and periodontal biotype. At 12 months, a significantly

Table II. PPD an CAL mean variations and radiographic mean bone gain (significant results are highlighted in bold).

Study sample teeth		EMD	No EMD	p-value ¹
Comparisons		Mean $\pm$ SD (mm)		
Mean PPD variation	T0 PPD vs 3-month PPD	-1.95 ± 1.73	-2.15 ± 2.80	0.52
	3-month PPD vs 12-month PPD	$+0.60\pm 1.05$	$+0.30\pm 1.42$	0.48
	T0 PPD vs 12-month PPD	-1.40 ± 1.73	-2.10 ± 2.77	0.21
Mean CAL variation	T0 CAL vs 3-month CAL	-0.15 ± 2.41	$+0.10\pm 4.78$	0.93
	3-month CAL vs 12-month CAL	-2.35 ± 2.06	-1.10 ± 2.20	0.0078
	T0 CAL vs 12-month CAL	-2.45 ± 1.85	-0.80 ± 3.62	0.0477
Radiographic mean bone gain	T0 xr vs 3-month xr	2.50 ± 1.51	2.40 ± 0.84	0.81
	3-month xr vs 12-month xr	1.70 ± 0.42	2.20 ± 1.40	0.38
	T0 xr vs 12-month xr	4.10 ± 0.88	4.60 ± 1.55	0.34

PPD: periodontal probing depth; CAL: clinical attachment level; T0: pre-operative measure; SD: standard deviation; EMD: Emdogain; xr: periapical radiograph; ¹: paired t-test (95% CI).

Table III. PPD and CAL mean variations per site (significant results are highlighted in bold).

		EMD	NO EMD	p-value ¹
PPD without the guide		MEDIA±SD (mm)		
T0 PPD vs 3-month PPD				
B	-0.80±1.73	-3.00±1.30	0.019	
DB	-2.20±1.48	-1.40±3.44	0.49	
DC	-1.20±1.30	-1.40±2.61	0.84	
DL	-3.40±1.67	-3.00±3.54	0.79	
T0 PPD vs 12-month PPD				
B	-0.60±1.14	-3.40±1.34	0.001	
DB	-1.20±2.39	-0.60±2.70	0.60	
DC	-1.20±1.10	-1.00±2.92	0.88	
DL	-2.80±1.30	-3.20±2.95	0.69	
3-month PPD vs 12-month PPD				
B	+0.20±0.84	-0.40±0.89	0.30	
DB	+0.60±1.34	+0.40±2.07	0.84	
DC	+0.40±1.14	+0.40±1.52	1.00	
DL	+0.20±0.84	-0.40±1.34	0.46	
CAL with the guide				
T0 CAL vs 3-month CAL				
B	-0.60±2.51	-0.60±5.64	1.00	
DB	+0.60±3.78	+0.80±4.97	0.89	
DC	+0.80±0.84	+2.20±3.03	0.24	
DL	-1.20±1.30	-1.40±4.28	0.92	
T0 CAL vs 12-month CAL				
B	-0.80±2.59	-0.80±4.66	1.00	
DB	-2.40±2.19	-1.60±3.36	0.34	
DC	-2.40±1.67	-1.00±3.16	0.40	
DL	-2.80±1.92	-0.20±2.59	0.23	
3-month CAL vs 12-month CAL				
B	-1.00±1.41	0.00±1.22	0.35	
DB	-3.40±2.70	-1.20±2.28	0.0042	
DC	-3.20±1.64	-2.60±1.65	0.42	
DL	-1.60±1.52	+1.00±1.41	0.0029	

PPD: periodontal probing depth; CAL: clinical attachment level; B: buccal; DB: disto-buccal; DC: disto-central; DL: disto-lingual, T0: pre-operative measure; SD: standard deviation; EMD: Emdogain; RX: periapical radiograph; ¹: paired t-test (95% CI).

higher average CAL was found for test vs control sites ($p<0.05$). Analyzing the RCTs that reported a similar average CAL at control sites, the results obtained with AMGs could be compared to those obtained using a resorbable membrane or bovine mineral bone (8, 9). This similarity could be due to similar 3-site probing measurement methods, and to the guide used in one study (8). Although some studies found beneficial effects in combining AMGs with different biomaterials, most studies did not show significant PPD and CAL improvements and the currently available results remain difficult to compare

because of significant problems related to the lack of standardization and the remarkable heterogeneity of results related to different factors such as patient selection methods, initial defect features and types of biomaterials used. It is also to be determined whether carriers are equally suitable and compatible to convey the interaction of AMGs with different biomaterial surface (4). Therefore, no reliable conclusions are available in this regard and studies are needed to clarify these preliminary findings. Although some authors hypothesized that most periodontal healing distal to the second molar is completed about 3 months after

third molar extraction (2, 11), statistically significant CAL changes between 3 and 12 months were presently found. Except for PPD, the mean bone gain values also showed an overall improvement, but nevertheless smaller than CAL and statistically not significant. Based on the present results, an intermediate 6-month recording time is advisable for future RCTs, to identify substantial changes or earlier result stabilization compared to 12-month measures. Even a follow-up of more than 1 year could prove useful, to confirm result stability over time. Wound healing assessment led to similar results for both study groups, possibly because of the limited number of cases and the single detection. In future studies, it should also be checked whether AMGs, besides affecting wound healing by improving soft tissue quality and thickness (12), are also able to influence, especially at the second molar distal area, the mean PPD reduction. Since the present study lasted 25 months due to the difficulty in finding patients who met the inclusion criteria and consented to be enrolled, the main study will possibly have to be multicentric, to reach more quickly the sample size required. Moreover, funding will be required for the main study to cover the EMD purchase cost.

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Inactivation of Influenza virus by a blend of essential plant oil vapour and aerosol

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To the Editor,

Influenza A virus (IAV) infections, a significant cause of morbidity and mortality worldwide, are responsible for numerous hospitalizations which result in a significant medical and economic burden. The best strategies to fight IAV are vaccination and antiviral drugs. The success of the vaccine may vary based on different factors, including the time needed to design and produce it and the genetic match between circulating and vaccine strains. Moreover, as frequent mutations in the structure of the viral genome often lead to resistance to licensed drugs, few satisfactory therapies are available for the control of Influenza (1). Hence, there is an urgent need to develop new approaches to control IAV infection.

In this view antimicrobial natural products, such as essential oils (EOs), could be crucial resources (2). The anti-IAV activity of EOs has been previously examined in Madin Darby Canine Kidney (MDCK) cells (3-5). It has been reported that *S. dentata* EO inhibits viral plaque formation (5) and EO blends are able to prevent both viral infection, reducing the expression of viral nucleoprotein, and cytokine production (3, 4). Though these studies demonstrate the great EO antiviral potential, further research is needed to identify the optimal administration routes. Inhalation represents one of the safest ways to administer EOs. For example, since many plants emit volatile biogenic compounds (VOCs), whose inhalation has many beneficial properties (6, 7),

it has been suggested that medical devices, such as nasal sprays containing VOCs, should be evaluated for their use against COVID-19 infection (6). So, due to the significant impact on human health, EO aerosols are an increasingly important research topic and inactivation of environmental viral particles could be a new strategy against viruses. In this study, the potential anti-IAV properties of an EO blend in vapour and in aerosol form, has been analysed.

MATERIALS AND METHODS

Cells and Viruses

Madin-Darby canine kidney (MDCK, ATCC, CRL-2936) cells were grown at 37°C in minimal essential medium (MEM, Corning Mediatech, Inc, Manassas, USA) containing 1.2 g/l NaHCO₃, and supplemented with 10% inactivated foetal calf serum (FCS, Corning Mediatech, Inc, Manassas, USA), 2 mM glutamine, nonessential amino acids, penicillin (100 IU/ml), and streptomycin (100 µg/ml).

The following Brisbane-like IAV strains, kindly provided by Dr. Isabella Donatelli (National Institute of Health, Rome) were used: A/Roma/ISS/02/08 H1N1 oseltamivir-sensitive virus and A/Parma/24/09 H1N1 oseltamivir-resistant virus. Viruses were propagated as previously described (8).

Cytotoxicity determination by MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay

Two-fold serial dilutions of a blend of essential oils (BEO) in culture medium were incubated at 37°C with MDCK cells

Key words: Essential oils, Vapour phase, Aerosol, Influenza virus, Respiratory infections, Viral inactivation

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grown in 96-well tissue culture microplates (Nalge Nunc Europe Ltd, Neerijse, Belgium). After 24, 48, and 72 h, cells were washed in phosphate buffered saline (PBS, pH 7.4) and 100 μ l of MTT solution (0.5 mg/ml in PBS) was added to each well. After 3 h at 37°C, dimethyl sulfoxide was added for 15 min at room temperature and absorbance at 570 nm was read using microplate reader (PerkinElmer Italia, Monza). BEO was kindly provided by G. Rapetti Mogol.

Virus cytopathic effect (CPE) titration by MTT assay

MDCK cells were infected with different dilutions of IAV for 1 h at 37°C. After viral adsorption, cells were incubated at 37°C for 24, 48 or 72 h in fresh medium containing 1 μ g/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin.

After each incubation period viral CPE was determined by MTT assay. Wells with CPE were scored as positive for virus growth and fifty-percent-tissue-culture-infective-dose (TCID₅₀) was determined by the method of Reed and Muench (9).

From dilutions to titres converting TCID₅₀ to plaque forming units (PFU): Poisson distribution

TCID₅₀ value indicates how much virus is needed to have CPE in 50% of the replicate wells. To know the actual amount of virus per ml, this value has to be converted into PFU per ml. This more precise estimate is obtained by applying the Poisson distribution. Where P(0) is the proportion of negative wells and m is the mean number of infectious units per volume (PFU/ml), $P(0) = e^{-m}$. For any titre expressed as a TCID₅₀, $P(0) = 0.5$. Thus $e^{-m} = 0.5$ and $m = -\ln 0.5$ which is ~ 0.7 , therefore, it is possible to predict the number of PFU/ml multiplying the TCID₅₀ titre (per ml) by 0.7.

In vitro antiviral activity of BEO towards IAV infection

MDCK cells, grown for 24 h, were incubated for 1 h at 37°C with different virus-BEO mixtures. After viral adsorption, cells were incubated at 37°C for 24 h in fresh medium, containing 1 μ g/ml TPCK-treated trypsin, in the presence or absence of BEO. CPE was measured by MTT assay and results were expressed as percentage of CPE inhibition by comparison with untreated infected control cultures. The inhibiting BEO titre has been reported as fifty-percent-effective-concentration (EC₅₀), the concentration at which 50% of cells were protected from the CPE.

Effect of oil vapours on viral infectivity

The antiviral activity of the BEO volatile phase was determined as a function of time using glass-airtight containers of known volume (165 ml). Two tubes of known volume (6 ml) were placed inside each container. One tube contained 1.5 ml of a known suspension of viral particles in MEM while the other tube contained 3 ml of a known dilution of BEO in H₂O or H₂O alone (control). The glass-airtight containers were incubated at 25°C. Every 24 h (up to 72 h), two containers were opened to withdraw one tube of viral suspension that had been in contact with the oil vapours and one tube of control viral suspension. The different viral samples were frozen at -80°C and the viral infectivity was titrated by the MTT test. Additional experiments were carried out by exposing the viral suspensions to more concentrated BEO vapours for shorter times (8 hours) and canola oil (CO) was used as a negative control (3).

Effect of oil nebulization on viral infectivity

For this type of study both an ultrasonic diffuser (i) and a nebulizing diffuser (ii) were used. The experiments were carried out in a closed environmental space of 76.5 m³ corresponding to 76,500 litres. Ultrasonic aroma humidifier diffusers use an oscillating plate that vibrate and, when the essential oils are added to the water, this vibration breaks them into small particles that are released into the air with the mist. Instead, cold and dry nebulization diffusers use neither heat nor water. They work by atomizing the essential oils into fine particles.

i) Experiments with ultrasonic aroma humidifier diffuser were carried out according to the following protocol:

Mock exposure: H₂O (200 ml total) aerosol was dispersed using an electric ultrasonic diffuser (Infinitoo.co.uk 500 ml Aromatherapy Oils Diffuser) on a small table about 65 cm away from different viral suspensions in culture medium (1.2 ml of each viral suspension was placed in a 3.5 cm diameter Petri dish). The thickness of the viral suspension layer was about 1.25 mm. The diffuser was activated for 2 h. The untreated control virus was subsequently collected and frozen at -80°C.

BEO exposure: the essential oil blend was diluted in H₂O (5%, 200 ml total) and the aerosol was dispersed using an electric ultrasonic diffuser (Infinitoo.co.uk 500 ml Aromatherapy Oils Diffuser) on a small table about 65 cm away from different viral suspensions as above described for mock exposure. CO at 5% in H₂O was used as an

internal negative control (3). After 2 h nebulization, the virus was collected and frozen at -80°C .

ii) Experiments with cold and dry nebulization diffuser were carried out according to the following protocol:

Mock exposure: 1.2 ml of different viral suspensions in culture medium were placed in 3.5 cm diameter Petri dishes. The thickness of the viral suspension layer was about 1.25 mm. The viral suspensions were exposed to the air in a closed environmental space of 76.5 m^3 . After 2 h, the viral suspensions were collected and frozen at -80°C .

BEO exposure: for dry and cold nebulization an essential oil diffuser (Pluzen, France) was used. The essential oil blend was put into the diffuser on a small table about 65 cm away from the viral suspensions (1.2 ml of each viral dilution was placed in a 3.5 cm diameter Petri dish). The thickness of the viral suspension layer was about 1.25 mm. The viral samples were exposed to the oil mixture for 2 hours. The exposure consisted in 4 spraying cycles divided as follows: 10 minutes of vaporization and 20 minute pause. CO was used as an internal negative control (3). After 2 h nebulization, the virus was collected and frozen at -80°C .

Transmission Electron Microscopy (TEM)

For TEM visualization, after 24 h exposure to BEO vapours, or 2 h exposure to BEO aerosol, unexposed (control) or exposed viral particles were absorbed onto carbon-formvar-coated, 400-mesh copper grids. Negative staining was carried out by utilizing 2% phosphotungstic acid (PTA) (pH 7.0) for 30 s, and samples were visualized by an EM 208 FEI transmission electron microscope at 80 kV.

Table I. *In vitro* antiviral activity of BEO towards A/RomaISS/02/08 H1N1 Influenza virus infection.

CC ₅₀	0.86%
EC ₅₀	0.081%
SI	10.62

CC₅₀ the concentration that reduced the cell viability by 50% when compared to untreated controls; EC₅₀ the concentration at which 50% of cells were protected from the virus induced killing; SI (selectivity index) the ratio between CC₅₀ and EC₅₀.

Statistical analysis

Data from three independent experiments were reported as the mean $\pm$ standard deviation (SD). A Student's t-test was used to determine whether there were significant differences between the controls and the BEO treatments (the reduction at each time exposure was compared to the reduction in the controls, if any). Differences were considered statistically significant if the resultant *P* value was ≤ 0.05 .

RESULTS

In vitro antiviral activity of BEO towards Influenza virus infection

In a first set of experiments, to assess the possible BEO cytotoxic effects, MDCK cells were incubated with different BEO concentrations for 24, 48 and 72 h and cell viability was measured by MTT assay. Results obtained showed that BEO induced a 100% cytotoxic effect at the concentration of 1.44% whereas it was not toxic at the concentration of 0.16% and the 50% cytotoxic concentration (CC₅₀) was 0.86%, regardless of the treatment time.

In order to evaluate the effect of BEO on viral replication, MDCK cells were infected for 1 h with A/RomaISS/02/08 H1N1 virus (m.o.i. 0.1) plus BEO and incubated with BEO for 24 h post-infection. Then viral cytopathic effect was evaluated by the MTT test. Results obtained are shown in Table I.

Effect of oil vapours on viral infectivity

A/RomaISS/02/08 H1N1 virus suspensions were exposed to BEO vapours for up to three days. Two glass tubes were placed inside different glass-airtight containers: one tube contained 1.5 ml of 1.05×10^5 A/RomaISS/02/08 H1N1 viral particles, corresponding to 1×10^5 TCID₅₀/ml, in MEM while the other tube contained 3 ml of 0.25% BEO in H₂O, 0.5% BEO in H₂O or H₂O alone (control). All containers were incubated at 25°C . Every 24 h (up to 72 h), three containers were opened to withdraw the tubes of viral suspensions that had been in contact with oil vapours and the tubes of control viral suspensions. CPE was assessed by the MTT test after infecting the cells with five-fold dilutions of the samples for 48 and 72 h. Under these experimental conditions a direct relationship was observed between BEO

concentration, exposure time and induced CPE in MDCK cells.

As shown in Figure 1, BEO exposure induced a marked inhibition of viral infectivity and, after 3 days of exposure, the virus was completely inactivated. Interestingly, BEO vapours inactivated viral suspensions and inhibited CPE in a dose dependent manner. Furthermore, it has been observed that the viral particles exposed to the vapours appeared to be weakened or, to some extent, damaged. In fact,

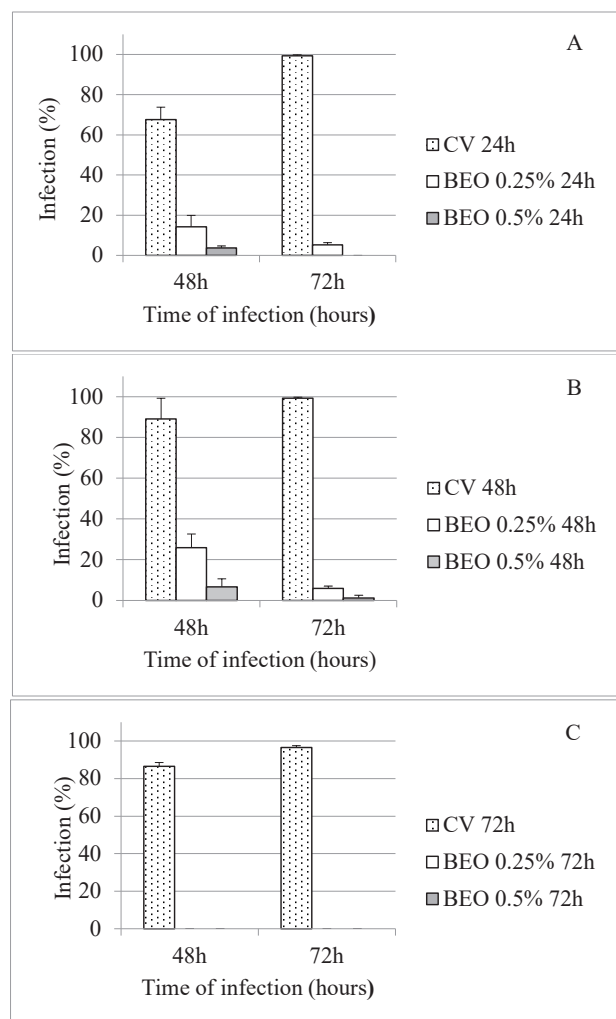


Fig 1. Determination of cytopathic effect (% of infected cells) induced by virus incubated for 24 (A), 48 (B), or 72 (C) h at 25°C in the absence (dotted bars) or presence of 0.25% or 0.5% BEO (white and grey bars, respectively). After the incubation period, viral samples were frozen at -80°C and, subsequently, the viral infectivity was titrated. MDCK cells were infected for 48 h or 72 h and CPE was determined by MTT assay. Each value is the means $\pm$ SD of three independent experiments, each run in triplicate.

compared to unexposed particles, these virions lost their ability to replicate over time, being less infectious after 72 h (Figure 1 C) compared to 48 h (Figure 1 B).

The emergence of drug resistant microorganisms is a serious threat to global health that requires new therapeutic strategies. Among them, essential oils are promising agents by fighting human pathogens in a “non-specific” way rather than targeting a single specific target (gene or protein).

Since the results we obtained suggested that BEO vapours could induce structural damage to the viral particles, we carried out further studies to verify whether BEO vapours were also active on a drug-resistant viral strain. For this purpose, the activity of the volatile phase of the oils was determined exposing suspensions of an oseltamivir-sensitive or an oseltamivir-resistant Influenza A/H1N1 viral strain to the BEO vapours for 8 h. In these experiments the negative controls consisted in CO and absence of oil. Two glass tubes were placed inside different glass-airtight containers: one tube contained 1.5 ml of oseltamivir-sensitive A/RomaISS/02/08 H1N1 (1×10^5 TCID₅₀/ml) or oseltamivir-resistant A/Parma/24/09 H1N1 (1×10^5 TCID₅₀/ml) in MEM, while the other tube contained 3 ml of 0.5% BEO in H₂O, 5% BEO in H₂O, or 0.5% CO in H₂O, 5% CO in H₂O, H₂O alone (as controls). After 8 h incubation at 25°C, all containers were opened to withdraw the tubes of viral suspensions that had been in contact with oil vapours and the tube of control viral suspensions.

CPE was assessed by the MTT test after infecting the cells with five-fold dilutions of the samples for 48 and 72 h (Fig. 2). As already observed, also in these experiments viral particles exposed to the BEO vapours appeared to be weakened or, to some extent, damaged. In fact, compared to unexposed or CO-exposed particles, these virions lost their ability to replicate over time, being less infectious after 72 h compared to 48 h. Regarding the sensitivity of the two different viral strains to BEO vapour, some differences were observed as the oseltamivir-resistant strain (Fig. 2B) was found to be slightly more affected by BEO than the oseltamivir-sensitive strain (Fig. 2A).

Results from these experiments were also used to calculate the titre of the treated and untreated virus suspensions expressed as TCID₅₀/ml (9). Both A/

RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1 viral strains exposed to BEO vapour were significantly less infectious, with about 2 log reduction in TCID₅₀/ml (1.69×10^4 vs 4.6×10^2 or 6.3×10^2 TCID₅₀/ml and 7.94×10^4 vs 4.6×10^2 or 6.76×10^2 TCID₅₀/ml, respectively). The negative control CO showed no activity on both viral strains at the concentrations tested. In fact, after exposure to 0.5% and 5% CO, the infectious titres of A/RomaISS/02/08 H1N1 strain were 1.71×10^4 and 1.69×10^4 , respectively, and the infectious titres of A/Parma/24/09 H1N1 were 8×10^4 and 7.95×10^4 , respectively.

The oseltamivir-resistant strain was found to be more susceptible to BEO vapour than the oseltamivir-

Table II. Percentage of infectivity inhibition after 8h exposure to CO or BEO vapours.

Concentration (%)		Infectivity inhibition (%)	
		A/RomaISS/02/08 H1N1	A/Parma/24/09 H1N1
CO	0.5	0	0
	5	0	0
BEO	0.5	97.3	99.4
	5	96.3	99.15

sensitive strain. The reduction in viral titre, expressed as % of infectivity inhibition, is shown in Table II. The reduction was statistically significant ($P \leq 0.05$) in comparison with the control (H₂O or CO vapour) at the same exposure time whereas no differences were observed between the two BEO concentrations (0.5 and 5%) used.

Viral inactivation assay: effect of BEO aerosol

The BEO aerosol activity was determined in experiments in which A/RomaISS/02/08 H1N1 or A/Parma/24/09 H1N1 viral suspension was exposed to BEO aerosol for two hours. The essential oil blend was diluted (5% in 200 ml H₂O) and the aerosol was dispersed using an electric ultrasonic diffuser (Infinitoo.co.uk 500 ml Aromatherapy Oils Diffuser). The negative control scenario consisted in CO or absence of oil: CO (5% in 200 ml H₂O) or H₂O (200 ml) aerosols were dispersed using the same electric ultrasonic diffuser. After 2 h, the differently exposed viral suspensions were frozen at -80°C and subsequently titrated. CPE was assessed by the MTT test after infecting the cells with five-fold dilutions of the samples for 48 and 72 h. Results obtained are reported in Fig. 3.

