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MiR-548ar-3p increases cigarette smoke extract-induced chronic obstructive pulmonary disease (COPD) injury through solute carrier family 17 member 9 (SLC17A9)

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Abstract: This study investigated the effect of microRNA mir-548ar-3p on cigarette smoke extract (CSE)-induced chronic obstructive pulmonary disease (COPD). High-throughput sequencing was performed on peripheral blood from smoking COPD patients and non-smoking individuals with normal pulmonary function, and miR-548ar-3p RNA, possessing large differential expression was selected. Experimental groups were divided into control, experimental model (EM), EM+mimic miRNA, negative control (NC) and EM+miR-548ar-3p groups; an empty vector or miR-548ar-3p mimic was transfected into human bronchial epithelial (HBE) cells. A COPD model was established by treating HBE cells with CSE. Cell viability, apoptosis and solute carrier family 17 member 9 (SLC17A9) protein expression were examined by cell counting kit-8, flow cytometry and Western blotting, respectively. Cell viability in the EM+miR-548ar-3p group decreased significantly, and the apoptosis rate and SLC17A9 protein expression increased significantly compared with the control ($P<0.05$, all groups). In smoking COPD patients, interferon (IFN)-γ and interleukin (IL)-17α expression detected by ELISA was significantly higher than in normal individuals. miR-548ar-3p expression was significantly lower ($P<0.05$, all groups). These findings suggest that miR-548ar-3p was expressed at a lower level in COPD patients. miR-548ar-3p may increase the extent of CSE-induced COPD injury through SLC17A9.

Keywords: chronic obstructive pulmonary disease (COPD); cigarette smoke extract (CSE); miR-548ar-3p; solute carrier family 17 member 9 (SLC17A9); high-throughput sequencing; human bronchial epithelial cells

INTRODUCTION

COPD is a lung disease causing continuous airflow restriction and airway remodeling, accompanied by a chronic inflammatory response of the lung and airways to toxic particles from the air. COPD is generally preventable and treatable [1,2]. Most COPD patients have clinical symptoms such as chronic obstructive bronchitis, emphysema and mucus blockage, which cause continuous obstruction of respiratory airflow [3]. COPD is the third leading cause of death in the world after heart disease and stroke [4]. There are many factors that lead to COPD, including environmental factors such as air pollution, harmful dust inhalation, as well as long-term smoking, crowded living conditions, genetic and individual risk factors, such as neonatal pulmonary dysplasia [5]. Smoking is generally considered an important independent risk factor for COPD, with more than 75% of COPD deaths related to smoking. Cigarette smoke extract (CSE) contains thousands of chemical components, most of which are important factors leading to COPD [6]. However, the pathogenesis of COPD caused by smoking is still unclear, and there are no effective treatments or prevention methods for COPD. Therefore, it is of great importance to study the mechanisms of COPD induced by smoking.

MicroRNAs (miRNA)s are a class of noncoding RNAs with 18-22 nucleotide sequences, which cannot be directly translated into proteins but can negatively

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regulate the expression of the target gene by binding to the three prime untranslated region (3'-UTR) of the target mRNAs [7]. miRNA can participate in many processes, such as cell proliferation, development, differentiation, apoptosis and aging [8]. miRNAs were shown to be involved in respiratory-related diseases. miR-141 [9], miR-21 [10] and miR-29a [11] play a role in non-small cell lung cancer. miR-92a [12] and miR-200 [13] regulate genes related to pulmonary fibrosis. miRNAs exhibit differential expression in COPD; for example, miR-21 increases autophagy and promotes apoptosis of bronchial epithelial cells [6,14], miR-191 and miR-126 can promote the release of ceramide-synthesis enzyme acid sphingomyelinase and thus lead to clearance of apoptotic cells in lung endothelial cells [15]. miR-210 can inhibit autophagy related 7 (*ATG7*) gene expression and promote myofibroblasts differentiation in lung fibroblasts [16].

COPD is accompanied by the abnormal expression of different miRNAs. The expression of miR-34a is significantly upregulated in lung tissue of COPD patients [17], the expression of miR-7 is upregulated, and the expression of miR-28-3p and miR-100 is downregulated in the serum of COPD patients [18]. miR-146a plays a potential role in the abnormal inflammatory response caused by COPD [19], indicating that the abnormal expression of miRNA is closely related to the occurrence and development of COPD.

The occurrence of COPD is also related to the accumulation and transport of adenosine triphosphate (ATP) [20]. Solute carrier family 17 member 9 (SLC17A9), also known as the vesicular nucleotide transporter (VNUT), is a member of the transmembrane protein family that can participate in the transport of small molecules [21]. It is mainly responsible for the transport of ATP. SLC17A9 regulates lysosomal proteolysis through the accumulation of ATP, which can attenuate the activity of proteases and promote cell death [22,23]. SLC17A9 is widely expressed in various organs and plays a key role in ATP transport and secretion in hepatocytes, alveolar epithelial cells, mesenteric epithelial cells and astrocytes [21, 24]. In lung cancer cell A549, ATP accumulates in vesicles through SLC17A9, induces ATP release and regulates cell autocrine signaling through transforming growth factor beta 1 (TGF-β1) [25].