Results from these experiments were also used to calculate the titre of the treated and untreated virus suspensions expressed as TCID₅₀/ml (9). Both A/RomaISS/02/08 H1N1 (Fig. 3A) and A/Parma/24/09 H1N1 (Fig. 3B) viral strains exposed to BEO aerosol were less infectious compared to the H₂O exposed control, showing about 1-2 log reduction in TCID₅₀/ml (3.2×10^3 vs 1.77×10^2 TCID₅₀/ml and 1.25×10^4 vs 4.35×10^2 TCID₅₀/ml, respectively). No significant differences were noticed between the two viral strains even if the oseltamivir-resistant strain was slightly

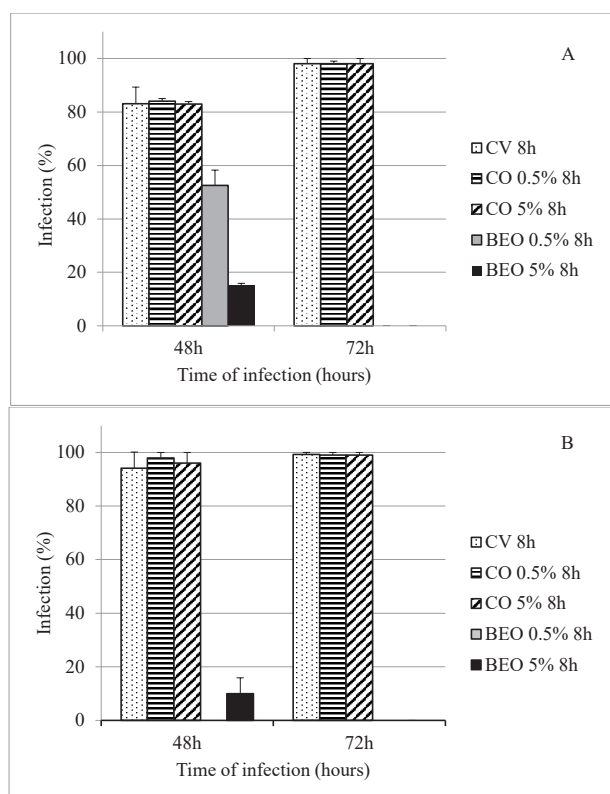


Fig. 2. Determination of cytopathic effect (% of infected cells) induced by A/RomaISS/02/08 H1N1 virus (A), or A/Parma/24/09 H1N1 virus (B), incubated for 8 h at 25°C in the absence (dotted bars), or presence of 0.5% CO (horizontal striped bars), 5% CO (diagonal striped bars), 0.5% BEO (grey bars), 5% BEO (black bars). After the incubation period, viral samples were frozen at -80°C and, subsequently, the viral infectivity was titrated. MDCK cells were infected for 48 h or 72 h and CPE was determined by MTT assay. Each value is the means $\pm$ SD of three independent experiments, each run in triplicate.

more susceptible than the oseltamivir-sensitive strain. As a matter of fact, the percentage of infectivity inhibition was 94.5 and 96.5 for A/RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1, respectively. The negative control CO showed no activity on both viral strains at the concentrations tested. In fact, after exposure to CO aerosol, the infectious titres of A/RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1 strains were 3.3×10^3 and 1.3×10^4 , respectively.

Both A/RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1 viruses were also exposed to BEO utilising a nebulizing diffuser that uses neither heat nor water diluent. The negative control scenario consisted in virus exposed to CO using the nebulizing diffuser or air-exposed virus. Infectious titres of treated and untreated viral suspensions, expressed as TCID₅₀/ml, were determined. Results obtained showed that both

viral strains exposed to BEO dry nebulization were less infectious compared to the air-exposed control, showing about 1 log reduction in TCID₅₀/ml (6.25×10^3 vs 6×10^2 TCID₅₀/ml and 2.5×10^4 vs 2.23×10^3 TCID₅₀/ml, respectively).

No significant differences were noticed between the two viral strains even if the oseltamivir-resistant strain was found to be slightly more susceptible than the oseltamivir-sensitive strain. As a matter of fact, the percentage of infectivity inhibition was 90.4 and 91.1 for A/RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1, respectively; also, in these experimental conditions the negative control CO showed no activity on both viral strains. In fact, after exposure to CO, the infectious titres of A/RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1 strains were 6.28×10^3 and 2.515×10^4 , respectively.

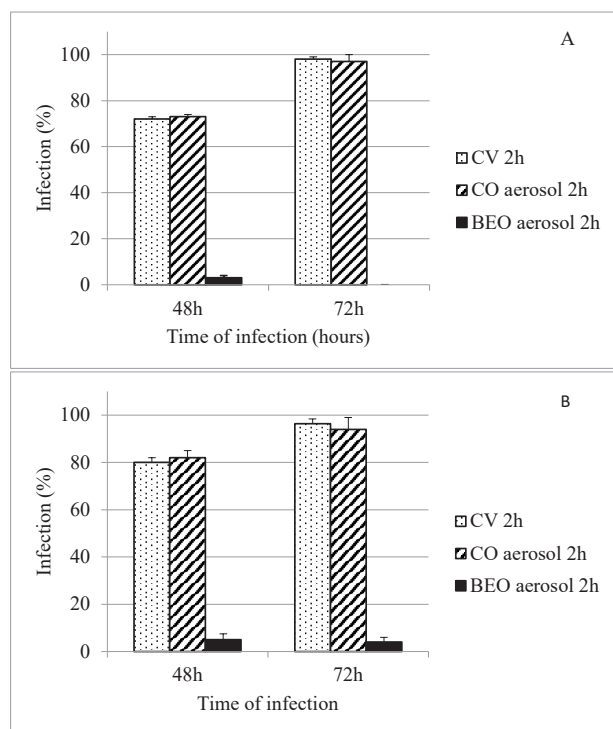


Fig. 3. Determination of cytopathic effect (% of infected cells) induced by A/RomaISS/02/08 H1N1 virus (A), or A/Parma/24/09 H1N1 virus (B), exposed for 2 h at 25°C in the absence (dotted bars) or presence of 5% CO (striped bars) or 5% BEO (black bars) aerosol. After the incubation period, viral samples were frozen at -80°C and, subsequently, the viral infectivity was titrated. MDCK cells were infected for 48 h or 72 h and CPE was determined by MTT assay. Each value is the means $\pm$ SD of three independent experiments, each run in triplicate.

Transmission Electron Microscopy (TEM)

Essential oils, being lipophilic, can intercalate in the lipid bilayer of the viral envelope, altering the fluidity of the membrane and possibly disrupting it. Since IAVs are enveloped by lipid membranes in which the viral surface proteins H and N are inserted, we hypothesized that the BEO vapour or aerosol most likely acted by non-specific intercalation in the lipid bilayer of the viral envelope thus disorganizing the protein projections. To verify this hypothesis, the morphological integrity of virus particles was checked by negative staining transmission electron microscopy. As shown in Fig. 4 and 5, structural changes of viral particles have been observed following exposure to both BEO vapour and BEO aerosol.

The TEM images of the control viral samples and the BEO vapour exposed viral particles are shown in Fig. 4.

Using TEM imaging it was possible to visualize the preservation of the morphology in the control viral particles and, at the same time, the structural changes in the viral particles exposed to BEO vapours. In fact, the control virions show well-ordered surface tips Fig. 4A, B inset A; conversely, in the samples exposed to the BEO vapour for 24 h the viral particles appeared partially decomposed in the membrane Fig. 4C, or they showed a disordered surface and the tips appeared discontinuous Fig. 4C, D, inset D.

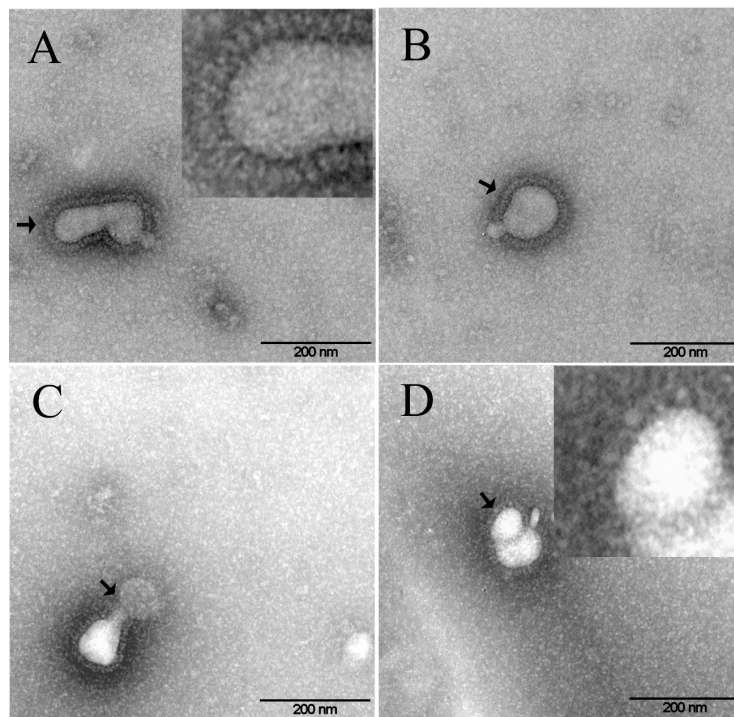


Fig. 4. Electron micrographs of A/RomaISS/02/08 H1N1 virus after negative staining with 2% PTA. (A, B) Two representative virus particles showing well-ordered surface tips (see arrows and inset A). (C, D) Virions exposed to BEO vapour for 24 hours showing a partially decomposed membrane (C, arrow) or a disordered surface and disorganised tips (see arrow and inset D). Magnification bars are built into the micrographs.

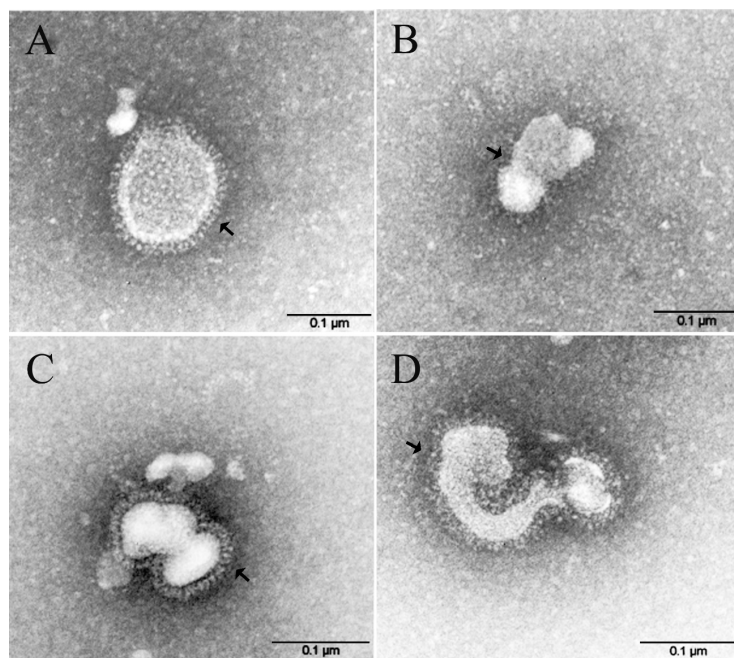


Fig. 5. Electron micrographs of Influenza virus particles after negative staining with 2% PTA. A representative control A/RomaISS/02/08 H1N1 virus particle showing well-organized surface spikes (arrow, A). A representative A/RomaISS/02/08 H1N1 viral particle exposed to BEO aerosol for 2 hours showing a disordered surface and hardly distinguishable spikes (arrow, B). A representative control A/Parma/24/09 H1N1 virus particle showing well-ordered surface tips (arrow, C). A representative A/Parma/24/09 H1N1 viral particle exposed to BEO aerosol for 2 hours showing an altered surface and the disruption of spike organization (arrow, D). Magnification bars are built into the micrographs.

The TEM images of the control viral samples and the BEO aerosol exposed viral particles are shown in Fig. 5A-D for A/RomaISS/02/08 H1N1 and A/Parma/24/09 H1N1, respectively.

These results strongly suggest that BEO, in both vapour and aerosol form, inhibits viral infectivity through structural damage of the viral particles.

DISCUSSION

Respiratory viruses cause serious problems for the whole population, due to frequent acute and chronic infections, occasional epidemics and, above all, very serious pandemics. The scarcity of available therapeutic agents is due both to the different replication strategies of these viruses, and consequently to the lack of a shared molecular target, and to the emergence of drug-resistant mutant strains. EOs, having broad-spectrum mechanisms of action and non-specific antiviral properties, are excellent candidates for the treatment and prevention of these infections and for their environmental control (10, 11). Indeed, the inactivation of viruses present in the environment represents the most efficient way to minimize the risk of direct contact with airborne infectious particles as well as infectious particles present on surfaces or re-aerosolized from them.

In the present study, the activity of a blend of different EOs, in vapour phase or in aerosol, has been demonstrated *in vitro* against an oseltamivir-sensitive and an oseltamivir-resistant IAV. TEM analysis suggests that, possibly due to their lipophilic nature, EOs can penetrate IAV envelope, easily leading to membrane rupture and inactivation of the virus regardless of the structure of its spike projections. This result confirms the use of EOs as antivirals effective against enveloped viruses and also against viruses resistant to licensed drugs, suggesting that BEO might also be effective on other enveloped respiratory viruses such as SARS-CoV-2. It is expected that a better understanding of the antiviral properties of EOs, will attract more and more attention from the nutraceutical and pharmaceutical industry. Indeed, the need for environmentally friendly antiviral drugs is increasing and EOs could play a key role in the development of new preventive and therapeutic approaches. In this

view it would be extremely important to verify BEO's activity against COVID-19.

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Cis-atracurium besylate during induction of general anaesthesia in patients with pneumoconiosis and hepatic insufficiency during simultaneous large-volume lavage of both lungs

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To the Editor,

Cis-atracurium besylate is a new type of non-depolarising mid-time muscle relaxant, the effect of which is similar to that of atracurium, but its potency is much stronger (1). In addition, the metabolism of cis-atracurium besylate does not depend on liver and kidney function and is mainly eliminated by the Hofmann product (2), the recovery index is not affected by the total amount administered or the method of administration, there is no accumulation in the body after repeated use (3), no difference to age or gender and its metabolites do not have the pharmacological effect (4) of a neuro-muscle block. Another difference between cis-atracurium besylate and atracurium is that there is no release of histamine, so the application of larger doses will not cause a drop in blood pressure (5). For patients with liver and kidney dysfunction, there is no need to adjust the dose because its pharmacodynamics and pharmacokinetic characteristics react similarly to patients with normal liver and kidney function (6-7).

Pneumoconiosis is a systematic disease (8). Respiratory failure can occur later in the patient, often accompanied by right-sided heart failure. In severe cases, myocardial damage leads to peripheral circulatory failure and blood pressure drop, which significantly affect liver and kidney function (9). Therefore, some patients with pneumoconiosis are often accompanied by liver and kidney dysfunction, poor physical condition (10), reduced tolerance to anaesthesia and surgery, and higher risk of surgical anaesthesia (11). This study hopes to optimise surgical medication plans and improve the safety of surgical anaesthesia through pharmacodynamic analysis of cis-atracurium besylate as an inducing muscle relaxant in patients with pneumoconiosis.

MATERIALS AND METHODS

General information

The study cohort comprised 62 patients admitted to the Qinhuangdao Beidaihe Rehabilitation Hospital from

Key words: simultaneous massive lavage of both lungs; pneumoconiosis; hepatic insufficiency; benzene sulfonic acid cis atracurium; muscle relaxant effect

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February 2011 to November 2019 who underwent the simultaneous large-volume lavage of both lungs for the treatment of second-stage pneumoconiosis with liver dysfunction. The patients were all male, aged 27 to 58 years, with an average age of (46.5 ± 6.6) years, American Society of Anesthesiologists (ASA) classification of II, body mass index (BMI) $<30 \text{ kg/m}^2$, and an average of $(22.4 \pm 2.7) \text{ kg/m}^2$. They were divided into two groups based on intraoperative muscle relaxation: a cis-atracurium besylate group (observation group) and a conventional muscle relaxant control group, with 31 patients in each group. Exclusion criteria for the patients were: heart and cerebrovascular diseases; kidney and other organ diseases; metabolic disorders; neuromuscular conduction diseases or other factors affecting neuromuscular conduction; drug allergy. There was no statistically significant difference between the two groups of patients in general information such as age, gender, ASA classification, and body mass index ($P > 0.05$), they were comparable. This study was approved by the ethics committee of the Qinhuangdao Beidaihe Rehabilitation Hospital, and all patients signed an informed consent form.

Anaesthesia

Both groups of patients were injected intramuscularly with 10 mg diazepam and 0.3 mg scopolamine 30 min before anaesthesia. After entering the operating room, they were kept in a calm state for 10 min. Anaesthesia was induced with remifentanyl $3\text{--}6 \text{ ng/ml}$ and propofol 3 ug/ml for a plasma target-controlled infusion, calibrated automatically with a muscle relaxation tester, and, after the induced plasma concentration reached the target, the cis-atracurium besylate 0.15 mg/kg was injected intravenously. When the muscle relaxation conduction block was at maximum, tracheal intubation was performed. During the operation, Respiratory rate (RR) was controlled at 10–12 times/min, Tidal volume (VT) was $8\text{--}10 \text{ kg}$, PETCO₂ was $35\text{--}45 \text{ mmHg}$, the temperature of the operating room was about 25°C , and the temperature of the detection part was not lower than 34°C .

The two groups that were given the muscle relaxant were tested using TOF-Watch SX accelerometers. The instrument was turned on after the patient lost consciousness, and it was automatically calibrated after which it displayed a single 0.1 Hz stimulation reading; the patient was given a muscle relaxant injection after 1

minute of stability. When the muscle relaxant conduction block was at maximum, it was converted to Train of Four (TOF) monitoring: a record onset time, T1 10% recovery time, T1 25% recovery time, T1 75% recovery time, recovery index, maximum effect duration, TOF ratio to T1 70% recovery time.

When T1 reached the lowest value, tracheal intubation and the evaluation of the conditions of tracheal intubation were carried out. It was considered: excellent, when the patient's glottis was open, the mandible was loose, and without a coughing response; good, when the vocal cord was mildly active, the jaw was loose, and without coughing response; and bad, when vocal cord movement was obvious, the lower jaw was tight, intubation was difficult, and a coughing response. If the patient's limbs were active, the vocal cords were retracted, and the mandible was tight, intubation would not be possible.

Statistical analysis

All data was analysed by SPSS19.0 statistical software. The count data is expressed in the form of the number of cases (percentage), and the statistical analysis used the *chi*-squared test; the measurement data was expressed by means $\pm$ standard deviation ($\bar{x} \pm s$) and statistical analysis used the *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Comparison of intubation conditions and maximum T1 block degree between the two groups

As shown in Table I, in both groups, there were no patients with poor intubation conditions, or who were unable to be intubated. The number of cases with excellent intubation conditions were 20 and 19 in the observation group and control group respectively, and the number of cases with good intubation conditions were 11 and 12 respectively, there was no significant difference in intubation conditions between the two groups ($P > 0.05$). The maximum degree of T1 block in the observation group and control group were 98.9 ± 5.0 and 98.2 ± 5.0 , respectively; the difference was not statistically significant ($P > 0.05$).

Comparison of muscle relaxation indicators between the two groups

The onset time of muscle relaxation in the

observation group (3.8 ± 0.8 min) was significantly longer than that of the control group (2.5 ± 0.6 min), and the difference was statistically significant ($P < 0.05$). There was no significant difference in T110% recovery time, T125% recovery time, T175% recovery time, recovery index, duration of maximum effect, and TOF ratio to 70% recovery time between the two groups ($P > 0.05$) (Table II).

DISCUSSION

In this study, we found that there were no significant differences in T110%, T125%, T175% recovery time, recovery index, duration of maximum effect, and TOF ratio to 70% recovery time between the two groups of patients ($P > 0.05$), indicating that the application of cis-atracurium besylate to the simultaneous large-volume lavage of both lungs to treat patients with pneumoconiosis and liver dysfunction during induction of general anaesthesia has no significant effect on anaesthesia. In addition, due to the slow onset time of cis-atracurium besylate,

the onset time of muscle relaxation in the observation group was significantly longer than that in the control group. Studies used pre-injection of small doses of cis-atracurium to shorten the onset time, and found that $50 \mu\text{g/kg}$ pre-injection can significantly shorten the onset time of the muscle relaxant, which can be used to optimize the induction of general anaesthesia for patients with pneumoconiosis and liver dysfunction.

It was found that cis-atracurium besylate has a certain clinical application value to the anaesthesia during surgery. With using cis-atracurium besylate in the treatment of pneumoconiosis with hepatic insufficiency during simultaneous large-volume lavage of both lungs during induction of general anaesthesia, the muscle relaxation onset time was significantly increased, and there were no significant differences in intubation conditions, maximum degree of block, duration of maximum muscle relaxation, and recovery time. In summary, cis-atracurium besylate has many advantages in the induction of patients with pneumoconiosis and

Table I. Comparison of intubation conditions and T1 maximum block degree between the two groups

Group	Case	Intubation conditions				T1 maximum degree of block
		Excellent	Fine	Bad	Unable to intubate	
Observation group	31	20	11	0	0	98.9 ± 5.0
Control group	31	19	12	0	0	98.2 ± 5.0
χ^2/t		-0.070				0.387
P		-0.790				0.700

Table II. Comparison of muscle relaxant indexes between the two groups

Group	Case	Effective time (min)	T110% Recovery time (min)	T125% Recovery time (min)	T175% Recovery time (min)	Recovery index	Maximum effect duration	TOF compared to 70% recovery time
Observation group	31	3.8 ± 0.8	41.7 ± 7.7	48.8 ± 9.3	58.4 ± 10.4	12.4 ± 2.8	34.6 ± 8.7	66.9 ± 8.4
Control group	31	2.5 ± 0.6	40.9 ± 7.5	46.7 ± 9.1	57.7 ± 9.7	13.1 ± 2.8	33.6 ± 8.3	64.8 ± 8.2
t	-	8.127	0.404	0.764	0.273	0.934	0.456	1.025
P	-	0.000	0.689	0.445	0.784	0.355	0.652	0.308

hepatic insufficiency, which increases the safety of anaesthesia and surgery and is therefore worthy of an extensive clinical promotion.

When cis-atracurium besylate was used to treat patients with pneumoconiosis and liver dysfunction during the simultaneous large-volume lavage of both lungs, the onset time of a single injection of muscle relaxant was significantly increased during induction of general anaesthesia. There were no significant differences in intubation conditions, maximum degree of block, duration of maximum muscle relaxation or recovery time.

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The value of scopolamine hydrobromide in simultaneous high volume lavage for both lungs in occupational pulmonary diseases

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To the Editor,

Scopolamine hydrobromide is an anticholinergic drug with strong peripheral effects, which can block the M-choline receptor (1, 2). Compared with atropine, scopolamine hydrobromide has a stronger peripheral effect, but a short maintenance time, and has a sedative, sleeping and excitatory respiratory effect on the cerebral cortex. In addition, scopolamine hydrobromide has been shown to inhibit glandular secretion, relieve capillary spasm, improve microcirculation, dilate bronchi, and relieve smooth muscle spasm (3, 4).

Pneumoconiosis is a systemic disease in which dust enters the lungs and causes inflammatory reactions, leading to diffuse fibrosis of the lung tissue. Large-volume whole lung lavage (WLL) removes dust deposited in the patient's lungs, secretions and sputum remaining in the respiratory tract through lavage. It can effectively relieve the clinical symptoms of patients, but there are problems such as hypoxia and increased airway pressure during the lavage process. Mechanical ventilation during the operation can also cause lung barotrauma (5).

At present, the clinical effect of scopolamine on WLL surgery in patients with pneumoconiosis

complicated with severe hypoxemia is still unclear. This study summarized the therapeutic effect of patients with pneumoconiosis who received scopolamine treatment with large-volume lavage of the lungs and hypoxemia in Qinhuangdao Beidaihe Rehabilitation Hospital. The purpose is to study the value of scopolamine in simultaneous large-volume lavage of both lungs in patients with pneumoconiosis.

MATERIALS AND METHODS

In this study, patients with pneumoconiosis complicated with hypoxemia during WLL surgery in Qinhuangdao Beidaihe Rehabilitation Hospital from January 2013 to April 2020 were treated with large volume lavage of both lungs at the same time. A total of 278 cases, including 189 males, accounting for 67.99%, and 89 females, accounting for 32.01%, with an average age of 53.4±10.6 years were enrolled in the study. The incidence of intraoperative hypoxemia, the improvement of oxygenation index after treatment with scopolamine hydrobromide, the time of withdrawal and extubation and clinical adverse reactions were observed. This study was in line with the Helsinki Declaration of the World Medical Association and was approved by the Ethics Committee of

Key words: pneumoconiosis; simultaneous large volume lavage of both lungs; scopolamine hydrobromide; hypoxemia; oxygenation index

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Qinhuangdao Beidaihe Rehabilitation Hospital (Beidaihe sanatorium for coal miners in China). All patients signed informed consent forms.

The inclusion criteria for the patients were: clearly diagnosed as pneumoconiosis; over 18 years of age; signed informed consent. Exclusion criteria were: patients who refused surgical treatment; glaucoma and severe heart disease; organic pyloric stenosis or paralytic intestinal infarction; patients who had taken antidepressant, psychotic and Parkinson's disease medication for a long time prior to the operation; incomplete case data.

Before surgery, endotracheal intubation and ventilator-assisted ventilation were performed under sedation and general anesthesia. Large-volume lavage operation for both lungs was carried out using lavage fluid 37°C normal saline; the lavage fluid suspension height was 60 cm from the axillary midline, the volume of lavage fluid each time was 1,000 mL, and the negative pressure after the lavage fluid was perfused After being aspirated until the lavage fluid was dripping out, mechanical positive pressure ventilation was performed every three times and the lavage fluid was sucked back under negative pressure until the lavage fluid was clear.

After the operation, the initial therapeutic dose of scopolamine hydrobromide 0.1-0.3mg/kg/h was administered by continuous intravenous pump. Blood pressure, heart rate, partial pressure of carbon dioxide and partial pressure of oxygen were monitored during medication. When the oxygenation index reached 300 mmHg, the pumping amount of scopolamine hydrobromide was halved. When the oxygenation index reached about 400 mmHg, the ventilator parameters were gradually reduced, and then the dosage of scopolamine hydrobromide was halved every half hour. When the support of the ventilator was not high and the blood pressure stable cut-off oxygenation index was significantly improved, the patients were extubated, and scopolamine hydrobromide was gradually stopped after extubation.

Observation index

During the operation, the patient's blood pressure, respiratory rate, oxygen concentration, peripheral blood oxygen saturation and partial pressure of oxygen were measured continuously. After the operation, ventilator-assisted ventilation was continued to monitor the partial pressure of carbon dioxide, finger oxygen saturation,

SPO₂%, partial pressure of arterial blood oxygen, partial pressure of arterial blood carbon dioxide, and a chest X-ray examination was carried out.

Statistical analysis

In this study, the data were processed by SPSS20.0 statistical software, and the measurement data were expressed by mean $\pm$ standard deviation. The counting data is expressed as a percentage (%). The difference was statistically significant ($P < 0.05$).

RESULTS

The results of this study showed that the lung wet rate of 278 patients significantly decreased and disappeared after treatment with scopolamine hydrobromide, and the pulmonary edema was significantly alleviated as shown by chest X-ray. In addition, the oxygenation index of the patients increased rapidly after treatment with scopolamine hydrobromide. The time required for oxygenation index to reach 300mmhg was about 4-6 hours, and the time for extubation was about 6-7 hours (Table I).

Adverse reactions

The results showed the only adverse reactions found were that 278 patients had varying degrees of abdominal distension, but this did not affect their breathing or oxygenation.

DISCUSSION

The conventional treatment of severe hypoxemia in pneumoconiosis surgery mainly includes prolonging the time of ventilator assistance, oral sildenafil hydrochloride, intravenous injection of prostaglandin E1, inhaled nitric oxide or ventavir (6-10). At present, there are few clinical studies on simultaneous large-volume lavage of both lungs due to severe hypoxia in WLL surgery. The results of this study show that the time required for the oxygenation index to reach 300mmHg after treatment is 4-6 hours, and the extubation time is 6-8 hours. The treatment of scopolamine hydrobromide in patients with severe hypoxemia during WLL surgery has a significant clinical efficacy. Scopolamine hydrobromide has

Table 1. Results of hyperoxia in 278 pneumoconiosis patients treated with scopolamine hydrobromide

Case	operation	Oxygenation index before administration mmHg Time	The oxygenation index reached 150mmHg Time	The oxygenation index reached 300mmHg Time	Extubation Time
278	WLL	130±2.25	2 ±0.5	4 ±0.5	6±0.5

an obvious sedative effect on a large number of patients and can relieve vascular smooth muscle spasm, expand pulmonary blood vessels, reduce pulmonary vascular resistance, and relieve pulmonary hypertension (11). It can also promote the redistribution of pulmonary blood flow and increase the ventilation blood flow ratio ($V_{max} Q$) to correct hypoxemia. In addition, it can inhibit gland secretion and reduce alveolar exudation, thereby reducing pulmonary interstitial and alveolar edema, and alleviating acute respiratory distress syndrome (ARDS). Finally, scopolamine hydrobromide can block the accumulation of inflammatory cells and the release of inflammatory factors, breaking the vicious circle of systemic inflammatory response syndrome (SIRS).

This is a retrospective study without a control group, and we look forward to conducting multi-center and large-sample randomized controlled trials in the future.

In conclusion, scopolamine hydrobromide could promptly increase the oxygenation index of patients with pneumoconiosis after WLL surgery, as well as improving the symptoms of emphysema and wet rales. Therefore its application is worthy of further research for future clinical promotion.

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Effect of miR-21a-5p on proliferation of mice airway smooth muscle cells by regulating superoxide dismutase 1

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To the Editor,

Studies have shown that oxidative stress can stimulate bronchial airway wall remodeling (1, 2). In bronchial asthma, oxidative stress products, such as a large number of reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2), are increased. Antioxidant products (such as hydrogen sulfide, reduced glutathione) are reduced, which results in increased airway inflammation and pathological proliferation of airway smooth muscle cells (ASMCs). As a result, the airway undergoes remodeling (1). As second messengers, ROS participate in regulating pro-oxidation molecular pathways, and can promote the proliferation of various cells, playing an important role in the pathological proliferation of ASMCs (2).

miRNA plays an important role in the occurrence and development of bronchial asthma by regulating oxidative stress (3). The overexpression of miR-21a-5p promotes the fibroblast activity of spinal cord fibroblasts after mechanical injury, enhances the proliferation of spinal cord fibroblasts, and attenuates their apoptosis (4). MicroRNA-21a-5p overexpression affects the mediator of extracellular matrix formation in uterine leiomyoma (5). Therefore, miR-21a-5p may also be involved in the occurrence and development of airway remodeling

in bronchial asthma. SOD1 is an antioxidant enzyme and is a suspicious regulatory target of miR-21a-5p through TargetScan. Therefore, this experiment studies whether miR-21a-5p can reduce airway oxidative stress and improve platelet derived growth factor (PDGF)-induced airway remodeling by regulating SOD1.

MATERIALS AND METHODS

ASMC cell culture and grouping

ASMCs (KeyGEN Bio TECH, Nanjing, China) were cultured in 1640 culture medium containing 10% fetal bovine serum (American Gibco Company). When the cell growth confluence reached 70%, they were put into a petri dish at a density of 1×10^4 /ml per well plate, cultured in a 5% CO_2 incubator at $37^\circ C$, digested with 0.25% trypsin (American Thermo Scientific Company), and cultured for 7 days at 1:2. The cells were inoculated in a 25 cm^2 culture flask at a density of 1×10^5 /ml. When the fusion rate reached about 60%, the cells were synchronized for 24 h and then grouped. Different concentrations of PDGF (10, 15, 20, and 30 ng/mL) were added and cultured for 48 h to observe the effect of PDGF on ASMC activity and cell proliferation. The cells were divided into normal control group, PDGF-induced group, PDGF+miR-21a-5p mimic group, and PDGF+miR-21a-5p inhibition group

Key words: miR-21a-5p; platelet derived growth factor (PDGF); primary airway smooth muscle cells (ASMCs); SOD1; proliferation

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to observe the effect of miR-21a-5p on PDGF-induced proliferation of ASMCs. The cells transfected with miR-21a-5p mimic or miR-21a-5p inhibitor were added with PDGF (20 ng/mL) and cultured for 48 h.

Activity of ASMCs was measured by CCK-8

The proliferation of ASMC cells in different treatment groups was measured by CCK-8 kit. ASMC cells were seeded in 96-well plates, and serum-free medium was changed when the cell growth confluence reached 70%. After different conditions were treated, 10 μ L of CCK-8 solution (Beijing Thinkfar Technology Co., Ltd.) was added to each well and incubated in the dark for 3 h, and the absorbance value (A450 nm) was measured at a wavelength of 450 nm using an RT-6100 enzyme analyzer (Shanghai Jumu Medical Instrument Co., Ltd. Shanghai, China).

Transfection of ASMC cells

Appropriate amounts of ASMC cells in logarithmic growth phase were inoculated into a 6-well plate. When the ASMC cells grew to 60–70% overnight, miR-21a-5p mimic, miR-21a-5p inhibitor, miR-21a-5p mimic control, and miR-21a-5p inhibitor control were transfected into ASMC cells according to the operation instructions of Lipofectamine 2000 transfection reagent. The transfection concentration was 50 nmol/L, the fresh medium was replaced after 6 h of transfection, and the cells were further cultured for 48 h. The cells were collected for RT-PCR and Western blot detection.

Determination of ASMC cell proliferation rate

The liquid from each well of the 96-well plate was absorbed and discarded, and 270 μ L DMEM medium was added. Then, 30 μ L 5 g/L MTT working solution was added, mixed well, and incubated at 37°C for 4 h. The culture medium in the well was discarded, and 270 μ L of dimethyl sulfoxide was added into each well and shaken for 10 min. The OD value of each well was measured by a microplate reader at 490 nm wavelength. Taking the OD value of the normal group as control, the cell proliferation rate (%) of ASMC cells in each well was calculated = OD value of each well/OD value of normal group $\times 100$ [%]. Cell proliferation inhibition rate = [1-(experimental group OD value-blank well OD value)/(control group OD value-blank well OD value)] $\times 100$ [%]. The process was repeated thrice, and the average value was taken.

Prediction of miR-21a-5p target gene

miR-base (<http://www.mirbase.org/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdc-berlin.de/>) software predicted that the miR-21a-5p target gene may be SOD1. Wild-type (wt)-SOD1 and mutant (mut)-SOD1 luciferase gene vectors were constructed by conventional methods, and these two plasmids were mixed with miR-21a-5p mimics, miR-21a-5p inhibitor, miR-21a-5p mimic control, and miR-21a-5p inhibitor control. They were co-transfected into ASMC cells and counted as Wt+miR-21a-5p mimics, Mut+miR-21a-5p inhibitor, Wt+negative control, and Mut+negative control. After 48 h in an incubator at 37°C and 5% CO₂, the activity of double luciferase was detected by double luciferase reporter gene kit (Nanjing Boen Biotechnology Co., Ltd. Nanjing, China). At the same time, the level of SOD1 in ASMC cells overexpressed miR-21a-5p was detected by RT-PCR and Western blot.

Real-time quantitative PCR

The ASMC cells of the treatment group were collected. Cell RNA with the total RNA extraction kit were extracted and reverse transcribed into cDNA according to the PrimeScript™ RT reagent kit instructions (Shanghai Sangon Biotech Co., Ltd.). The expression of SOD1 mRNA and miR-21a-5p in cells was detected by real-time quantitative PCR. The reaction system was as follows: SYBR Premix Ex Taq™II 10 μ L, PCR primers 2 μ L, DNA template 2 μ L, nuclease-free water (DEPC) 6 μ L, and total reaction system 20 μ L. Each sample was inserted into three parallel multiple holes. The reaction system was completed on a PCR instrument. The TaqMan MicroRNA Assays kit (Applied Biosystems, Foster City, CA, USA) was used to detect the level of miR-21a-5p. The miR-21a-5p expression level was determined by $2^{-\Delta\Delta Ct}$. Reaction conditions were as follows: predenaturation at 95°C for 30 s, reaction at 95°C for 5 s, annealing at 60°C for 30 s, and 40 cycles. The primer series was as follows: miR-21a-5p: F-5'-GCGGGCTAATACTGCCTGG-3', R-5'-ATCCAGTCCAGGGTCCGAAA-3'; U6: F-5'-CTCGCTTCGGCAGCAC-3', R-5'-AACGCTTCACGAATTTGCGT-3'; SOD1mRNA: F-5'-TTATGATGGGCAGGCAAAGAA-3', R-5'-ACTGCCTTTAGAGAACCAGCC-3'; β -actin: F-5'-ATGGTGAAGGTCCGGTGTG-3', R-5'-AACTTGCCGTGGGTAGAG-3'. U6 was used as the internal reference of miR-21a-5p, and β -actin was used as the internal reference of SOD1 gene expression. After the reaction, the cycle

threshold (Ct) of each gene was calculated and compared with the internal reference gene U6 to determine the expression level.

Western blot analysis of cell protein expression level

Cell lysate, protease inhibitor, and phosphatase inhibitor were mixed at a ratio of 8:1:1, respectively, to prepare cell lysate buffer. Next, 150 μ L of RIPA cell lysis buffer was added to each well and placed in an ice bath for 30 min to extract the total protein of each group of cells. Centrifugation was carried out at 4°C for 15 min, and the protein purity was detected by BCA protein quantitative kit. Then, 50 μ g of protein extract was separated by 10% SDS-PAGE electrophoresis, and 5% skim milk was added to incubate and block for 1.5 h. The corresponding SOD1, connective tissue growth factor (CTGF), and TGF- β 1 primary antibodies (1:1,000, British Abcam Company) were added and left overnight at 4°C, and HRP-labeled goat anti-rabbit IgG (volume dilution ratio 1:3,000, Wuhan Guge Biological Co., Ltd.) was added after washing the membrane with TBST three times the next day and reacting at room temperature for 1 h. Following, the film was washed with TBST thrice, each time for 10 min, and ECL chemiluminescence solution was added to develop color. Image Lab software was used to analyze the gray value of the band, and β -actin was used as an internal reference.

Detection of intracellular ROS level

DCFH-DA was diluted in serum-free DMEM/F12 medium with a final concentration of 10 μ mol/L. After cell culture, the culture medium was discarded; 100 μ L of diluted DCFH-DA was added to each well and incubated in a CO₂ incubator at 37°C for 20 min. The cells were washed gently with serum-free DMEM/F12 culture medium thrice to remove DCFH-DA that did not enter the cells, taking care not to suck out the cells. Flow cytometry was used for detection. An excitation wavelength of 488 nm and an emission wavelength of 525 nm were used to detect the intensity of fluorescence before and after the drug action.

Detection of SOD, GSH activity, and MDA content

The cells of each group were homogenized and lysed on ice, and the supernatant was collected by centrifugation. The activities of superoxide dismutase (SOD) and GSH were detected by SOD and GSH activity detection kit (Nanjing Jiancheng Biological Engineering Research Institute Co., Ltd. Nanjing, China), and the content of

MDA was determined by MDA content detection kit (Nanjing Jiancheng Biological Engineering Research Institute Co., Ltd. Nanjing, China).

Statistical analysis

GraphPad Prism 6 was used for statistical analysis. The experimental data were expressed by mean $\pm$ standard deviation. One-way ANOVA was used to compare between groups, and LSD was used to compare the significance between groups. $P < 0.05$ means that the difference is statistically significant.

RESULTS

Effect of PDGF on oxidative stress, proliferation and activity in ASMC cells

To observe the effect of PDGF on the proliferation and activity of ASMC cells, different concentrations of PDGF were added to ASMC cells for 48 h, and the activities of SOD1, GSH, ROS, and MDA were determined according to the instructions of the kit. The proliferation and activity of ASMC cells were determined by MTT and CCK-8, respectively. Tables I and II show that PDGF increased the activities of ROS and MDA, and decreased proliferation activity

Table I. Effects of different doses of PDGF on proliferation and activity of ASMC cells

Group	Cell activity	Cell proliferation inhibition rate
Control group	0.16 $\pm$ 0.051	0.62 $\pm$ 0.28
PDGF (10 ng/mL)	0.23 $\pm$ 0.074	0.53 $\pm$ 0.17
PDGF (15 ng/mL)	0.34 $\pm$ 0.094*	0.41 $\pm$ 0.14*
PDGF (20 ng/mL)	0.46 $\pm$ 0.17*	0.34 $\pm$ 0.13*
PDGF (30 ng/mL)	0.58 $\pm$ 0.28*	0.27 $\pm$ 0.12*

*Different concentrations of PDGF were added to ASMC cells and cultured for 48 hours. The proliferation and activity of ASMC cells were determined by MTT and CCK-8, respectively. The experimental data are expressed as mean $\pm$ standard deviation. * $P < 0.05$ compared with the control group.*

and the activities of SOD1 and GSH of ASMC cells in a dose-dependent manner.

Effect of miR-21a-5p inhibitor on PDGF-induced activity and proliferation of ASMC cells

To observe the effect of miR-21a-5p on PDGF-induced proliferation of ASMCs, the cells were divided into normal control group, PDGF-induced group, PDGF+miR-21a-5p mimic group, and PDGF+miR-21a-5p inhibition group. PDGF (20 ng/ml) was added to+miR-21a-5p mimic or miR-

21a-5p inhibitor transfected ASMC cells and cultured for 48 hours. The proliferation and activity of ASMC cells were determined by MTT and CCK-8, respectively. Fig. 1 suggests that miR-21a-5p inhibitor reduced the PDGF-induced ASMC cell activity and increased the proliferation inhibition rate of ASMC cells (Fig. 1 A, B).

Effect of miR-21a-5p inhibitor on oxidative stress induced by PDGF in ASMC cells

To observe the effect of miR-21a-5p on PDGF-

Table II. Effects of different doses of PDGF on oxidative stress of ASMC cells

Group	SOD1 (U/mg protein)	GSH (U/mg protein)	ROS DCF (fluorescence intensity)	MDA (nmol/mg protein)
Control group	158.74±67.83	139.82±51.37	54.95±21.83	2.81±1.04
PDGF (10 ng/mL)	144.98±53.12	127.63±47.82	61.18±23.59	3.23±1.36
PDGF (15 ng/mL)	132.83±56.36*	114.56±41.74*	78.64±29.76*	4.18±1.52*
PDGF (20 ng/mL)	98.94±43.49*	99.29±38.58*	89.16±34.84*	6.37±2.94*
PDGF (30 ng/mL)	74.43±22.65*	90.48±35.64*	104.67±41.48*	7.82±3.17*

Different concentrations of PDGF were added to ASMC cells for 48 hours, and the activities of SOD1, GSH, ROS, and MDA were determined according to the instructions of the kit. The experimental data were expressed as mean ± standard deviation. * $P < 0.05$ compared with the control group.

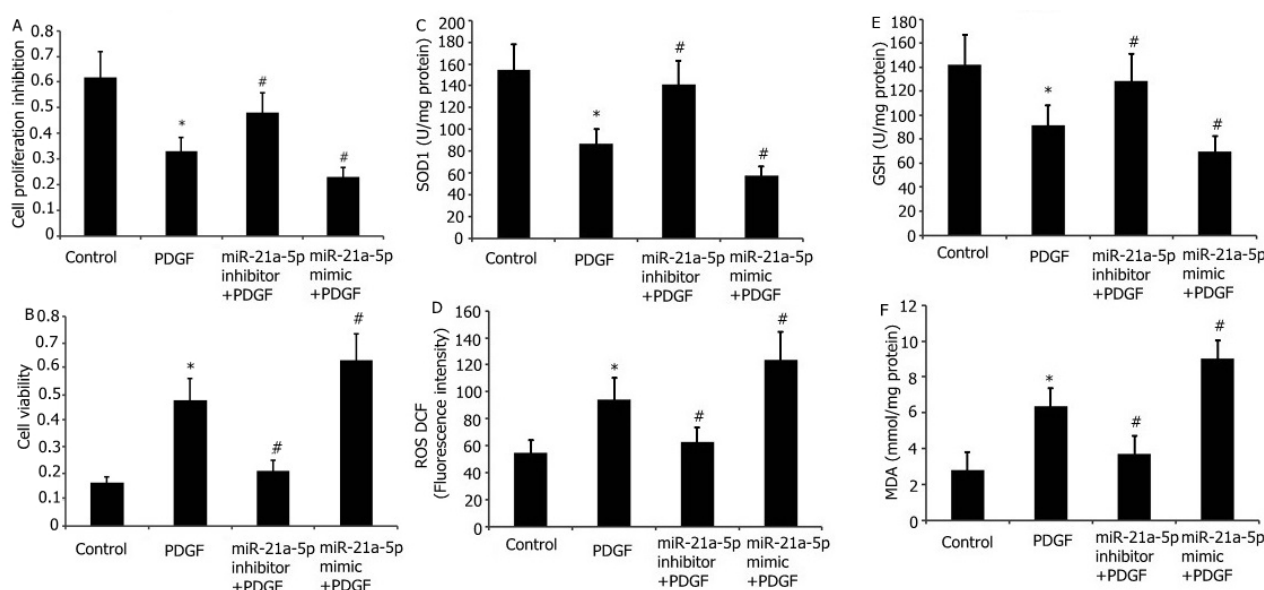


Fig. 1. Effect of miR-21a-5p inhibitor on PDGF-induced oxidative stress activity and proliferation in ASMC cells. PDGF (20 ng/ml) was added to+miR-21a-5p mimic or miR-21a-5p inhibitor transfected ASMC cells and cultured for 48 hours. **A, B)** The proliferation and activity of ASMC cells were determined by MTT and CCK-8, respectively. **C-F)** The activities of SOD1, GSH, ROS, and MDA were determined according to the instructions of the kit. The experimental data were expressed as mean ± standard deviation. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with model group (PDGF group).

induced proliferation of ASMCs, the cells were divided into normal control group, PDGF-induced group, PDGF+miR-21a-5p mimic group, and PDGF+miR-21a-5p inhibitor group. PDGF (20 ng/mL) was added to +miR-21a-5p mimic or miR-21a-5p inhibitor transfected ASMC cells and cultured for

48 h. The activities of SOD1, GSH, ROS, and MDA were determined according to the kit instructions. Fig. 1 shows that miR-21a-5p inhibitor reduced PDGF-induced ROS and MDA activities, and increased PDGF-induced SOD1 and GSH activities; miR-21a-5p mimic increased the activity of ROS and

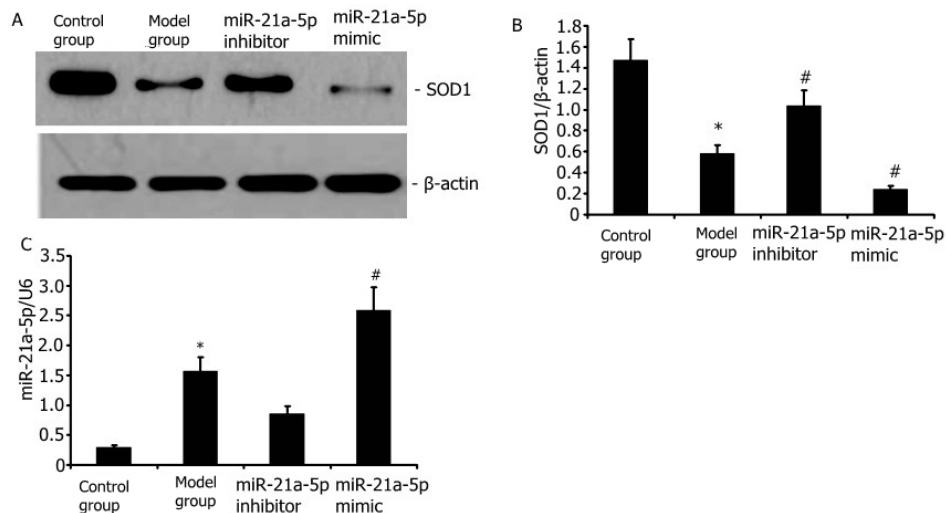


Fig. 2. Effects of miR-21a-5p inhibitor on miR-21a-5p and SOD1. PDGF (20 ng/ml) was added to +miR-21a-5p mimic or miR-21a-5p inhibitor transfected ASMC cells and cultured for 48 hours. The expressions of miR-21a-5p and SOD1 were determined by RT-PCR and Western blot, respectively. The experimental data were expressed as mean $\pm$ standard deviation. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the model group (PDGF group). **A, B)** Western blot to detect the expression of SOD1 protein in ASMC cells and quantitative analysis **C)** Quantitative analysis of miR-21a-5p expression in ASMCs by RT-PCR.