Although there are many studies on miRNAs in respiratory system-related diseases and COPD, the molecular regulation of COPD and miRNAs is incomplete and needs further exploration. In the present study, miRNA with differential expression was selected by high-throughput sequencing of peripheral blood from smokers with COPD and non-smoking individuals with normal pulmonary function. A COPD model was established by treating human bronchial epithelial (HBE) cells with CSE, and the effect of the selected miRNA on cell proliferation, apoptosis, and SLC17A9 protein expression was examined with the aim of obtaining a theoretical basis for the pathogenesis, treatment, and prevention of COPD. The findings of this study may help to develop a novel therapeutic approach for the treatment and prevention of COPD.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Institutional Review Board of the Third Affiliated Hospital of Zunyi Medical University (The First People's Hospital of Zunyi). Informed consent was obtained from all subjects.

Cells, hsa-miR-548ar-3p and reagents

HBE cells (HUM-iCell-a005) were obtained from iCell Bioscience Inc., Shanghai, P.R. China. Mimic hsa-miR-548ar-3p (RX004775) and empty vector (RX004775) were purchased from General Biosystems (Anhui) Corp. Ltd., Anhui, P.R. China. The following reagents were used: Dulbecco's Modified Eagle's Medium (DMEM) complete medium (KGM12800S), cell counting kit (CCK)-8 (KGA317, NanJing KeyGen Biotech Co., Ltd., Jiangsu, P.R. China); lipofectamine 3000 transfection reagent (L3000015, Invitrogen, Waltham, MA, USA); interferon gamma (INF-γ) enzyme-linked immunosorbent assay (ELISA) kit (MM-0033H1), interleukin (IL)-17α ELISA kit (MM-2035H1), miRNA cDNA synthesis kit (CW2141S), miRNA purification kit (CW0627S), TRIzol reagent (CW0580S), HiFiScript cDNA synthesis kit (CW2569M), bicinchoninic acid (BCA) protein assay kit (CW0014S) (Beijing ComWin Biotech Co., Ltd.,

Beijing, P.R. China); annexin V-FITC/PI apoptosis kit (AP101-100-kit, MultiSciences (Lianke) Biotech Co., Ltd., Zhejiang, P.R. China); polyvinylidene difluoride (PVDF) membrane (IPVH00010, MilliporeSigma, MA, USA); mouse monoclonal anti-actin (TA-09), horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin G (IgG) (H+L) (ZB-2305), horseradish peroxidase (HRP)-labeled goat anti-rabbit (IgG) (H+L) (ZB-2301) (Beijing ZSGB-BIO Co., Ltd., Beijing, P.R. China); SLC17A9 antibody (OM175445, Omnimabs, CA, USA).

Instruments

The following instruments were used: fluorescent quantitative polymerase chain reaction (PCR) instrument (CFX Connect™), ultra-high sensitivity chemiluminescence imaging system (Chemi Doc™ XRS+), Bio-Rad Laboratories (Shanghai) Co., Ltd., Shanghai, P.R. China); microplate reader (RT-6100, Rayto, Shenzhen, Guangdong, P.R. China), vertical protein electrophoresis instrument (DYY-6C, Beijing Liuyi Instrument Factory, Beijing, P.R. China); microscope (CX41, Olympus Corp., Shinjuku, Tokyo, Japan).

Experimental groups

For selection of miRNA, the subjects were allocated into non-smoking individuals with normal pulmonary function, designated as the Control group, and smoking patients with COPD (COPD group) (n=6/group). Peripheral blood samples were obtained from the subjects. For transfection efficiency verification, HBE cells were randomly divided as follows: the Control group comprising HBE cells with no intervention; the mimic negative control (NC) group containing HBE cells that were transfected with empty vector for 48 h; the miR-548ar-3p group containing HBE cells transfected with miR-548ar-3p for 48 h. To study the effect of the selected miRNA on the COPD cell model, HBE cells were randomly allocated as follows: the Control group (HBE cells with no intervention); experimental model (EM) group (HBE cells treated with 2.5% CSE for 48 h); EM+mimic NC group (HBE cells transfected with empty vector for 48 h and treated with 2.5% CSE for 48 h); EM+miR-548ar-3p group (HBE cells transfected with miR-548ar-3p for 48 h and then treated with 2.5% CSE for 48 h).

miR-548ar-3p expression detection by fluorescence quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from cells with TRIzol reagent and reverse-transcribed with the cDNA synthesis kit as per the manufacturer's instructions. Quantifications of target gene mRNA was performed using the $2^{-\Delta\Delta Ct}$ method. The operating system was as follows: 9.5 μL RNase-free deionized H_2O, 1 μL cDNA/DNA, 1 μL upstream primer, 1 μL downstream primer, 12.5 μL 2xULtraSYBR mixture. The reaction procedure was as follows: three-step pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 57°C for 30 s, extension at 72°C for 30s, cycle 40. Melting curve analysis: 95°C 15 s, 58°C 1 min, 95°C 15 s, 58°C 15 s, 58°C 15s, 95°C 0.5°C. The primers were as follows: U6 F: GCTTCGGCAGCACATATACTAAAAT, primer length 25 bp; U6 R CGCTTCACGAATTTGCGTGTCAT, primer length 23 bp. The product lengths were 91 bp, and the annealing temperature was 61.5°C.