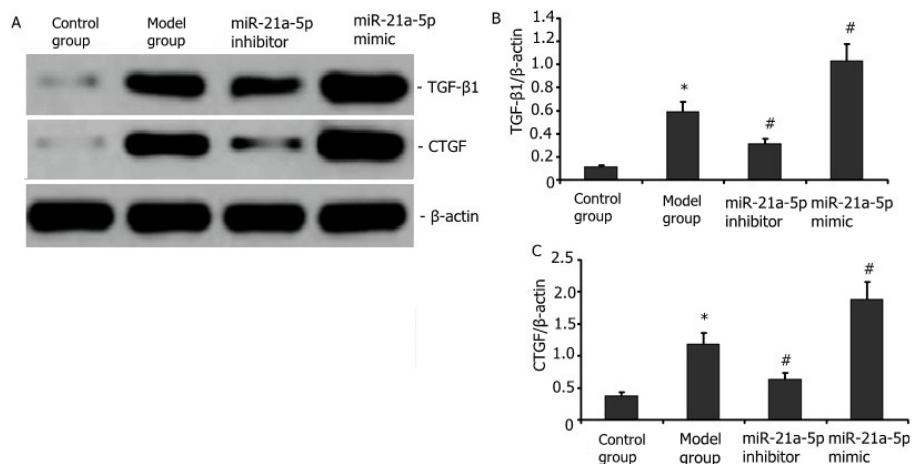


Fig. 3. Effects of miR-21a-5p inhibitor on CTGF and TGF-β1. PDGF (20 ng/ml) was added to +miR-21a-5p mimic or miR-21a-5p inhibitor transfected ASMC cells and cultured for 48 hours. The expression of CTGF and TGF-β1 was determined by Western blot, and the experimental data were expressed as mean $\pm$ standard deviation. * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the model group (PDGF group). **A–C)** Western blot to detect the expression of CTGF and TGF-β1 protein in ASMC cells and quantitative analysis.

MDA induced by PDGF but decreased the activity of SOD1 and GSH induced by PDGF (Fig. 1 C-F).

Effects of miR-21a-5p inhibitor on miR-21a-5p and SOD1

To observe the effect of miR-21a-5p on PDGF-induced proliferation of ASMCs, the cells were divided into normal control group, PDGF-induced group, PDGF+miR-21a-5p mimic group, and PDGF+miR-21a-5p inhibition group. PDGF (20 ng/mL) was added to+miR-21a-5p mimic or miR-21a-5p inhibitor transfected ASMC cells and cultured for 48 h. The expressions of miR-21a-5p and SOD1 were determined by RT-PCR and Western blot. Fig. 2 shows that miR-21a-5p inhibitor reduced the expression of miR-21a-5p induced by PDGF and increased the expression of SOD1 induced by PDGF; miR-21a-5p mimic increased the expression of miR-21a-5p induced by PDGF and decreased the activity of SOD1 and GSH induced by PDGF. Moreover, miR-21a-5p inhibitor and miR-21a-5p mimic were successfully transfected into ASMCs (Fig. 2).

Effects of miR-21a-5p inhibitor on CTGF and TGF-1

To observe the effect of miR-21a-5p on PDGF-induced CTGF and TGF- β 1, the cells were divided into normal control group, PDGF-induced group, PDGF+miR-21a-5p mimic group, and PDGF+miR-21a-5p inhibition group. PDGF (20 ng/mL) was added to+miR-21a-5p mimic or miR-21a-5p inhibitor transfected ASMC cells and cultured for 48 h. The expressions of CTGF and TGF-1 were determined by Western blot. miR-21a-5p inhibitor decreased PDGF-induced CTGF and TGF- β 1 expression; miR-21a-5p mimic increased the expression of CTGF and TGF- β 1 induced by PDGF (Fig. 3).

SOD1 is the regulatory target of miR-21a-5p

Studies have shown that SOD1 is an important regulatory protein of ASMC cell proliferation induced by PDGF. miR-base (<http://www.mirbase.org/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdc-berlin.de/>) databases were used to predict possible regulatory targets of miR-21a-5p and determine how miR-21a-5p affects PDGF-induced

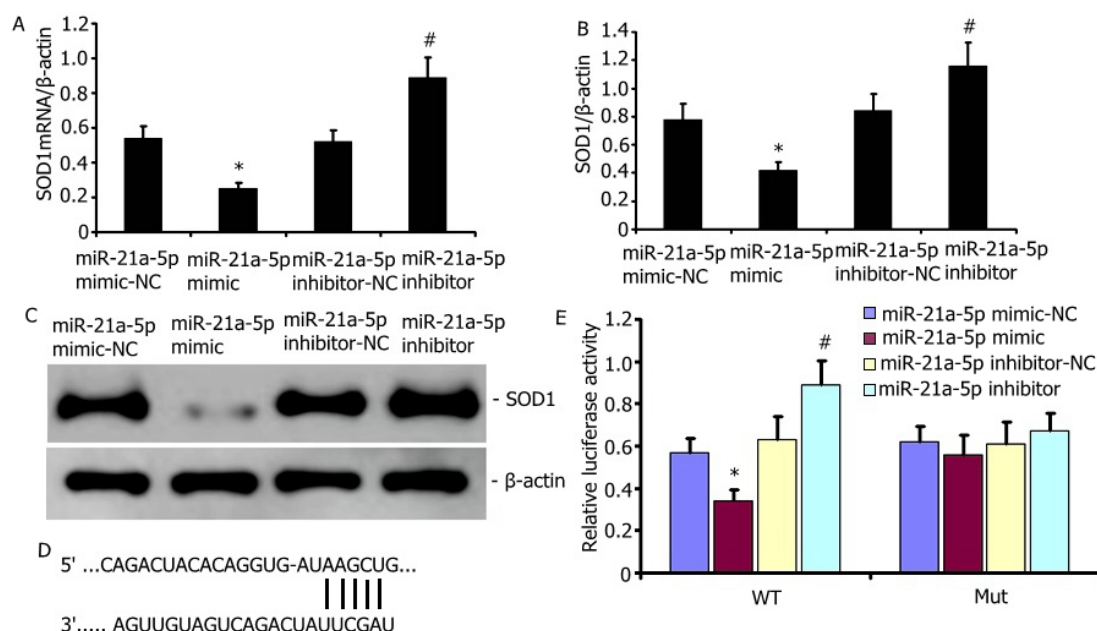


Fig. 4. SOD1 is a regulatory target for miR-21a-5p. **A)** Effects of miR-21a-5p mimic and inhibitor on SOD1 mRNA expression. **B, C)** Effect of miR-21a-5p mimic and inhibitor on SOD1 expression. **D)** Prediction by TargetScan that SOD1 is that regulatory target of miR-21a-5p. **E)** Test miR-21a-5p bound to (wt)-SOD1 but not to (mut)-SOD1 in dual luciferase reporter gene analysis.

ASMC cell proliferation. Analysis showed that after miR-21a-5p overexpression, SOD1 mRNA and protein levels were significantly reduced, as exhibited in Fig. 4 A, B, D, E. (Wt)-SOD1 and (mut)-SOD1 were constructed for TEST in double luciferase reporter gene analysis to verify the interaction between miR-21a-5p and SOD1 further (Fig. 4 C, F). As assumed, miR-21a-5p bound to (wt)-SOD1 but not to (mut)-GSK3 β (Fig. 4 D, E).

DISCUSSION

Recent studies have found that a variety of miRNAs play an important role in the proliferation, hypertrophy and contraction of ASMCs (6). The downregulation of miR-133a expression in asthmatic airway smooth muscle can mediate calcium ion-induced excessive contraction, abnormal proliferation, and apoptosis disorder of airway smooth muscle by upregulating RhoA expression (7). The combination of miR-26a and 3'-UTR of glycogen synthase kinase-3 β (GSK-3 β) reduces the level of endogenous GSK-3 protein and stimulates the hyperplasia and hypertrophy of airway smooth muscle. Knockout of miR-26a reduces the expression of endogenous GSK-3 protein and then inhibits the hyperplasia and hypertrophy of airway smooth muscle (8). Increasing the expression of miR-10a inhibits the proliferation and hypertrophy of ASMCs. Inhibiting the expression of miR-10a significantly reduces the proliferation of ASMCs (9). Studies have found that TGF β 1 can promote the expression of miR-21 in ASMCs by regulating TGF β /Smad signaling pathway and stimulate airway remodeling (10). miR-21 can downregulate Smad7, regulate TGF β /Smad signaling pathway, and stimulate airway remodeling (10). Increasing the expression of miR-21a-5p inhibits the expression and activity of SOD1, increases the activity of ROS, stimulates the activity and proliferation of ASMC cells, and promotes airway remodeling. By contrast, inhibiting the expression of miR-21a-5p increases the expression and activity of SOD1, reduces the activity of ROS, inhibits the activity and proliferation of ASMC cells, and reduces airway remodeling.

Furthermore, the double luciferase activity was

detected by the double luciferase reporter gene kit to confirm that SOD1 is the regulatory target of miR-21a-5p, suggesting that miR-21a-5p regulates the activity and expression of SOD1, regulates the activity and proliferation of ASMC cells induced by PDGF, and regulates airway remodeling.

In conclusion, this study reveals that miR-21a-5p regulates the proliferation and activity of ASMC cells induced by PDGF by regulating oxidative stress and further regulates airway remodeling induced by bronchial asthma.

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Effect of placental polypeptides on immune function in patients with acute exacerbation of chronic obstructive pulmonary disease

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Acute exacerbation of chronic obstructive pulmonary disease (AECOPD) is one of the most common respiratory diseases in clinical practice. It is mainly caused by repeated infections of pathogenic bacteria in the respiratory tract, causing repeated aggravation of the disease, prolonged non-healing, and serious damage to the immune function of the body (1). The decline in immune function makes the patient more susceptible to infection and aggravates the condition. Therefore, improving the patient's immune function may help reduce the incidence of patient infection and delay the occurrence and development of chronic obstructive pulmonary disease. Placental peptides are small-molecule peptides with biological activity, and they are extracted from healthy human placenta (2). Based on modern pharmacological studies, placental peptides can enhance cellular immune response, activate T cell expression, enhance patient immunity, and resist tumors (3). They can also promote the proliferation of bone marrow hematopoietic cells and effectively alleviate leukopenia caused by chemotherapy (4, 5). Placental polypeptides also have anti-cell mutation, anti-inflammatory, and antioxidant effects (5). Therefore, in the present study, we used placental peptides to treat patients with AECOPD by fighting infection, reducing phlegm, and relieving asthma. This study aims to improve the immune status

of patients with AECOPD and improve the ventilation function and prognosis of such patients.

MATERIALS AND METHODS

Drug

Placental peptides were purchased from Guiyang Qianfeng Biological Products Co., Ltd (Guiyang, China) (National Medicine Standard: H20046260).

Patients

This study included 100 patients with acute exacerbation of chronic obstructive pulmonary disease who were admitted to the Department of Emergency Medicine and Department of Respiratory and Critical Care Medicine, the First Affiliated Hospital of Kunming Medical University, and Shunde Lunjiao Hospital of Foshan City from February 2018 to February 2020. A total of 60 patients were randomly grouped into the placental peptide group (treatment group: conventional treatment + placental peptide), while 40 patients were placed in the placebo group (conventional treatment + placebo). The treatment group included 42 males and 18 females aged 51–88 years with an average age of 68.23 ± 18.04 years, course of disease of 1–20 years, and average course of disease of 12.32 ± 2.97 years. The placebo group included 28 males and 12 females age 53–91 years old with an average age of 70.54 ± 14.76 years, course

Key words: *Placental peptides; acute exacerbation of chronic obstruction; T cell subsets; Lung function*

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of disease of 1–25 years, and average course of disease of 12.85 ± 3.14 years. No significant difference was observed in the general information between the two groups of patients ($P > 0.05$).

Inclusion criteria and exclusion criteria

Inclusion criteria:

First, the inclusion criteria are in accordance with (6) AECOPD diagnosis and treatment criteria. Second, no immunomodulators and glucocorticoids have been used in the past three months. Finally, the patients can tolerate placental peptides.

Exclusion criteria:

First, the condition is combined with malignant tumors, severe dysfunction of vital organs such as liver and kidney, diabetes, hyperthyroidism, and other diseases. Second, the condition is combined with bronchiectasis, active tuberculosis, and bronchial asthma. Third, the patients have immune system diseases. Finally, the patients have occupational dust and chemical substance exposure history.

Randomization

We used an independent interactive web response system and adopt the method of competition to enter the group to complete the randomization. The ratio of the number of participants in the treatment and placebo groups is approximately 1:1.

When a sub-center accepts an eligible participant, the investigators will enter the baseline information (e.g., subject's screening number, abbreviated name, age, gender, and medical history) in the central randomization system. Then, the central randomization system will assign an identification code (SSID, 6 digits) and a random number (4 digits), which are unique for each participant.

The drug administrators will log into the central randomization system after the random number is generated and assign the research medication to the nurses according to the group information based on the random number.

Power calculation and statistical analyses

To estimate the sample size, we assumed the improvement of the rate of low immunity to be 70% in the placebo group and 80% in the treatment group based on previous studies (6). The study needed 81 participants to achieve 80% power with a two-sided α level of 0.05. Assuming a 10% dropout rate, 15 patients are required per group.

Treatment method

Placebo group: After diagnosis, on the basis of conventional treatment (e.g., continuous low-flow oxygen inhalation, anti-infection, fluid rehydration, antispasmodic, anti-asthmatic, anti-cough, and expectorant treatments), the patients were administered with placebo (250 ml of 0.9% sodium chloride injection) by intravenous drip once a day for 14 days.

Treatment group: After diagnosis, based on conventional treatment (continuous low-flow oxygen inhalation, anti-infection, fluid rehydration, antispasmodic, anti-asthmatic, anti-cough, and expectorant treatments), the patients were administered with injection of 8 ml of placental polypeptide, which is dissolved in 250 ml of 0.9% sodium chloride injection, by intravenous drip once a day for 14 days.

Observation index

In the morning, 5 mL of fasting venous blood was extracted, the levels of peripheral blood T lymphocyte subsets (CD3+, CD4+, CD8+, NK cells, and CD4+/CD8+) were measured by flow cytometry, and IL-2, IL-4, and IFN- γ were measured by ELISA. The pulmonary function was measured using the German Jaeger advanced combined pulmonary function tester (Type MasterScreen-Body/Diff). The parameters were measured at the time of admission and at 2 weeks after treatment. A walking distance of 6 min, takes the average of two distances, was measured at the time of admission and at 1 and 3 months after treatment.

Quality of Life Score

The COPD assessment test scale was used to measure the patient's quality of life score at the time of admission and at 1 and 3 months after treatment, with a score of 0–40 points. It measures sleep, energy, confidence in going out, housework, the feeling of going up a flight of stairs or climbing, chest tightness, and sputum expectoration. Cough includes eight items, and the higher the score, the worse the quality of life.

Efficacy evaluation

Significantly effective: the condition is relatively stable, the pulmonary rales are significantly reduced, the activity tolerance is improved, the number of attacks is reduced, the severity is reduced, and the general condition is improved;

Effective: Symptoms are alleviated, and lung signs have no obvious improvement;

Invalid: No improvement or aggravation of clinical symptoms and signs, and no obvious improvement in the number and degree of attacks.

Statistical analysis

We used the SPSS 10.0 software for data analysis. Measurement data were expressed as mean $\pm$ standard deviation and analyzed using *t* test. Counting data is expressed as rate (%) and analyzed using χ^2 test. $P < 0.05$ is considered statistically significant.

RESULTS

Changes in the effective rate of treatment between the two groups of patients

After 14 days of treatment with placental peptide, the total effective rate of the treatment group was 91.7%, which was significantly higher than that of the placebo group (71.5%, $P < 0.05$). The significantly effective of the treatment group was 66.7%, which was significantly higher than that of the placebo group (40.0%, $P < 0.05$), as shown in Table I.

Effects of placental polypeptides on T lymphocyte subsets in patients with AECOPD

No significant difference was observed in the CD4⁺, CD8⁺, and NK cell levels and CD4⁺/CD8⁺ between the treatment and placebo group before treatment with placental polypeptides ($P > 0.05$, Fig. 1A–B). After 14 days of treatment with placental polypeptides, the CD4⁺ level of the placental peptide group was significantly higher, the CD8⁺ level was significantly lower, the NK cell level was significantly higher, and the ratio of CD4⁺/CD8⁺ was significantly higher than that of the placebo group ($P < 0.05$), as shown in Fig. 1C–D.

Effects of placental polypeptides on Th1, Th2, and HLA-DR14 in patients with AECOPD

No significant differences in Th1, Th2, and HLA-DR14 were observed between the treatment and placebo group before treatment with placental polypeptides ($P > 0.05$, Fig. 2A–C). After 14 days of treatment with placental polypeptides, the Th1, Th2, and HLA-DR14 levels of the treatment group were significantly higher than those of the placebo group ($P < 0.05$, Fig. 2D–F).

Changes in lung function indicators before and after treatment in the two groups of patients

No significant difference was observed in the lung function FEV1, FEV1/FVC, and PEF between the two groups of patients before treatment with placental polypeptides ($P > 0.05$, Fig. 2G–K). After 14 days of treatment with placental polypeptides, the FEV1, FEV1/FVC, and PEF of the treatment group were significantly higher than those in the placebo group ($P < 0.05$, Fig. 2L–P).

Effects of placental polypeptides on quality of life and 6-min walking distance in patients with COPD

No significant difference was observed in the quality of life and 6-min walking distance between the two groups of patients before treatment with placental polypeptides ($P > 0.05$). After 1 and 3 months of treatment with placental polypeptides for the treatment group of patients, placental polypeptides significantly improved the quality of life and 6-min walking distance ($P < 0.05$, Table II).

Comparison of adverse reactions between the two groups

After treatment with placental polypeptides, three

Table I. Changes in the effective rate of treatment between the two groups of patients.

Group	<i>n</i>	Significantly effective	Effective	Invalid	Overall effective
Treatment group	60	40 (66.7)	15 (25.0)	5 (8.3)	55 (91.7)*
Placebo group	40	16 (40.0)	13 (32.5)	11 (27.5)	29 (72.5)

After 14 days of treatment for the two groups of patients, the effective rate of the two groups of patients was determined. The data were expressed in percentages (* $P < 0.05$, compared with the placebo group).

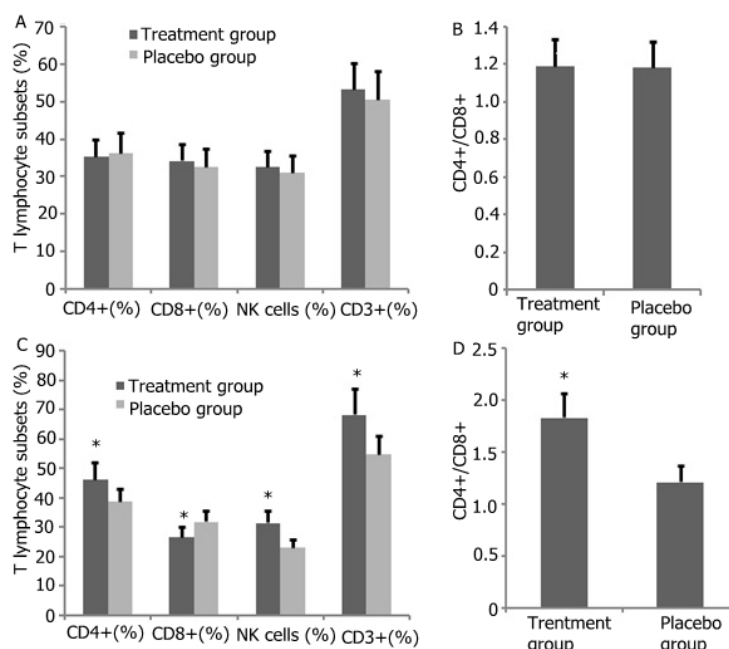


Fig. 1. Changes of T lymphocyte subsets between the two groups of patients before and after treatment. **A-B**): Before treatment, the CD4+, CD8+, NK cells, and CD4+/CD8+ levels of peripheral venous blood of the treatment group and the placebo group of the two groups of patients were measured by flow cytometry, and the data were expressed as mean±standard deviation ($P>0.05$, compared with the placebo group). **C-D**): After 14 days of treatment for the two groups of patients, the CD4+, CD8+, NK cells, and CD4+/CD8+ levels in the peripheral venous blood of the treatment and placebo group were measured by flow cytometry. The data were expressed as mean±standard deviation (* $P<0.05$, compared with the placebo group).

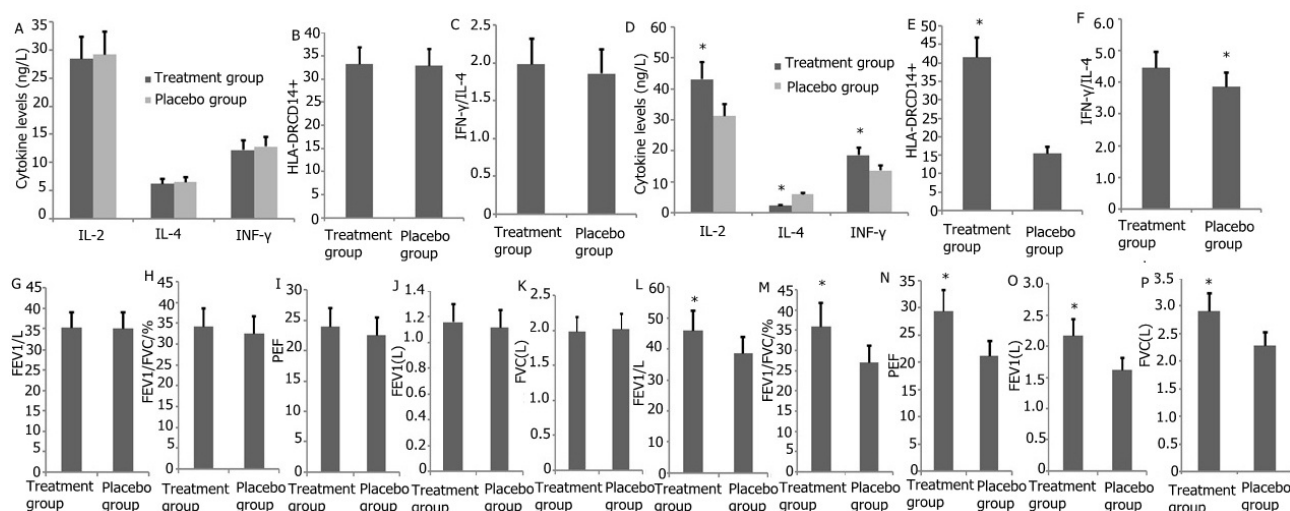


Fig. 2. Changes in the Th1, Th2, and HLA-DR14 levels and lung function indices between the two groups of patients before and after treatment. **A-C**): Before treatment, the levels of Th1 (IL-2; INF-γ), Th2 (IL-4), and IFN-γ/IL-4 in the treatment and the placebo group were measured by ELISA, and HLA-DRCD14+ was measured by flow cytometry. The data are expressed as mean±standard deviation ($P>0.05$, compared with the placebo group); **D-F**): After 14 days of treatment for the two groups of patients, the Th1 (IL-2; INF-γ), Th2 (IL-4), and IFN-γ/IL-4 levels in the peripheral venous blood of the treatment group and the placebo group were measured by ELISA. The HLA-DRCD14+ level was measured by flow cytometry. The data are expressed as mean±standard deviation (* $P<0.05$, compared with the placebo group); **G-K**): Before treatment, the lung function FEV1, FEV1/FVC, and PEF indices of the two groups of patients were measured, and the data are expressed as mean±standard deviation ($P>0.05$, compared with the placebo group); **L-P**): After 14 days of treatment for the two groups of patients, lung function FEV1, FEV1/FVC, and PEF were measured, and the data were expressed as mean±standard deviation (* $P<0.05$, compared with the placebo group).

Table II. *Changes in Quality of life and 6 min walking distance of the two groups of patients before and after treatment.*

Group	6 min walking distance (m)			Quality of life (points)		
	Before treatment	1 month after treatment	3 months after treatment	Before treatment	1 month after treatment	3 months after treatment
Placebo group	175.4±15.3	225.5±23.6	376.8±28.0	23.9±7.7	18.2±6.0	17.1±6.5
Treatment group	177.6±14.7	286.6±24.7*	450.9±34.8*	24.2±7.9	12.4±2.4*	10.1±1.5*

*After 1 and 3 months of treatment for the two groups of patients, Quality of life and 6 min walking distance were measured, and the data were expressed as mean±standard deviation (*P<0.05, after 1 and 3 months of treatment compared with the placebo group).*

patients in the treatment group developed nausea and vomiting, and one case of digestive tract ulcer was recorded. In the placebo group, two patients experienced nausea and vomiting, and two patients developed peptic ulcer. No significant difference was observed in adverse reactions ($P > 0.05$). After the proton pump inhibitor was added, the symptoms were gradually relieved.

DISCUSSION

Cellular immunity plays an important role in the process of anti-infection immunity in AECOPD (7, 8). In the present study, we found that the levels of CD3+, CD4+, CD8+, NK cells, and D4+/CD8+ in chronic obstructive pulmonary disease and CD14+ monocytes HLA-DR+ are reduced, indicating that the body immunity is reduced. After treatment with placental peptides, the levels of CD3+, CD4+, CD8+, NK cells, and D4+/CD8+ in chronic obstructive pulmonary disease significantly increased, CD14+ monocyte HLA-DR+ level increased, and the body's immunity is improved. The immune regulatory function of helper T cells is realized by secreting related cytokines, which mainly include Th1 and Th2. Th1 mainly mediates cellular immunity and secretes IFN- γ (9). Th2 cells secrete cytokines represented by IL-4, promote humoral immune response, inhibit the

maturation of Th1 cells, and produce corresponding cytokines (10). IL-2 is a major cytokine secreted by Th1 cells. It acts as a key cytokine in the immune regulatory network. By binding to IL-2 receptors on the surface of target cells, it promotes the proliferation and differentiation of target cells and participates in regulating the body's immune response (11, 12). In the present study, after treatment with placental peptides, the secretion of IFN- γ and IL-2 increased, whereas the secretion of IL-4 decreased. Moreover, the Th1/Th2 ratio increased, and the body's immunity was enhanced.

In conclusion, the placental peptides used in the treatment of patients with AECOPD can increase the levels of T cell subsets CD4+, CD3+, NK cells, and CD4+/CD8+ levels in patients. It improves the immune function of the immune body, treatment effect, and lung function of the patient.

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DECLARATION

Ethics approval and consent to participate.

This study was approved by the First Affiliated Hospital of Kunming Medical University and Shunde Lunjiao Hospital of Foshan City Research Ethics Boards.

Written informed consent for participation in the study was obtained from patients and parents.

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Long noncoding RNA NORAD/miR-942-5p/Prdx6 axis modulates osteogenic differentiation of bone marrow mesenchymal stem cells in postmenopausal osteoporosis

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Due to the estrogen deficiency mediated imbalance of bone formation and bone resorption, postmenopausal osteoporosis (PMOP) has become a prevalent bone disease all over the world (1). It has been reported that there are about 200 million cases with osteoporosis (OP) worldwide, and the incidence of OP was 40% to 50% in women more than 60 years old in China (2). Patients with OP often suffer from reduced bone mass and bone strength as well as the microstructure degradation of bone tissues (3). Therefore, identifying novel biomarkers in treating PMOP may provide an insight into preventing and treating PMOP.

Peroxiredoxins (Prdx) are antioxidant enzymes with six subtypes (Prdx 1–6), among which, Prdx1, Prdx2, and Prdx5 have been reported to play a vital role in osteoporosis (4). In addition, Prdx6 has been identified to be implicated in the development of a variety of diseases. For example, Prdx6 regulates the development of angiotensin II-mediated endotheliocyte via modulating vascular inflammation, oxidative stress, and endothelial dysfunction (5). Prdx6 modulates the pathogenesis of Alzheimer's disease through mediating the astrocytes in A β proteostasis (6). Nevertheless, whether Prdx6 participated in the progression of PMOP remains elusive.

In this study, we identified that lncRNA NORAD/miR-942-5p/Prdx6 axis regulated osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMMSCs) in PMOP. The findings of the work might offer an insight into preventing and treating PMOP.

MATERIALS AND METHODS

Samples

Forty cases of PMOP patients and forty healthy controls at the age of 60–80 years were enrolled in our study. 3 ml venous peripheral blood was collected and put in anticoagulation tubes containing EDTA. The experiments were approved by the Ethical Committee of Changzhou Hospital of Traditional Chinese Medicine. The written consent was obtained from all participants.

Cell culture and transfection

The hBMMSCs (Cat. No. SCSP-405) and HEK-293T cells (Cat. No. GNHu43) were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum, penicillin (100 μ g/ml) and streptomycin (100 μ g/ml) (Thermo Fisher Scientific, USA) with 5% CO₂ (37°C). The

Key words: NORAD, miR-942-5p, Prdx6, osteogenic differentiation, osteoporosis

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miR-942-5p mimics, NC mimics, small hairpin shRNA (shRNA) targeting NORAD (sh-NORAD), shRNA targeting Prdx6 (sh-Prdx6), sh-NC, overexpression vector for Prdx6 (oe-Prdx6) and oe-NC vectors were purchased from the RiboBio (Guangzhou, China). Transfections were performed via Lipofectamine 2000 (Thermo Fisher Scientific).

Osteogenic differentiation

The osteogenic medium was supplemented with osteogenic inducers: ascorbic acid (50 µg/mL), dexamethasone (Dex, 10 mol/L) and Glycerolphosphate (Gly, 10 mmol/L) (Sigma, USA). The hBMMSCs were cultured in a 12-well plate and when reaching 80% confluence, the medium was changed into an osteogenic medium. Every 3 days, the osteogenic medium was renewed. The induced hBMMSCs were harvested and analyzed after 14 days.

Alkaline phosphatase (ALP) activity

ALP activity was detected after cell culture, using the Alkaline Phosphatase Assay Kit (Abcam). Briefly, after collected and rinsed with PBS, hBMMSCs were incubated and lysed with RIPA buffer (Beyotime, Shanghai, China). The cell lysate was centrifuged with 10,000 rpm/min for 5 min, and the supernatant was used to measure ALP activity.

Alizarin red S (ARS) staining

Cell matrix mineralization was assessed by ARS staining. After osteogenic differentiation for 14 days, the hBMMSCs were washed by PBS and fixed in 4% formaldehyde for 10 min. Post washing by diH₂O, hBMMSCs were subjected to ARS solution (pH = 4.2) for 30 min. Subsequently, hBMMSCs were observed through a camera.

Luciferase reporter assay

Luciferase reporter assay was utilized for validating whether lncRNA NORAD could bind with miR-942-5p and miR-942-5p could target Prdx6. The wild type NORAD (NORAD-WT), wild type 3'UTR of Prdx6 (Prdx6-WT), mutant NORAD (NORAD-Mut) and mutant 3'UTR of Prdx6 (Prdx6-Mut) were synthesized and cloned into the pmirGLO3 Basic luciferase vectors (Promega, UK). HEK-293T cells were cultured and then miR-942-5p mimics and NC mimics were transfected into them. The luciferase activity was detected using a Dual-Luciferase Reporter assay system (Promega Corporation).

Western blot analysis

To determine the levels of protein, hBMMSCs were lysed by RIPA buffer. The total protein concentrations in cell lysates were quantified with a BCA protein assay kit (Pierce, Thermo Fisher Scientific, USA) and then resolved by SDS-PAGE followed by transferring onto PVDF membranes. The membrane was then probed with primary antibodies including anti-Prdx6 (ab133348; Abcam, Shanghai, China), anti-OPN (ab75285; Abcam), anti-Runx-2 (ab192256; Abcam) and anti-GAPDH (ab181602; Abcam). Membranes were then subjected to incubation with a secondary antibody. Finally, protein bands were visualized using an ECL kit (Thermo Fisher Scientific, Inc.).

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from hBMMSCs and peripheral blood of patients with the TRIzol reagent (Invitrogen). Reverse transcription was performed with the M-MLV Reverse Transcriptase (Invitrogen) to produce the first-strand cDNA. RT-qPCR was conducted by use of the SYBR Premix Ex Taq II (Takara, Dalian, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The expression of miR-942-5p was detected by RT-qPCR with TaqMan MicroRNA Assay (Applied Biosystems). GAPDH was used as an endogenous control for lncRNA NORAD and Prdx6 while U6 served as an endogenous control for miR-942-5p. The expression of NORAD, Prdx6 and miR-942-5p was calculated by using $2^{-\Delta\Delta C_t}$ method.

RNA pull-down assay

The biotin-labeled Bio-miR-942-5p and Bio-NC were synthesized by RiboBio (Guangzhou). In brief, hBMMSCs were transfected with Bio-miR-942-5p or Bio-NC and incubated for 48h. Then hBMMSCs were lysed and cultured with magnetic beads conjoined with streptavidin (Life Technologies, Carlsbad, USA). Further, the biotin-coupled RNA complex was subjected to proteinase K and the levels of Prdx6 or lncRNA NORAD were assessed by RT-qPCR.

Statistical analysis

Data analyses were subjected to SPSS 21.0 software (IBM, NY, USA). The measurement data were displayed as mean ± SD. Comparisons between two groups were done

according to the student's t-test. Differences among more than two groups were done in one-way analysis of variance (ANOVA). Pearson's correlation analysis analyzed the correlation between the expression of miR-942-5p and Prdx6 (or NORAD), lncRNA NORAD and Prdx6. $P < 0.05$ was an indicator of statistical significance.

RESULTS

Prdx6 was elevated in PMOP and regulated osteogenic differentiation

In order to explore the function of Prdx6 in PMOP, the Prdx6 expression in the peripheral blood of patients

with PMOP was measured. The data delineated that Prdx6 was distinctly elevated in the peripheral blood of patients with PMOP (Fig. 1A). Next, the mRNA and protein levels of Prdx6 and bone formation and osteogenic differentiation-related proteins (OPN and Runx-2) during the osteogenic differentiation of hBMMSCs were evaluated. The results revealed that the mRNA and protein levels of Prdx6 were decreased while the levels of OPN and Runx-2 were increased during the osteogenic differentiation of hBMMSCs (Fig. 1B and C). Furthermore, Prdx6 was knockdown in hBMMSCs and more mineralized nodules formation was detected due to Prdx6 depletion (Fig. 1D).

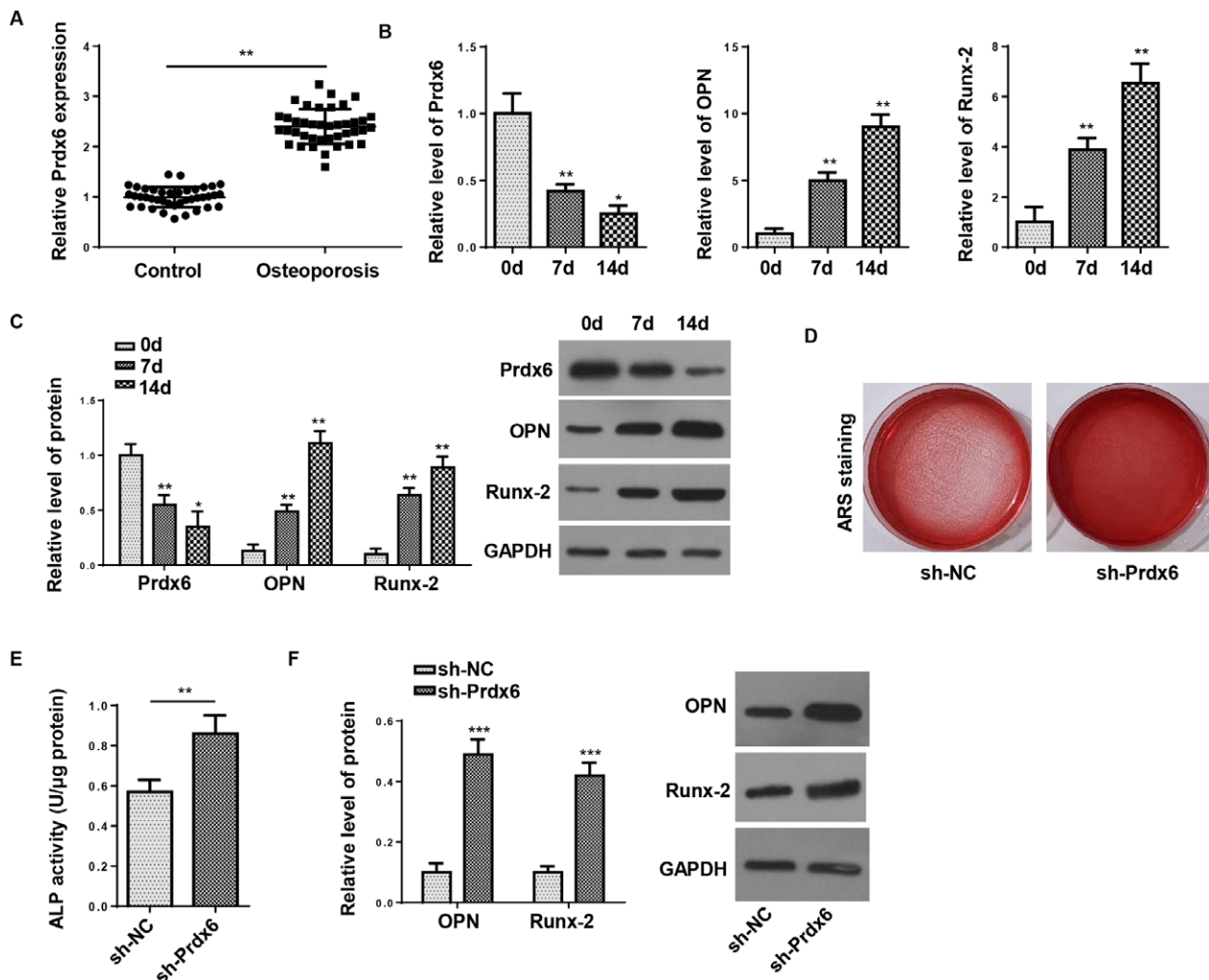


Fig. 1. Prdx6 was elevated in PMOP and regulated osteogenic differentiation. (A): The expression of Prdx6 was assessed by RT-qPCR; (B-C): RT-qPCR and western blot analysis were implemented to measure the protein expression of Prdx6, OPN and Runx-2; (D-E): The osteogenic differentiation was evaluated by ARS staining and ALP activity assays; (F): The protein levels of OPN and Runx-2 were tested by western blot analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Moreover, the ALP activity was also increased as a result of Prdx6 silencing (Fig. 1E). In addition, the protein levels of OPN and Runx-2 were upregulated due to Prdx6 silencing (Fig. 1F). To conclude, Prdx6 was elevated in PMOP and modulated osteogenic differentiation in PMOP.

Prdx6 served as a target of miR-942-5p

Afterwards, the mechanisms of Prdx6 in the modulation in the development of PMOP were investigated. According to the starBase database, the miRNAs targeting Prdx6 were searched, showing that miR-942-5p was the only miRNA had binding sites for Prdx6 with high stringency (≥ 3) and RT-qPCR unveiled that miR-942-5p expression was evidently decreased in the peripheral blood of patients with postmenopausal PMOP (Fig. 2A). Then RNA pull-down assay and luciferase reporter assay were implemented to identify whether miR-942-5p could bind with Prdx6. As exhibited in Fig. 2B, RNA pull-down assay depicted that Prdx6

was enriched in Bio-miR-942-5p group rather than Bio-NC group. Moreover, the luciferase activity of Prdx6-WT was evidently declined due to miR-942-5p upregulation while the luciferase activity of Prdx6-Mut had no change in both groups (Fig. 2C). Next, the mRNA and protein levels of Prdx6 were distinctly suppressed on account of miR-942-5p overexpression (Fig. 2D).

Pearson's correlation analysis demonstrated that miR-942-5p expression in peripheral blood of patients was negatively correlated with Prdx6 expression (Fig. 2E). Taken together, Prdx6 served as a target of miR-942-5p.

LncRNA NORAD sponged miR-942-5p

As ceRNA hypothesis was frequently proposed in recent years, which means that miRNAs can be sponged by lncRNAs to increase the expression of target genes to regulate the progression of diseases (7). Furthermore, the upstream regulator of miR-942-5p was searched in the starBase database. The

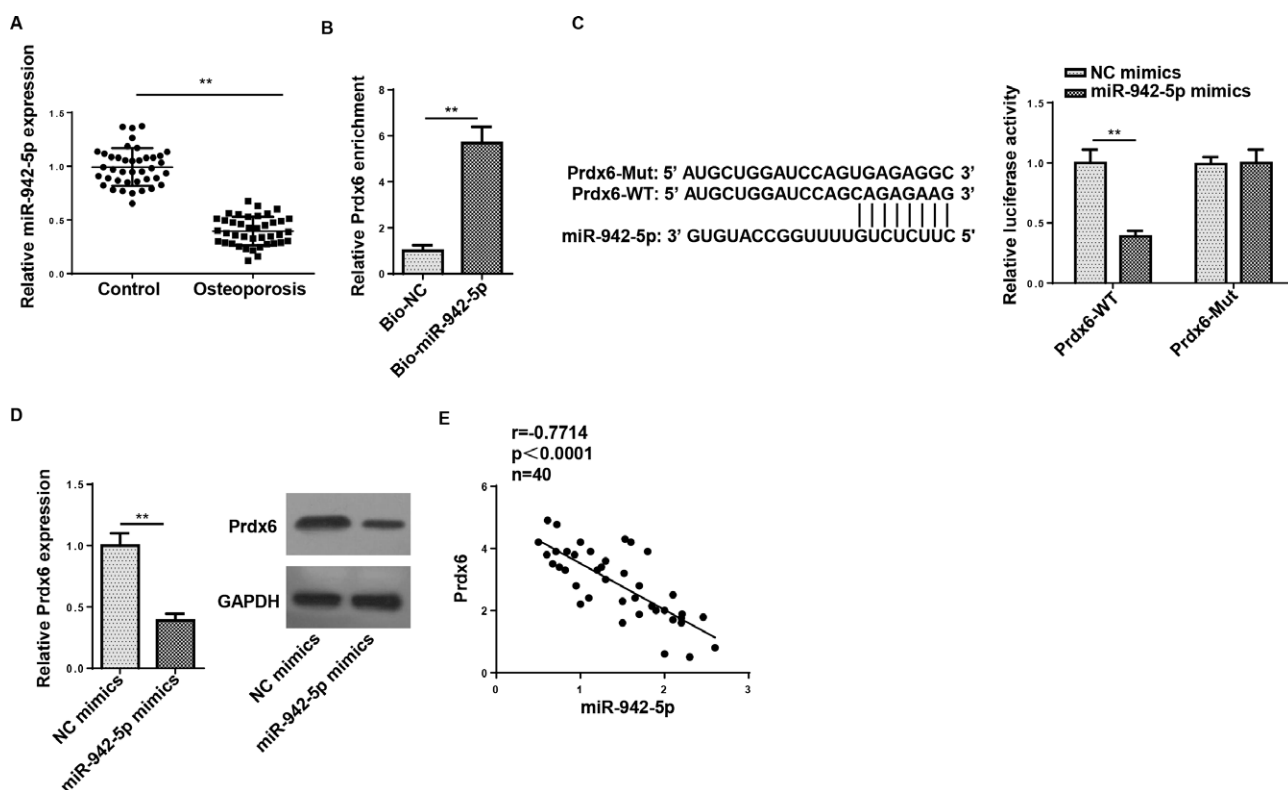


Fig. 2. Prdx6 served as a target of miR-942-5p. (A): RT-qPCR was used to evaluate the expression of miR-942-5p; (B-C): The binding possibility of miR-942-5p and Prdx6 was measured by RNA pull down and luciferase reporter assays; (D): The mRNA and protein levels of Prdx6 were examined by RT-qPCR and western blot assay; (E): Pearson's correlation analysis revealed the association between miR-942-5p and Prdx6. ** $P < 0.01$.

expression of NORAD in peripheral blood of PMOP was measured by RT-qPCR, depicting that NORAD was strikingly elevated in PMOP patients (Fig. 3A). We found there were two lncRNAs (MALAT1 and NORAD) that had binding sites for miR-942-5p (medium stringency ≥ 2) and RNA pull-down assay revealed that lncRNA NORAD was more enriched in Bio-942-5p than lncRNA MALAT1 (Fig. 3B). Besides, the enhancement of miR-842-5p dramatically decreased the luciferase activity of NORAD-WT reporters instead of NORAD-Mut reporters (Fig. 3C). Additionally, silencing of

NORAD evidently increased the expression of miR-942-5p (Fig. 3D). Similarly, there was a negative correlation between NORAD and miR-942-5p expression, and a positive correlation between NORAD and Prdx6 expression in peripheral blood of patients (Fig. 3E). In a word, lncRNA NORAD sponged miR-942-5p.

LncRNA NORAD/miR-942-5p/Prdx6 axis regulated the development of PMOP

Subsequently, rescue assays were implemented to identify whether NORAD/miR-942-5p/Prdx6 axis

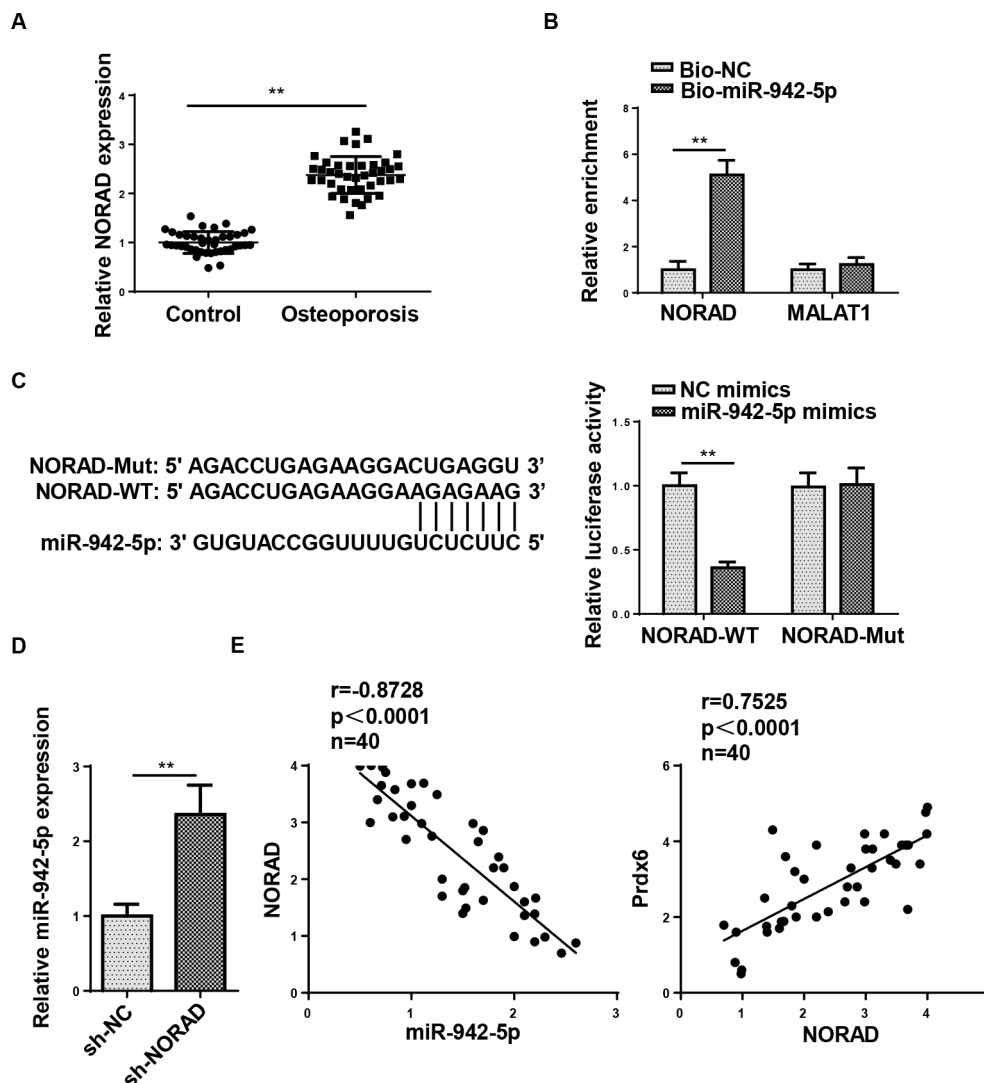


Fig. 3. LncRNA NORAD sponged miR-942-5p. (A): The expression of NORAD in peripheral blood of PMOP patients was evaluated by RT-qPCR; (B-C): RNA pull down and luciferase reporter assays were employed to identify the binding affinity of NORAD and miR-942-5p; (D): The expression of miR-942-2p was measured by RT-qPCR after suppressing NORAD; (E): The associations between NORAD and miR-942-5p, Prdx6 and NORAD were explored by Pearson's correlation analysis. ** $P < 0.01$.

could regulate the development of PMOP. Prior to all, NORAD was knocked down while Prdx6 was overexpressed in hBMMSCs. The data delineated that NORAD was distinctly decreased by sh-NORAD transfection while Prdx6 was overtly increased by oe-Prdx6 transfection (Fig. 4A). What's more,

downregulation of NORAD induced the decreased expression of Prdx6 (Fig. 4B). Silencing of NORAD depletion could increase the mineralized nodules formation, but these effects were reversed by Prdx6 upregulation (Fig. 4C). Besides, the increased ALP activity resulted from NORAD inhibition was offset

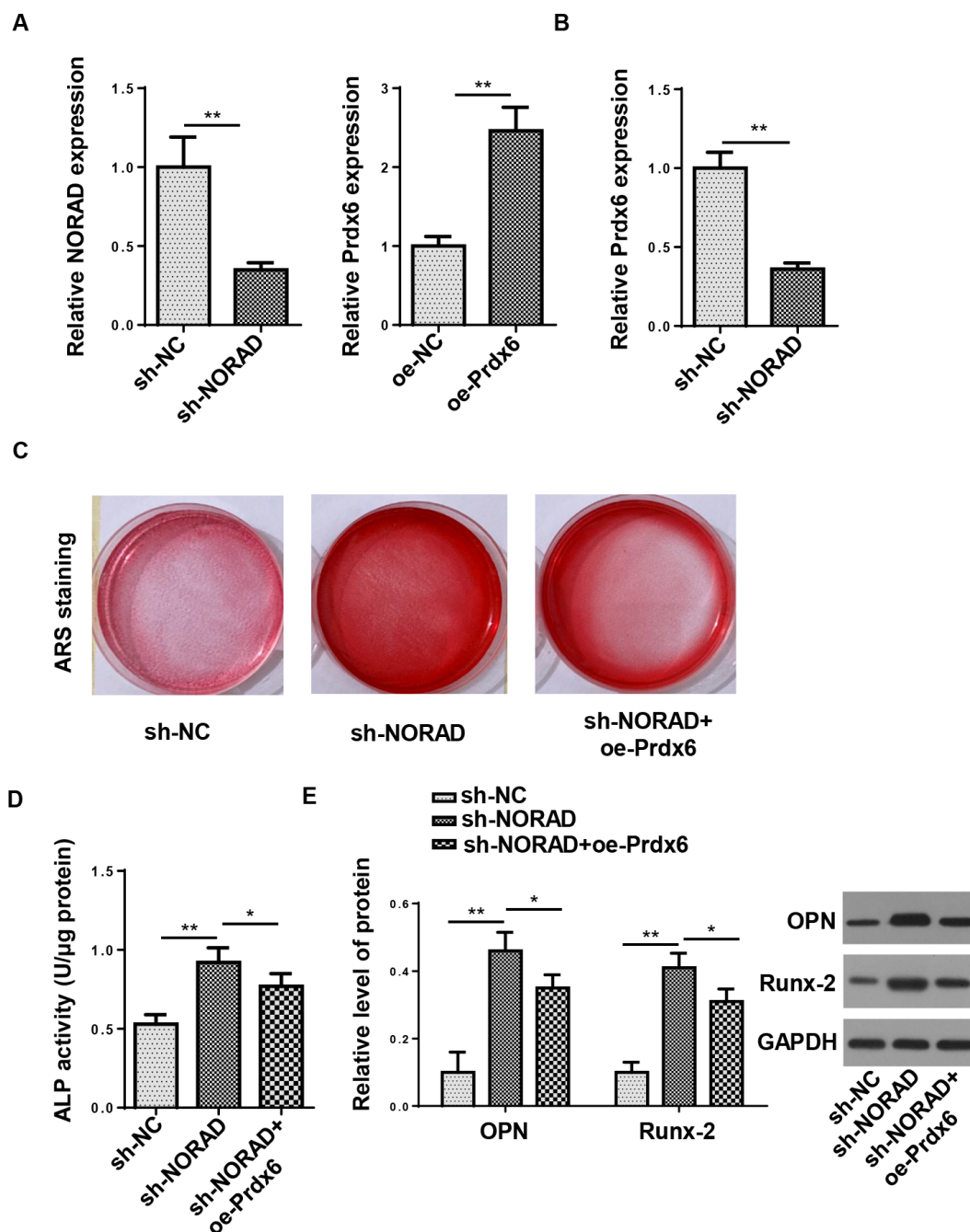


Fig. 4. LncRNA NORAD/miR-942-5p/Prdx6 axis regulated the development of PMOP (A): The expression of lncRNA NORAD and Prdx6 were investigated by RT-qPCR; (B): The expression of Prdx6 was measured through RT-qPCR after repressing NORAD; (C-D): The osteogenic differentiation was measured through ARS staining and ALP activity assays; (E): The protein levels of OPN and Runx-2 were examined via western blot analysis. * $P < 0.05$, ** $P < 0.01$.

by Prdx6 elevation (Fig. 4D). In addition, the OPN and Runx-2 levels were increased as a result of NORAD silencing but counteracted by the upregulation of Prdx6 (Fig. 4E). All in all, lncRNA NORAD regulated the development of PMOP through Prdx6.

DISCUSSION

PMOP is a common metabolic bone disease resulted from the failure of ovary and the decrease of estrogen (8). Prdx, a family of peroxidases, is involved in the pathogenesis of various diseases, including osteoporosis (9). In this study, we found Prdx6 expression was increased in PMOP and the knockdown of Prdx6 restrained the development of PMOP.

miR-942-5p has been reported to modulate the osteogenesis-angiogenesis coupling process in bone mesenchymal stem cells (BMSCs) (10). Herein, it was demonstrated that miR-942-5p expression was decreased in PMOP patients' peripheral blood. Furthermore, miR-942-5p was validated to target Prdx6 and the expression of miR-942-5p was negatively correlated with Prdx6.

There was evidence depicting that lncRNA NORAD may affect the development of several diseases. For instance, NORAD is involved in the pathogenesis of high glucose-induced diabetic retinopathy by sponging miR-125 (11). NORAD binds with miR-26a-5p to modulate the differentiation and proliferation of BMSCs in steroid-mediated osteonecrosis of the femoral head (12). In our study, functional assays revealed that the upregulation of Prdx6 could reverse the NORAD knockdown mediated osteogenic differentiation of hBMSCs in PMOP.

In conclusion, the present work suggested that NORAD/miR-942-5p/Prdx6 axis might be involved in the osteogenic differentiation of hBMSCs in PMOP, which might help to improve the prevention and control of PMOP.

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Umbilical cord-derived mesenchymal stem cell-derived exosomal MiR-133a-3p protects against myocardial infarction by inhibiting apoptosis

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To the Editor,

Electron microscopy showed that exosome (Exo) is a lipid bilayer with a round or characteristic cup shape with a diameter of 30-150 nm (1) mainly derived from polycystic vesicles formed by intracellular lysosomal microparticles. Vesicle nucleic acids mainly include mRNA, microRNA, long non-coding RNA and circular RNA (1). Many cells, including mesenchymal stem cells (MSCs), can secrete Exo (2). Exo was associated with cell communication, gene regulation, immune regulation, substance metabolism, cell differentiation and migration, angiogenesis, injury and repair, tumor cell growth and other processes (3).

MiRNA is a new class of non-coding single-stranded small RNAs (20-24 nt) that inhibit translation by negatively regulating gene expression at the post-transcriptional level by binding to target gene mRNA (4). Large amounts of miRNAs are expressed in the human cardiovascular system, where they play important roles in normal physiological activities and the occurrence, development and prognosis of cardiovascular diseases. Many studies have shown that miR-133a-3p plays an important regulatory role in the process of alleviating myocardial injury after acute myocardial infarction (AMI) (5).

Among the various types of stem cells, MSCs are the most studied by researchers because of their unique advantages, such as strong self-renewal, easy to be isolated, cultured, amplified and purified, little immune exclusion (6). Umbilical cord-derived mesenchymal stem cell (UMSC) has stem cell characteristics as well as broad differentiation potential and low immunogenicity, and does not involve ethical issues (7). Their acquisition is also very effective, which means that UMSC-derived exosomes are promising candidates for regenerative medicine for allogeneic treatment.

Therefore, we propose the hypothesis that has-miR-133a-3p in Exo secreted by human UMSCs (huMSCs) inhibits myocardial senescence and plays a protective role in ischemic myocardium.

MATERIALS AND METHODS

This study was approved by the ethics committee of the Affiliated Zhongshan Hospital of Dalian University and was conducted in accordance with the Declaration of Helsinki. huMSCs from healthy adults were purchased from Cambrex BioScience. The huMSCs were cultured with Dulbecco's Modified Eagle Medium (DMEM;

Key words: uMSC; exosome; miR-133a-3p; FOSL2; apoptosis

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Hyclone, South Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin and streptomycin. Cell supernatant was collected after 48 h of culture in exo-free culture medium instead of cell culture medium, and the Exo Isolation Reagent was used (Ribo, Guangzhou, China). Surface markers CD73, CD90, CD105, CD34 and CD45 were detected by flow cytometry. The ultrastructure of particles was detected by electron microscopy. Western blot (WB) was used to detect the surface markers.

Firstly, huMSCs were treated with exome-free *fetal bovine serum* (Gibco) for 2 days, then 5 ml cell culture medium was centrifuged at $1500 \times g$ for 30 min to remove cells and debris. 1.5 ml RiboTM exosome isolation reagent was added to the cell-free media. These mixtures were centrifuged at $2000 \times g$ for 30 min. The supernatant was aspirated and the Exo resuspended in 100 μ L PBS.

When the fusion degree of myocardial cells reached 50%, Lipofectamine[®] RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. Specific experimental methods were as follows: 150 μ L Opti-MEM was added into two EP tubes, and Lipofectamine[®] RNAiMAX Reagent 9 μ L were added into one 150 μ L Opti-MEM and shaken. Meanwhile, 3 μ L microRNA-133a-3p mimic/NC was added into another 150 μ L Opti-MEM and shaken. The two tubes were mixed with Opti-MEM with RNAiMAX Reagent and miRNA-133A-3p mimic/NC, respectively. After 5 min of incubation at room temperature, the mixture of a total of 300 μ L was added into 1.5 mL of medium. The medium was added into a culture hole of a 6-well plate and placed in a cell culture box for 2 days for analysis of cultured cells.

The viability of H9C2 cells was detected by CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan). The cells were plated on 96-well plates with 5×10^3 cells per well. After incubation for 72 h, CCK-8 reagent was added at 10 μ L per well and incubated for another 2 h. The absorbance at 450 nm was measured by a Microplate Reader (Bio-Rad, Hercules, CA, USA).

H9C2 cells were placed in a Petri dish for ischemia and hypoxia treatment. The cells were collected and washed with phosphate buffered saline (PBS) and resuspended in binding buffer. Staining solutions (BB-4101-2; BestBio Science., Shanghai, China) were added to the cells for 30 min in the dark. Apoptotic cells were detected by flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

After extracting cells or RNA from Sprague-Dawley (SD) rats, reverse transcription was performed into complementary deoxyribose nucleic acid (cDNA), and target gene 3' untranslated region (3' UTR) fragment was amplified by Polymerase Chain Reaction (PCR) with corresponding primers of target gene and then subcloned to pRL-CMA expression vector as wide-type. Meanwhile, the 3'UTR mutation of the target gene was selected into the pRL-CMA expression vector as a mutant. 100ng wide-type target gene, mutant target gene, 10 μ L mir-133a-3p mimic and NC were transfected with lipofectamine RNAiMAX transfection reagent into hek-293 cells cultured *in vitro* and in 24-well plates, respectively. Fluorescence reporter genes of each group were detected 48 h after transfection.

After anesthesia, SD rats were fixed on the operating table and intubated under general anesthesia. The skin, subcutaneous tissue, intercostal muscle and pleura were separated step by step in the fourth rib space, and the pericardium was opened. The AMI model was established by ligation at the distal 1/3 of the anterior descending segment of the coronary artery. According to the needs of the experimental group, 50 μ L agomir-133a-3p was locally injected around myocardial infarction tissue at a concentration of 2×10^3 mol/L for each rat. Penicillin was injected intramuscularly to prevent infection.

SD rat tissues were fixed with 10% paraformaldehyde in paraffin (Yunqing, Changzhou, China). The embedding block was cut into (5 μ m thick) slices with a microtome (Sino, Beijing, China) and the sections were observed under a microscope. The slices were dewaxed with xylene, fractionated by ethanol, rehydrated, stained with hematoxylin for 5 min, and stained with 0.5% eosin for 2 min. The morphological changes of cardiomyocytes injury were observed under a microscope after sealing. For Masson staining, the cardiomyocytes tissue slides were stained with Ponceau S acid fuchsin for 5-10 min, followed by incubation with a molybdophosphoric acid solution for 3 min and staining with aniline blue for 5 min, and finally observed with a camera (EOS 80D; Canon, Inc., Tokyo, Japan).

To detect the cardiac function of three groups: Sham, AMI, AMI + exo, we used echocardiography. The anesthetic was isoflurane (induction concentration of 3.5%, maintenance concentration of 2.5%). Two-dimensional echocardiography was used to detect the parameters of left ventricular end-systolic diameter

(LVSD), left ventricular end-diastolic diameter (LVED%) and left ventricular ejection fraction (LVEF%) in the rats.

RNA was extracted with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and the reverse transcription kit was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) and used as the template of PCR. After the customized quantitative PCR target primer, 1 μ L reverse transcription product was taken, and 40 cycles were amplified by ABI7000 system (Applied Biosystems, Foster City, CA, USA) with fluorescence quantitative PCR kit. Finally, the relative amount of target gene was calculated according to Ct value. Using GAPDH as an internal reference, the corresponding $\Delta\Delta$ Ct values in each group of cells were calculated, and the quantitative analysis was performed based on the quantitative amount of the target factor ($2^{-\Delta\Delta$ Ct). All the primers are listed in Table I.

After tissue protein extraction, Bicinchoninic acid (BCA) Protein Assay kit was used to detect the protein concentration, and 20 μ g protein was used for electrophoresis in sodium dodecyl sulphate (SDS)-page, followed by electrical transfer to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing, primary antibody was added and incubated at 4°C overnight. Housekeeping protein GAPDH was used as an internal reference. Horseradish peroxidase (HRP) labeled secondary antibody was used to identify primary antibody, ECL luminescent solution was used for color rendering, and film exposure was used for preservation.

Statistical analysis

All data are presented as mean $\pm$ SD (standard deviation). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was made using One-way ANOVA test followed by Post Hoc Test (least significant difference). All data were analyzed by GraphPad Prism 7.0 software (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of myocardial damage by uMSC-derived exosomes

Firstly, exosomes were isolated from umbilical cord-derived mesenchymal stem cell (uMSC). To identify uMSC, Flow cytometry was used to confirm the identity of the collected uMSCs. The results showed that uMSCs were positive for surface markers of CD73, CD105 and negative for surface markers of CD34 and CD45 (Fig. 1A). Subsequently, a transmission electron microscope (TEM) showed the regular morphology of isolated MSC-derived exosomes was like a cup-shaped structure (Fig. 1B). Using western blot analysis, the expression of three exosome markers, CD63, CD81 and Tsg101 were analyzed, and they had a high expression in exosomes, but (Fig. 1C). All these results combined can show that the isolation of uMSCs was successful. To determinate the effect of UMSC-derived exosomes

Table I. Real time PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
miR-133a-3p	ACACTCCAGCTGGGAGCGGAAA AGGA	CTCAACTGGTGTCTGGAGTCGGC AATTCAGTTGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
FOSL2	CACGCCGAGTCCTACTCCA	GTGGGCTGTACCATCCACTG
GAPDH	ACAACCTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR: quantitative reverse-transcription polymerase chain reaction.

on the injured cardiomyocytes after AMI, we co-cultured H9C2 cells with exosomes for 48 h.

Immunofluorescence showed that the exos were indeed ingested by cardiomyocytes (Fig. 2A). CCK8 assay was used to detect the viability of cells in all three groups: control, hypoxia and hypoxia + exos. Compared with hypoxia group, cell viability showed significantly higher in hypoxia + exos group (Fig. 2B). Flow cytometry to detect the apoptosis of H9C2 cells showed that in the exo group, the apoptosis rate dramatically decreased (Fig. 2C). Western blot assay was also conducted. Bax expression decreased in the exo group compared with hypoxia group, on the contrary, the expression of Bcl-2 was high in the exo group (Fig. 2D). Therefore, all these studies showed that exo can protect H9C2 cells against apoptosis.

uMSC-derived exosomes inhibit myocardial damage and improve cardiac function

By HE staining, the cardiomyocytes of the control group were normal and with no edema,

while the cardiomyocytes size of the AMI group was significantly changed, the cardiomyocytes edema was obvious, and the interstitial fibrosis was severe. Exo treatment significantly attenuated cardiomyocytes structural changes caused by AMI. The features of myocardial tissues were further analyzed using Masson staining, and irregular alteration of cell shape and lining in the rat myocardium after the induction of AMI were found. When injected with uMSC-derived exosomes, abnormal cellular alterations could be mitigated (Fig. 3A). Echocardiography was used to detect cardiac function in rats (Fig. 3B). Compared with sham operation rats, LVEF% and LVFS% decreased significantly in AMI rats, and recovered significantly after exo injection (Fig. 3 C, D). The expression of Bcl-2 protein in border zone was detected by Western blot. Bcl-2 expression was found upregulated in the exo group, while the expression of Bax decreased (Fig. 3E). These data together supported that exo improves cardiac function in AMI rats.

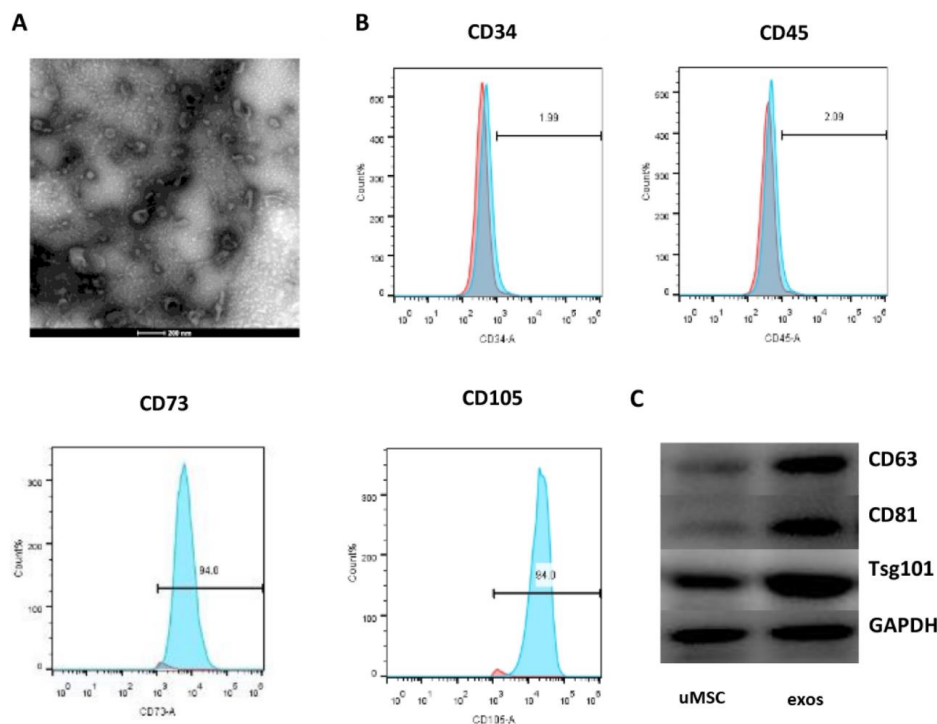


Fig. 1. Umbilical cord-derived mesenchymal stem cells and exosome isolation. **A)** TEM (magnification, $\times 49,000$) of the morphological properties of uMSC derived exosomes. **B)** Flow cytometry analysis of uMSCs, confirmed by the positive expression of CD73, CD105 combined with lower expression of CD34 and CD45CD. **C)** Western blot analysis of CD63, CD81 and Tsg101 protein expression in uMSCs and exosomes. GAPDH was used as an internal control. uMSC, Umbilical cord-derived mesenchymal stem cells; TEM, transmission electron microscopy; Tsg101, Tumor susceptibility gene 101 protein.

Exo-miR-133a-3p secreted by huMSCs inhibits cardiomyocyte apoptosis

Many studies have confirmed that after AMI, the expression of miR-133a-3p can obviously change in the cardiomyocytes, which can protect the cardiac function. Therefore, we wonder whether the uMSC derived exos have high expression of miR-133a-3p. The expression of miR-133a-3p increased in the exo group compared with the AMI group by RT-PCR (Fig. 4A). Firstly, miR-133a-3p was overexpressed with miR-133a-3p mimics in uMSC (Fig. 4B), and then, exos isolated from the uMSC were co-cultured

with H9C2 cells for 48 hours. Flow cytometry assay was conducted to detect the apoptosis rate. The result was similar to previous evidence: the apoptosis rate dramatically decreased in the hypoxia+miR-133a-3p mimics group compared with hypoxia+miR-133a-3p NC group (Fig. 4C). The protein expression of Bax decreased in the hypoxia+miR-133a-3p mimics group, while Bcl-2 expression increased compared with the hypoxia+miR-133a-3p NC group. P-Ampk expression increased in the exo group (Fig. 4D). All results showed that exo-miR-133a-3p secreted by huMSCs inhibits cardiomyocyte apoptosis.

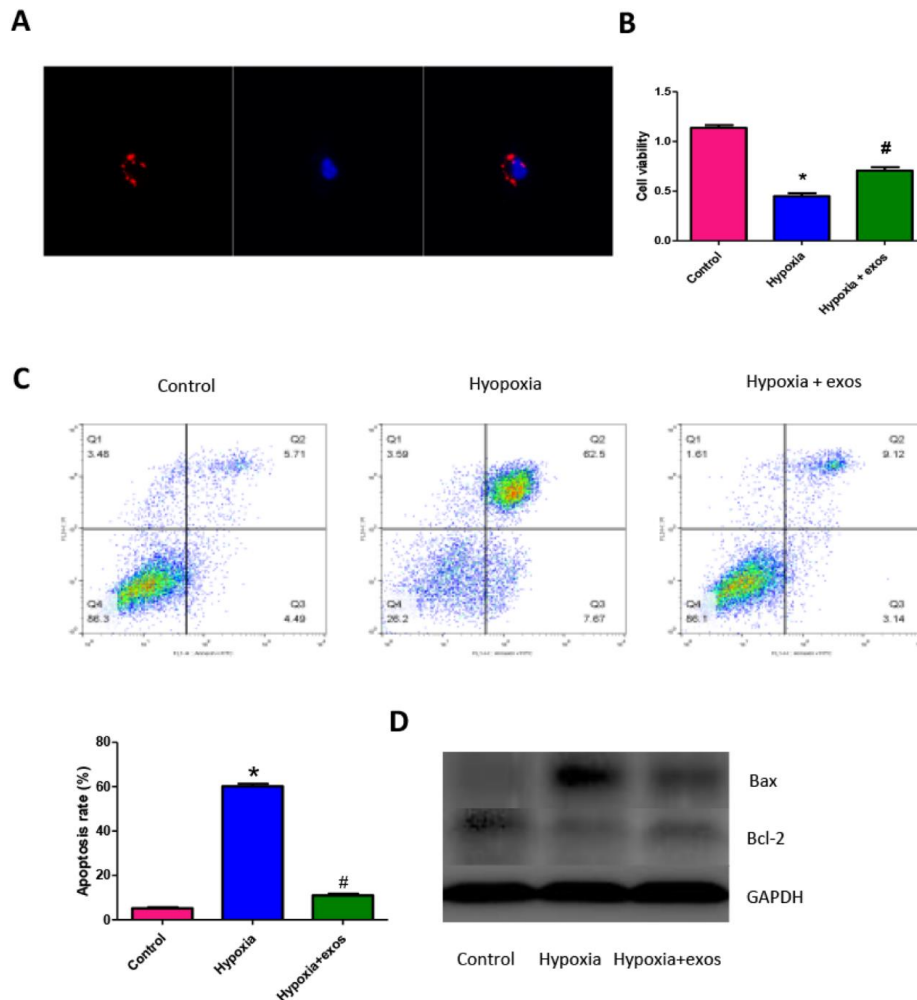


Fig. 2. UMSC derived exosomes prevented hypoxia-induced cardiomyocyte apoptosis. **A)** Immunofluorescence shows the exosomes in the H9C2 cells. Cell nuclei were stained using DAPI (magnification, $\times 120$), blue; Exos were stain using Dil (magnification, $\times 120$), red. **B)** CCK-8 assay detects the viability of H9C2 cells co-cultured with exosomes for 48 hours. (“*” $P < 0.05$ vs control, “#” $P < 0.05$ vs hypoxia, $n = 3$). **C)** The representative images of flow cytometry using Annexin V-FITC and PI staining. **D)** Western blot bands of Bax and Bcl-2 in control, hypoxia and hypoxia+exo group. (“*” $P < 0.05$ vs control, “#” $P < 0.05$ vs hypoxia, $n = 3$).

MiR-133a-3p inhibits H9C2 apoptosis induced by hypoxia by targeting FOSL2

To predict downstream target genes, we used miRDB (<http://mirdb.org/>), (http://www.targetscan.org/vert_72/) and miRanda (<http://www.microrna.org/microrna/home.do>). The results showed that Fosl2 was possibility the target gene of miR-133a-3p (Fig. 5A). When transfected with miR-133a-3p mimics, the expression of miR-133a-3p increased dramatically, in H9C2 cells. We next assessed whether FOSL2 is a true target of miR-133a-3p. We next assessed whether FOSL2 is a direct target of miR-133a-3p. The mRNA expression

of FOSL2 decreased in the miR-133a-3p mimics group compared with NC group (Fig. 5B). Western blot assay also showed the expression of FOSL2 decreased in the miR-133a-3p mimics group (Fig. 5C). Finally, we constructed luciferase reporter plasmids containing the 3'-UTR of FOSL2, then co-transfected with miR-133a-3p and the reporter plasmids into HEK293T cells. The results show that miR-133a-3p overexpression obviously inhibited luciferase activity in the wild-type group, however, which was failed to inhibit activity in the mutant 3'-UTR (Fig. 5D). These results confirmed the target gene of miR-133a-3p is FOSL2.

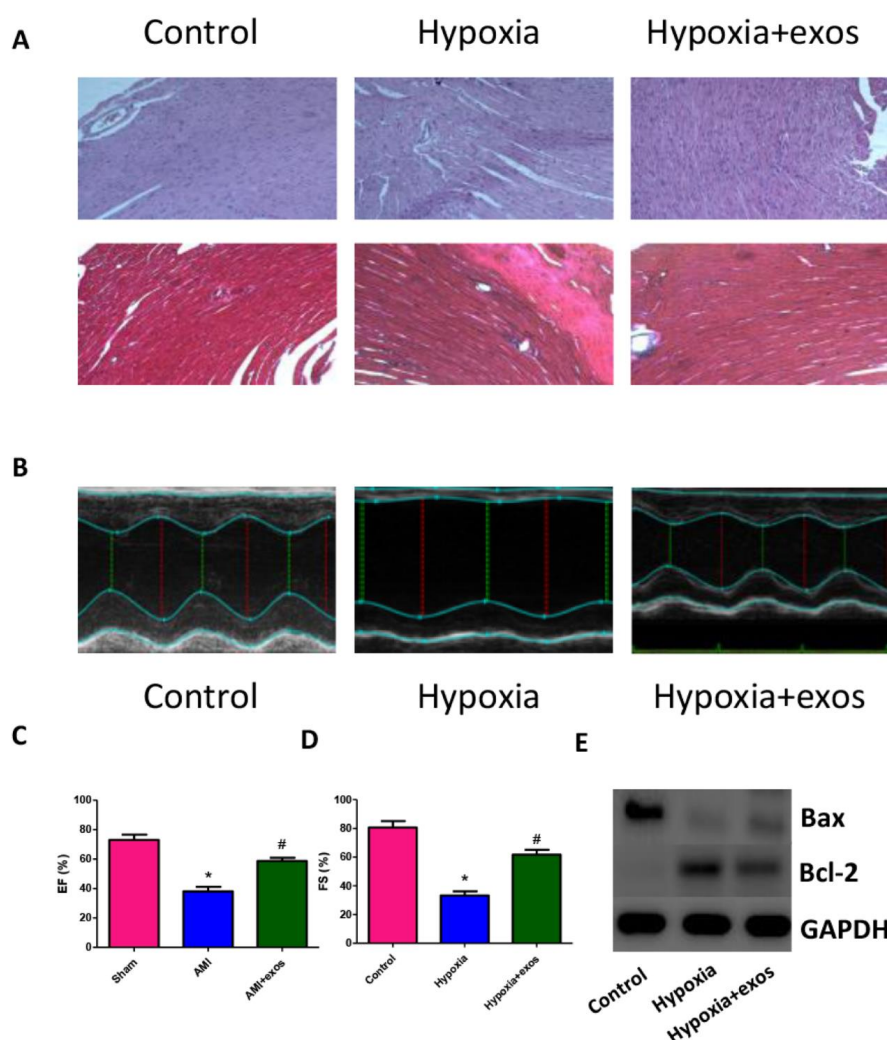


Fig. 3. Inhibition of rat myocardial damages by MSC-derived exosomes. **A)** H&E and Masson staining of the Sham, AMI and AMI+Exo groups (magnification, $\times 400$). **B)** Representative photographs in rat echocardiography. **C)** Ejection fractions (EF) and **(D)** Fractional shortening (FS). **E)** Western blot bands of Bax and Bcl-2 in sham, AMI and AMI + exo rat hearts. H&E, hematoxylin and eosin; AMI, acute myocardial infarction; Exo, exosomes.

DISCUSSION

This study is innovative in research ideas, mainly focusing on the effect and molecular mechanism of the inhibition of myocardial apoptosis by exo-miR-133a-3p secreted by huMSCs, which is embodied in the following aspects: 1) to clarify the effect of exo-miR-133a-3p on the apoptosis of cardiomyocytes; 2) to clarify the protective effect of exo-miR-133a-

3p on ischemic myocardium. AMI is an important factor in the death of cardiovascular patients (8). This article shows that exo-miR-133a-3p secreted by huMSCs can reduce myocardial apoptosis caused by acute ischemia and improve cardiac function. The protective effect of miR-133a-3p is achieved by targeting FOSL2. This suggests that overexpression of miR-133a-3p may be an effective treatment for myocardial infarction. As a new therapeutic measure,

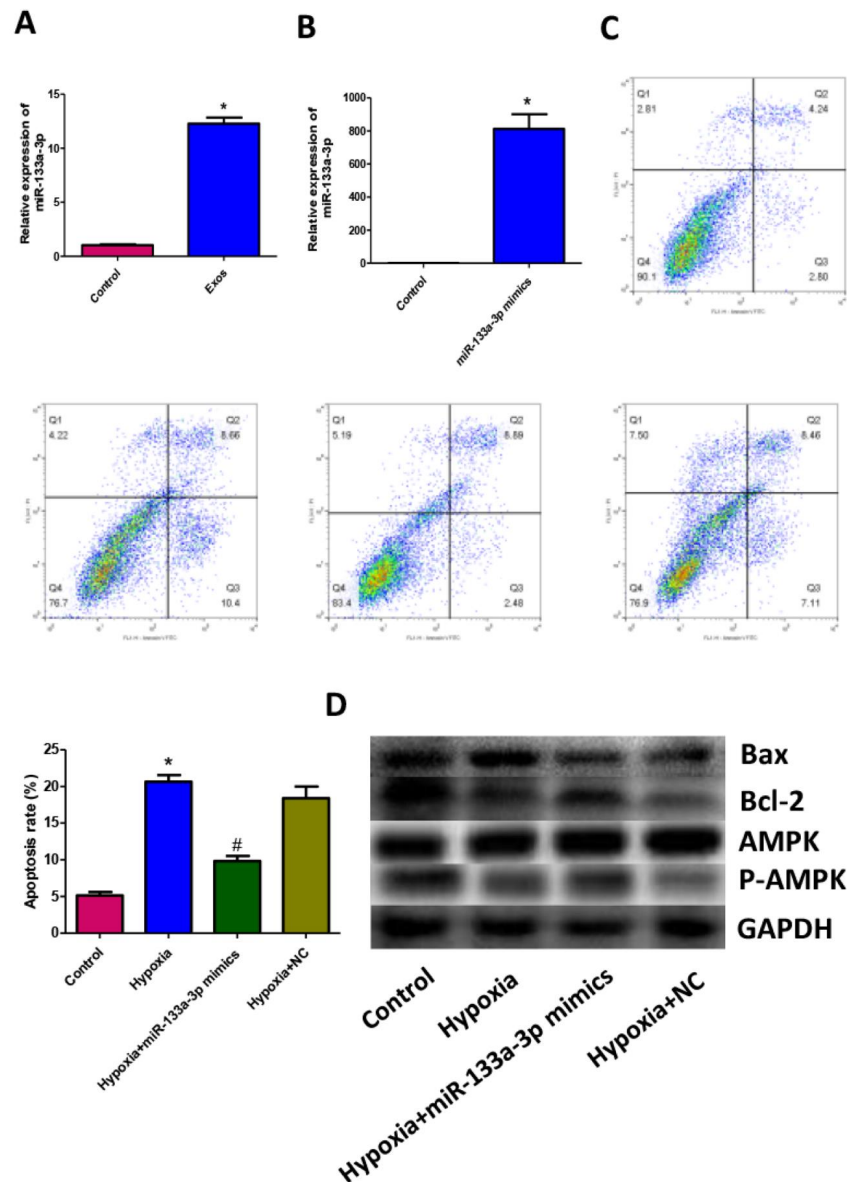


Fig. 4. Overexpression of miR-133a-3p in the uMSC inhibiting the apoptosis of H9C2. **A)** MiR-133a-5p expression level was increased in exosomes after hypoxia (“*” $P < 0.05$ vs control, $n = 3$). **B)** Overexpression of miR-133a-3p in uMSC, RT-PCR detect the relative expression of miR-133a-3p (“*” $P < 0.05$ vs control, $n = 3$). **C)** The representative images of flow cytometry using Annexin V-FITC and PI staining. **D)** Western blot bands of Bax, Bcl-2, AMPK and p-AMPK in control, AMI and AMI + miR-133a-3p mimics + uMSC.

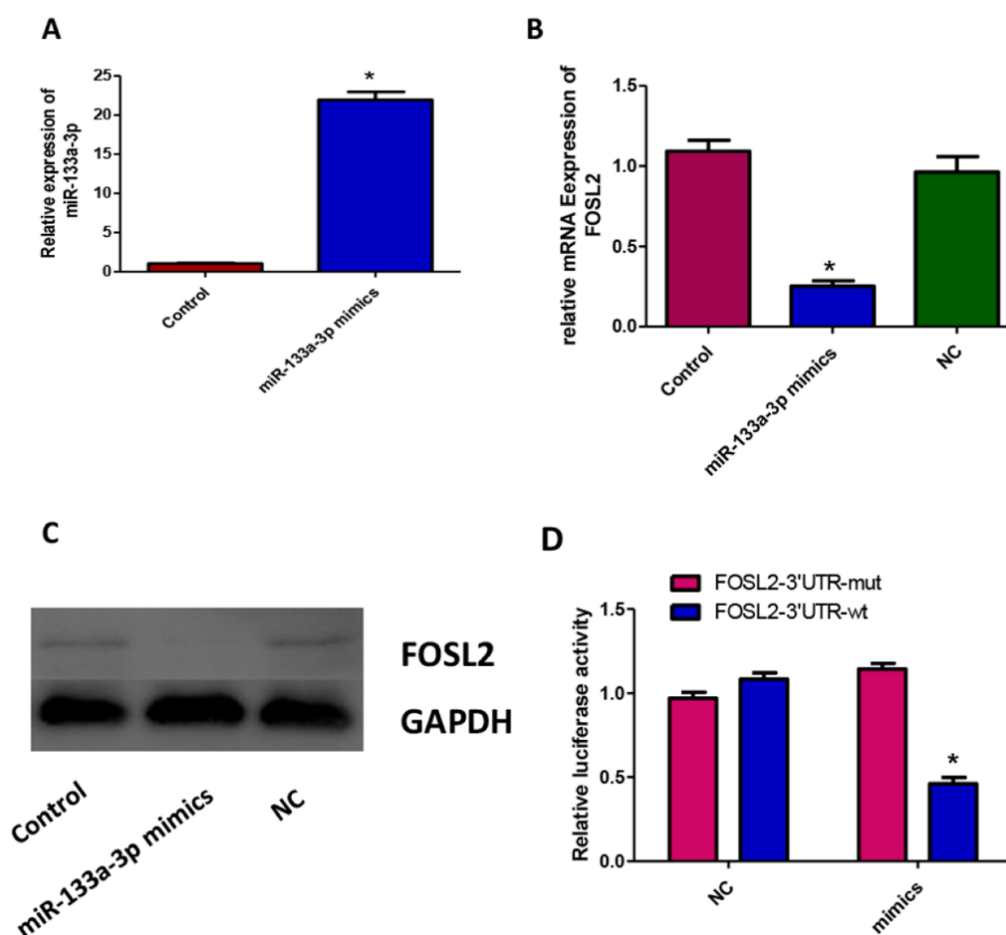


Fig. 5. MiR-133a-3p target FOSL2. **A)** Overexpression of miR-133a-3p in H9C2 cells. **B)** Statistical results of mRNA expression level of FOSL2 (“*” $P < 0.05$ v. NC, $n = 3$). **C)** Western blot bands of FOSL2. **D)** Luciferase reporter containing WT or mutant 3'-UTR of FOSL2 mRNA with miR-133a-3p mimics or NC into HEK293T cells for Dual Luciferase reporter assay. MiR-133a-3p overexpression significantly decreased the relative luciferase activity of FOSL2 mRNA WT 3'-UTR, but did not decrease the relative luciferase activity of mutant 3'-UTR (“*” $P < 0.05$ vs FOSL2-WT+NC, $n = 3$).

exosomes have developed rapidly in recent years (9). Compared with stem cells, exosomes are safer. In this paper, we co-cultured exosomes in cardiomyocytes and found that they can reduce apoptosis of H9C2 cells after ischemia and hypoxia. Then, exosomes were injected into SD rats and the exosome group was found to significantly improve cardiac function. Overexpression of mir-133a-3p in uMSC also inhibited apoptosis, and FOSL2 is the downstream target gene and AMPK pathway were found to play a role. This protein is also involved in many processes such as cancer, fibrosis, differentiation of T cells, bones' development and differentiation (10). We found that FOSL2 also have the function on the

heart, and decreasing it can improve the cardiac function. However, there are also some limitations in this study, firstly, we didn't have the rats injected with the agomiR-133a-3p; secondly, the AMPK pathway needs to be further studied.

In conclusion, Umbilical cord-derived mesenchymal stem cell-derived exosomal miR-133a-3p can protect against AMI by inhibiting apoptosis by targeting FOSL2 through AMPK pathway.

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Relationship between serum 25(OH)D levels at birth and respiratory disease in preterm infants

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To the Editor,

Respiratory distress syndrome (RDS) and bronchopulmonary dysplasia (BPD) are the main pulmonary complications of premature infants. Despite the improvement of treatment, RDS is still a severe, highly fatal disease in the extremely premature infant, and a considerable number of the surviving infants may be accompanied by BPD (1). RDS and BPD are associated with long-term oxygen use and high mortality, seriously affecting the quality of life of premature infants. In recent years, the relationship between vitamin D and lung diseases in infancy has gained increasing attention (2). Al-Beltagi et al. (3) have found that 25-hydroxyvitamin D (25[OH]D) was deficient in most preterm infants, especially those with RDS, and administration of vitamin D was associated with significant decrease in severity, rate of complications, and duration of hospital stay. Serum 25(OH)D is the intermediate product of vitamin D metabolism, which has a long half-life and is not affected by blood calcium, blood phosphorus, or parathyroid hormone, so it can accurately reflect vitamin D nutriture of the body. Therefore, we hypothesized that serum 25(OH)D deficiency may increase the risk of RDS and BPD occurrence. In this paper, the effects of serum 25(OH)D levels on RDS and BPD in preterm infants were analysed to provide a new idea for the prevention and treatment of lung diseases, so as to reduce the occurrence of RDS and BPD in preterm infants.

MATERIALS AND METHODS

Participants

All the cases between January 1, 2018 and December 31, 2019 in this study were recruited from the Neonatal Intensive Care Unit (NICU) at Zhengzhou First People's Hospital, which is a large, three A-level, general hospital in China. The inclusion criteria were as follows: preterm infants with a gestational age <34 weeks; admission within 24 hours of birth and venous blood collection for 25-(OH) D detection; complete case data for ascertainment of RDS and BPD diagnoses. Exclusion criteria were to have: severe congenital malformations, metabolic diseases and incomplete clinical data; liver or kidney disease, as well as hypothyroidism confirmed by screening tests for neonatal diseases; failure to complete treatment after birth or transfer to another hospital; maternal diseases or drugs that might affect the vitamin D level of preterm infants, such as chronic liver and kidney diseases, thyroid diseases, bone metabolic diseases, and the use of anticonvulsant or antiepileptic drugs during pregnancy that affected vitamin D metabolism. A total of 118 patients were eligible for inclusion and were evaluated for RDS and BPD diagnosis. According to whether neonatal RDS and BPD occurred, all the patients were divided into an RDS group (n=82) and a non-RDS group (n=36), and the RDS group was subsequently divided into a BPD group (n=17) and a non-BPD group (n=65).

This study was approved by the Ethics Committee

Key words: preterm infants; serum 25(OH)D; respiratory distress syndrome; bronchopulmonary dysplasia; multivariate logistic regression

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and Review Board of Zhengzhou First People's Hospital (No: 20171124-02). The informed consent for the minors to participate in this study was obtained from the patient's guardians, and the study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Clinical data collection

Clinical data of all groups were collected, including delivery patterns, pregnancy complications (such as gestational hypertension, gestational diabetes) and use of prenatal hormone. For premature infants, the gestational age, birth weight, sex, birth season, Apgar score also were recorded.

Blood sample collection and 25(OH)D level detection

Blood samples of all the included patients were collected at the time of the first intravenous blood collection within 2 hours after admission. The collected blood samples were centrifuged with 3,500 r/min for 10 min to obtain the serum. The serum 25(OH)D level detection, a combination of 25(OH)D₂ and 25(OH)D₃, which represent the best analytes for overall vitamin D status, was performed by specialists in the laboratory. The test was conducted following the manufacturer's protocol for the serum 25(OH)D kit (DiaSorin, USA), which uses a chemiluminescence immunoassay to determine serum concentrations of 25(OH)D.

Vitamin D levels in the different groups were assessed. Serum 25(OH)D <20 ng/mL was defined as vitamin D deficiency, and serum 25(OH)D <10 ng/mL was defined as severe deficiency, serum 25(OH)D between 10-20 ng/mL was defined as mild deficiency. Furthermore, serum 25(OH)D between 20-30 ng/mL represented vitamin D was insufficient, and serum 25(OH)D ≥30 ng/mL represented vitamin D was adequate (4).

Assessment of RDS and BPD

RDS was defined according to the latest guideline-2019 European Consensus Guidelines on the Management of Respiratory Distress Syndrome (5). RDS was diagnosed in a premature infant when there was respiratory distress requiring more than 30% oxygen delivered by positive pressure using either nasal continuous positive airway or an endotracheal tube with a chest radiograph that had diffuse infiltrates with a ground glass granular appearance and air bronchograms (3).

BPD was defined as a requirement for supplemental oxygen or mechanical ventilation at the infant's corrected gestational age of 36 or above. Although there was no agreement on the best definition of BPD in the literature, the information on respiratory support in the first 28 days of life was collected to re-analyse our data using the National Institutes of Health consensus definition of BPD (6).

Statistical analysis

Statistical analysis was carried out using the software SPSS 20.0. The measurement data in accordance with the normal distribution were expressed as mean ± standard deviation, and the comparison between the two groups was analysed by *t*-test. The non-normal distribution measurement data were expressed as median, and the comparison between groups was analysed by rank sum test. The counting data were expressed as percentage or rate, and the comparison between groups was analysed by *chi*-squared test. After application of these methods above, the statistically significant factors were screened out for subsequent analysis. RDS and BPD influencing factors of preterm infants were analysed by multivariate logistic regression. The occurrence of RDS or BPD was set as outcome, and the statistically significant factors were considered as covariates in our regression models. Significant differences were considered as $P < 0.05$.

RESULTS

Relationship of serum 25(OH)D with RDS and BPD

During the study period, 129 premature infants with gestational age less than 37 weeks were hospitalized; 118 preterm infants met the inclusion criteria, and their average gestational age was 31.06 ± 1.26 weeks, and the male to female ratio was approximately 1.41:1. Among the 118 preterm infants, the serum 25(OH)D was generally insufficient or scarce, and the average level was 12.83 ± 6.10 ng/mL. Forty-six infants had severe vitamin D deficiency (39.0%, 46/118), of whom 39 were complicated with RDS (84.8%, 39/46). In the 39 infants, 13 were complicated with BPD (33.3%, 13/39). Fifty-one infants had mild vitamin D deficiency (43.2%, 51/118), of whom 38 were complicated with RDS (74.5%, 38/51). In the 38 infants, 4 were complicated with BPD (10.5%, 4/38). Twenty-one infants had insufficient vitamin D (17.8%, 21/118), of

whom 5 were complicated with RDS (23.8%, 5/21). In the 5 infants, none was complicated with BPD. Compared with those with insufficient vitamin D, the infants with severe and mild vitamin D deficiency had a significantly higher probability of concurrent RDS ($\chi^2=23.776$, $P<0.05$; $\chi^2=15.896$, $P<0.05$). But there was no significant difference between the infants with severe and mild vitamin D deficiency ($\chi^2=1.559$, $P=0.212$). As for the probability of concurrent BPD, there was no significant difference between patients with mild vitamin D deficiency and insufficient vitamin D ($\chi^2=1.744$, $P=0.187$), but the probability of concurrent BPD in patients with severe vitamin D deficiency was significantly higher than those with mild vitamin D deficiency and insufficient vitamin D ($\chi^2=7.364$, $P<0.05$; $\chi^2=6.976$, $P<0.05$) (Fig. 1).

Comparison of the different groups

The 118 eligible preterm infants (69 males and 49 females) were divided into the RDS group (n=82) and the non-RDS group (n=36) according

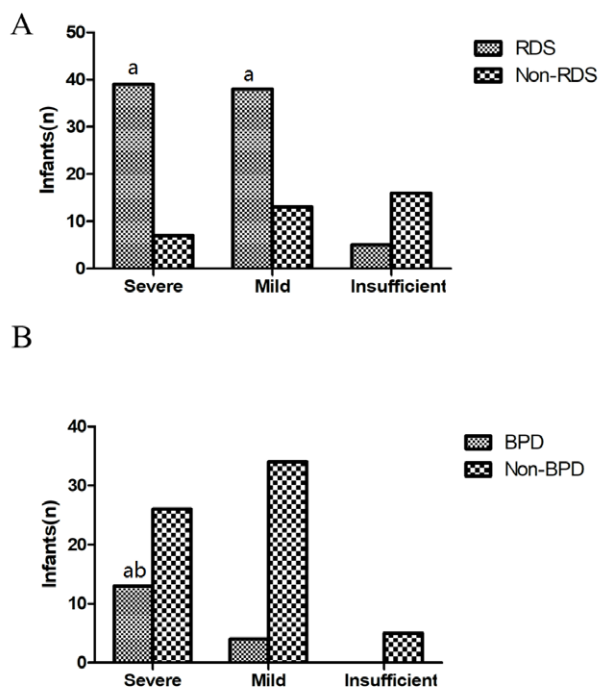


Fig. 1. Incidence of RDS and BPD at different serum 25(OH) D levels. **A)** RDS; **B)** BPD. ^a significant difference compared with those with insufficient vitamin D; ^b significant difference compared with those with mild vitamin D deficiency.

to the occurrence of RDS. In the RDS group, the birth gestational age, birth weight and Apgar scores at 1 min were significantly lower than those in the non-RDS group ($P<0.05$). There were no significant differences in cesarean birth, sex, birth season, Apgar scores at 5 min, prenatal use of hormones, maternal gestational diabetes or severe preeclampsia between the two groups (Table I). The average serum 25(OH)D level of the preterm infants in the RDS group was 10.84 ± 4.63 ng/mL, which was significantly lower than that in the non-RDS group of 17.33 ± 6.68 ng/mL ($P<0.05$) (Fig. 2A). The factors affecting the RDS occurrence, including gestational age, birth weight, Apgar scores at 1 min and 25(OH)D level, were considered as covariates and analysed by multivariate logistic regression. Small gestational age and low serum 25(OH)D level were independent risk factors for the RDS occurrence ($P<0.05$), as shown in Table II. The odds of RDS occurrence were 8.841 times higher for preterm infants with small gestational age, compared to those with great gestational age. Moreover, the odds of RDS occurrence for preterm infants with low 25(OH)D level were 1.206 times higher than those with high serum 25(OH)D level.

There were 17 preterm infants complicated with BPD in the RDS group. The comparison of the BPD group (n=17) and non-BPD group (n=65) showed that the gestational age of the preterm infants in the BPD group was significantly lower than that in the non-BPD group ($P<0.05$) (Table I). The average serum 25(OH)D level in children with concurrent BPD was 8.18 ± 2.64 ng/mL, which was lower than that in the non-BPD group (11.53 ± 4.80 ng/mL) with a statistically significant difference ($P<0.05$), as shown in Fig. 2B. The multivariate regression analysis, with gestational age and 25(OH)D level as covariates, showed that low 25(OH)D level was an independent risk factor for BPD in preterm infants. The odds of BPD occurrence were 1.221 times higher for preterm infants with low serum 25(OH)D level, compared to those with high 25(OH)D level. With the average gestational age of the preterm infants being 30.55 ± 1.07 weeks, the BPD occurrence had no significant relationship with gestational age, as shown in Table II.

Table I. Comparison of the general characteristics between the different groups.

Variable	RDS group (n=82)	non-RDS group (n=36)	P value	BPD group (n=17)	non-BPD group (n=65)	P value
Gestational age (week)	30.55±1.07	32.23±0.81	<0.001	30.08±1.34	30.68±0.96	0.039
Birth weight (g)	1341.16±356.83	1755.56±276.16	<0.001	1215.88±378.50	1373.92±346.52	0.104
Cesarean section (n, %)	38(46.3)	19(52.7)	0.519	8(47.1)	30(46.2)	0.947
Sex (male/female)	50/32	19/17	0.405	11/6	39/26	0.723
Apgar score 1 min	5.43±1.82	6.28±1.50	0.015	5.47±1.81	5.42±1.83	0.912
Apgar score 5 min	7.32±1.13	7.53±0.84	0.319	6.94±1.34	7.41±1.06	0.125
Birth season (spring/summer/fall/winter)	10/26/22/24	9/8/9/10	0.337	3/6/7/1	7/20/15/23	0.100
Prenatal use of hormones (n, %)	25(30.5)	11(30.5)	0.994	2(11.8)	23(35.4)	0.060
Gestational diabetes (n, %)	11(30.3)	4(11.1)	0.729	1(5.9)	10(15.4)	0.306
Severe preeclampsia (n, %)	8(9.5)	4(11.1)	0.823	0(0.0)	8(12.3)	0.128

DISCUSSION

The placental transport of vitamin D occurs mainly in late pregnancy, so preterm babies are more likely to suffer from vitamin D deficiency at birth (7). The vitamin D deficiency rate was 82.2%, consistent with 64% in the United States and 83% in India (8). Maternal vitamin D deficiency affects placental

development and weight, leading to an increased RDS and BPD incidence (9). In the present study, the RDS and BPD incidences in infants with severe and mild vitamin D deficiency were significantly higher than those with insufficient vitamin D.

Studies have shown that the risk of adverse respiratory consequences in newborns increases when maternal vitamin D levels are low (10). The

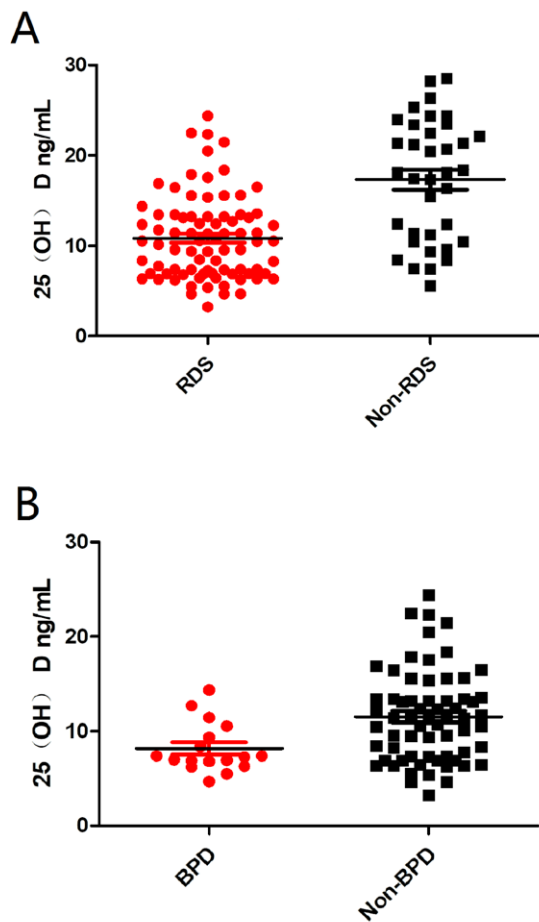


Fig. 2. Comparison of serum 25(OH)D levels. *A)* RDS and non-RDS groups; *B)* BPD and non-BPD groups.

level of 25(OH)D in the RDS group was significantly lower than that in the non-RDS group. It is reported that vitamin D deficiency is an independent risk factor for the occurrence of RDS (11), which is consistent with our results. In the 82 RDS infants, 17 had BPD and no BPD occurred in the non-RDS group, which also indicated that BPD was based on severe RDS as the primary disease. Previous studies have shown that serum levels of 25(OH)D were negatively correlated with the incidence of BPD, and the severity of the disease was negatively correlated with 25(OH)D in BPD infants (12), which also coincide with our study. Furthermore, this study showed that the occurrence of BPD had no significant relationship with gestational age, which might be related to the small range of gestational age and small sample size in this study group.

In summary, vitamin D deficiency is common in preterm infants and increases the risk of RDS and BPD, which would be prevented by appropriate vitamin D supplementation during pregnancy.

ACKNOWLEDGEMENTS

We wish to thank the staff in the Department of Pediatrics of Zhengzhou First People's Hospital for their assistance.

Table II. Multivariate logistic regression analysis of factors affecting the occurrence of RDS and BPD.

Subject	Factor	β	S.E	Wald statistic	Odds ratio	P value	95% confidence interval
Occurrence of RDS	Gestational age	2.179	0.560	15.165	8.841	0.000	[2.952,26.476]
	Birth weight	-0.001	0.001	0.661	0.999	0.416	[0.997, 1.001]
	Apgar score 1 min	0.216	0.185	1.365	1.241	0.243	[0.864, 1.782]
	25(OH)D	-0.187	0.060	9.760	1.206	0.002	[1.072,1.356]
Occurrence of BPD	Gestational age	0.421	0.275	2.342	1.523	0.126	[0.889, 2.612]
	25(OH)D	0.200	0.087	5.252	1.221	0.022	[1.029, 1.449]

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The role of ultrasound in detection of a lesion of the peripheral nerve: a case consideration

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To the Editor,

Foot drop is a syndrome characterized by deficit of dorsiflexion and eversion of the foot and toe extension with consequent drooping and intra-rotated foot (equino-varus deformity) and steppage or equine gait: the subject tends to walk with an exaggerated flexion of the hip and knee to prevent the toe of the falling foot from crawling on the floor. Foot drop can be caused by a variety of lesions or disorders with central or peripheral localization along the neuromotor pathway that ends in the dorsiflexor muscles of the foot (1, 2). Therefore, the differential diagnosis includes lesions involving the first and second motor neurons, the L5 root, the lumbosacral plexus, the sciatic nerve or the common peroneal.

Peroneal neuropathy is the most frequent cause of foot drop as well as the most frequent mononeuropathy of the lower limb and 15% of mononeuropathies in adults (2, 3). The superficial position of the nerve and adjacent to the bone surface makes it susceptible to external compression, particularly at the level of the head and the fibular neck near which it becomes superficial. Conditions favoring nerve compression are habitual postures such as crossing the legs and squatting, prolonged immobilization or weight loss that causes the depletion of adipose tissue that acts as a protection for the nerve at the level of the fibula

head (1, 4). Other causes of peroneal neuropathy include traumatic or iatrogenic injuries of the nerve, nerve entrapment at the level of the fibrous tunnel below the peroneus longus muscle, space-occupying intrinsic or extrinsic lesions to the nerve, vasculitis, diabetes mellitus and hereditary neuropathies such as Hereditary Neuropathy with Liability to Pressure palsies (HNPP) (5-8).

CASE REPORT

We report the case of a 36-year-old male patient who comes to our observation with foot drop following weight loss and intense exercise. In the last 7 months, the patient reported difficulty in lifting the right forefoot associated with pain and paresthesias in the anterior leg. The disorder has increased in intensity over the past 2 months. On neurological physical examination, right steppage, squatting and regular ascent were observed. Cranial nerves were normal. Upper limbs showed normal tone, strength and tropism. Lower limbs hyposthenia of the anterior tibial muscle and extensor of the right big toe, regulating the remaining tropism, tone and strength. On deep and superficial sensitivity, tactile hypoesthesia and puncture of the dorsum of the foot and the anterior lodge of the right leg are noted.

Key words: nervous ultrasound, electromyography, neurography, peripheral neuropathy

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During an ultrasound scan (US) performed with a high-frequency linear probe, there was a marked echo-structural subversion and thinning of about 50% of the external popliteal sciatic nerve at the level of the fibula head. The P. Doppler signal showed nerve hyperemia, and during the dynamic maneuvers, a further reduction in the caliber of the nerve was detected due to neuro-muscular impingement. No compressive musculoskeletal expansive formations were noted. Electromyographic and electroneuronographic examinations, the latter with

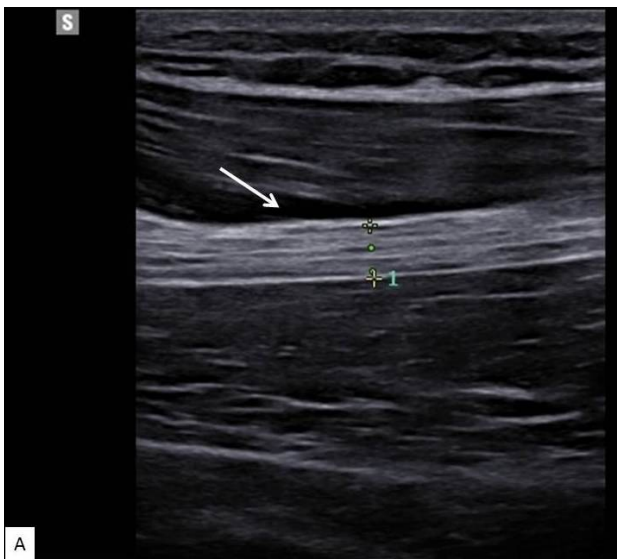


Fig. 1. Long axis ultrasound examination of the sciatic nerve showing regular thickness and echostructure (1-arrow).

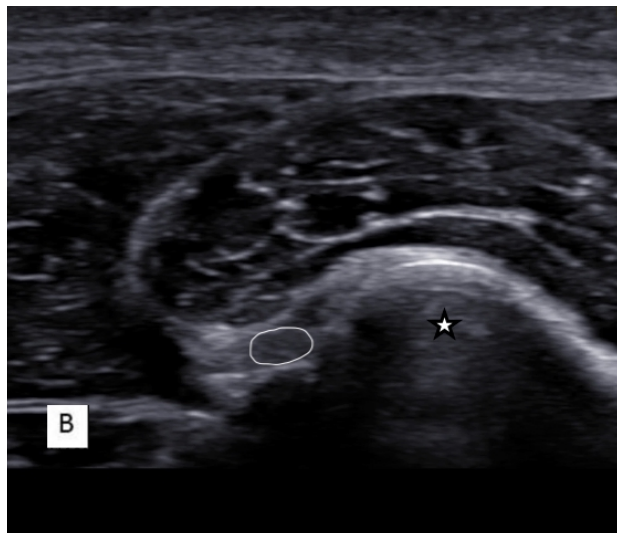


Fig. 2. Short axis ultrasound examination of the external popliteal nerve (circle) which presents regular echo structure and thickness at this level of the fibula head (star).

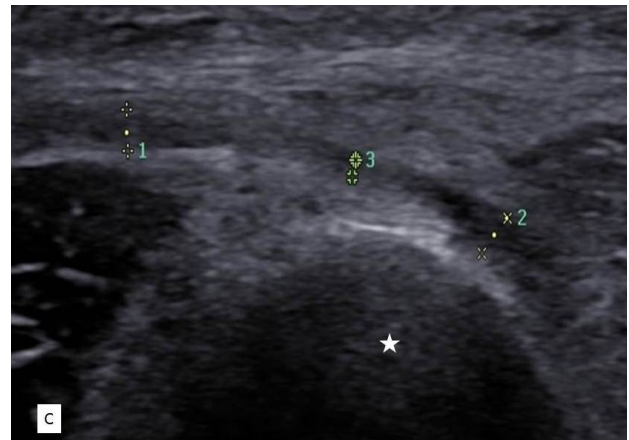


Fig. 3. Ultrasound examination of the long axis of the external popliteal sciatic nerve showing regular thickness and ecostructure (1-2) and reduced thickness and inhomogeneous echo structure (3) at the head of the fibula (star).

reduced nerve conduction velocity, confirmed the presence of a motor lesion of the external popliteal sciatic nerve. The patient underwent a cycle of neuro-protectors and neurological rehabilitation with a moderate functional recovery after 3 months of treatment (Fig. 1-3).

DISCUSSION

The diagnosis of fibular neuropathy is based on physical examination and electrophysiologic studies. A careful anamnestic evaluation allows to identify causes or favoring factors. Upon physical examination, atrophy of the anterolateral compartment of the leg (mainly the anterior tibialis) can occur and more often the sensory symptoms are limited to the back of the foot and toes (1-3). In addition to those of the anterolateral compartment (anterior tibialis, peroneal and extensor fingers) it is also important to examine the function of the gluteal muscles, the hamstrings and the leg in particular the posterior tibialis to exclude proximal pathologies such as L5 radiculopathy, lumbar plexopathy and a lesion of the lumbosacral trunk in which we also find deficiencies of these muscles (7).

Evocation of local pain and Tinel's sign positivity following palpation of the common peroneal nerve at the level of the head and neck of the fibula are

signs of common peroneal nerve neuropathy. Electrodiagnostic studies, which include nerve conduction assessment and electromyography, are the first-line tests for diagnosing fibular neuropathy, differentiating it from other injuries and providing an estimate of injury severity and prognosis (2, 9). Imaging, in particular US and magnetic resonance imaging (MRI), can identify in addition to the structural anomalies of the nerve also the cause of the disease, thus influencing management. X-ray and computerized tomography scan (CT) of the knee and ankle can be useful for identifying fractures, dislocations, or bone injuries. CT can additionally highlight space-occupying lesions that cause compression on the nerve (10). MRI and US may show signs of suffering from peripheral nerves such as morphological (swelling or thinning) and structural (loss of fascicular architecture) abnormalities as well as variations in normal echogenicity or signal intensity. They can also highlight the extent of damage, abnormal course of the nerve, deviation by masses extrinsic or intrinsic to the nerve.

MRI compared to the US provides a more panoramic assessment of the surrounding soft tissues and bone structures that may contribute to nerve entrapment. MRI allows advancing diagnostic hypotheses about the nature of any intrinsic or extrinsic lesions because of its excellent contrast resolution, multiparametricity, and use of contrast medium (9-10). In addition, it can detect muscle abnormalities caused by denervation such as muscle edema and fat infiltration (10).

The US has numerous advantages including cost-effectiveness, wide availability in the area, better patient compliance by avoiding the need for prolonged and in some cases painful immobilization. The use of color Doppler can give additional information. Usually, the presence of internal vascular signals to the peripheral nerves is to be considered pathological. Color Doppler can facilitate the distinction of the nerve from vascular structures or can determine the presence of vascular signals from intrinsic or extrinsic masses (11-12). The US exceeds the limit of MRI as a static exam, allowing for a dynamic evaluation that can show alterations in real-time, such as transient nerve entrapment. It also

allows for an immediate comparative examination with the unaffected side (8-11).

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