Detection of inflammatory factors IFN-γ and IL-17α by ELISA

The contents of IFN-γ and IL-17α in the peripheral blood of the Control group and the COPD group were determined using an ELISA kit according to the manufacturer's instructions. The absorbance (OD value) of each well was measured at 450 nm.

HBE cell culture

After the HBE cells were 90% confluent, the cell culture supernatant was discarded. The cells were washed with pre-warmed phosphate buffered saline (PBS) at 37°C, digested with trypsin, centrifuged and subcultured at a ratio of 1:3. The cells were placed in a 5% CO2, 37°C constant temperature incubator and the medium was changed every other day. The cell culture medium was DMEM complete medium containing 10% fetal bovine serum and 1% double antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin).

MicroRNA transient transfection

HBE cells in the logarithmic growth phase were plated, and 2×10^5 cells were seeded in 6-well plates. The culture medium was replaced with incomplete DMEM medium the next day. Mimic NC (25 nM) or miR-548ar-3p mimic (25 nM) were diluted in opti-MEM and incubated at room temperature for 5 min. Lipofectamine 3000 was diluted with opti-MEM, and incubated at room temperature for 5 min. The two opti-MEM dilutions were mixed evenly, incubated at room temperature for 15 min, added to the cells, cultured for 6 h, and replaced with fresh DMEM complete medium. Subsequent experiments were performed after 48h.

Preparation of CSE and establishment of the experimental COPD cell model

According to a previous study [26], a cigarette was connected to a designated suction filter device for preparing CSE. The cigarette was lit and smoke was continuously drawn into the device at -5 cm H_2O (about -0.49 kPa) for 5 min. The inhaled smoke was introduced into 10 mL PBS buffer through an inlet of the vacuum container to make a suspension. The suspension was adjusted to pH 7.4 with 1 M NaOH solution and filtered through a 0.22 μm microporous membrane for the CSE stock solution. The prepared CSE solution was used within 1 h. Establishment of the experimental COPD cell model: the cell culture medium that had been cultured was replaced with medium containing 2.5% CSE solution and cultured for 48 h to obtain the COPD cell model.

Cell viability detection

Cell suspension (100 μL, 7×10^3 cells/well) was seeded in a 96-well plate and the plate was incubated in a humidified incubator at 37°C with 5% CO_2 for 24 h. Ten μL of CCK-8 solution were added to each well of the plate using a repeating pipettor. The plate was incubated for 2 h in the incubator. The absorbance was measured at 450 nm using a microplate reader.

Apoptosis detection by flow cytometry

Apoptosis analysis was performed using the Annexin V-FITC analysis kit according to the manufacturer's instruction. Cells in each group were collected, digested with trypsin without ethylenediaminetetraacetic acid (EDTA) and washed twice with ice-cold PBS; 1×10^6 cells/mL were collected by centrifugation at 151×g for 3 min and resuspended in 300 μL 1xbinding buffer. Three μL of Annexin V-FITC and 5 μL of 7-aminoactinomycin D (7-AAD) were added according to the manufacturer's protocol. After 10 min of incubation at 4°C in the dark, 200 μL of precooled 1×binding buffer was added. The apoptosis rate was detected using flow cytometry and the experiment was performed three times.

SLC17A9 protein detection by Western blotting

The cells were harvested and lysed with cell lysis buffer. The supernatant was collected after centrifugation at 9705×g for 10 min at 4°C. Total proteins were extracted, and the protein concentration was determined using bicinchoninic acid (BCA) according to the manufacturer's instruction. Proteins (50 μg per lane) were separated using 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was rinsed with Tris-buffered saline (TBS) for 10-15 min, placed in Tris-buffered saline with Tween (TBST) blocking buffer containing 5% (w/v) skimmed milk powder. The membrane was incubated at 4°C overnight following the addition of primary antibodies (internal reference anti-actin (1:2000), and target protein SLC17A9 antibody (1:500)). The membrane was then rinsed with TBST three times and incubated at room temperature for 2 h with secondary antibodies [(HRP-labeled goat anti-mouse (IgG) (H+L) (1:2000), and HRP-labeled goat anti-rabbit (IgG) (H+L) (1:2000)]. Protein bands were detected using an enhanced chemiluminescence kit (PerkinElmer Inc., MA, USA) and quantified as the ratio to actin. Quantification was performed using Quantity One software. The formula for the calculation of relative protein expression was: [IOD/Area (ratio of gray value to area) of target protein (SLC17A9)]/[IOD/Area (ratio of gray value to area) of internal reference protein (actin)].

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). All

experiments were performed 3 times. An independent sample t-test was used for quantitative value comparison between two groups. The mean values of multiple groups were compared by one-way ANOVA and the Student-Newman-Keuls (S-N-K) method was used for pairwise comparison. P<0.05 was considered statistically significant.

RESULTS

Expression of miR-548ar-3p in the peripheral blood of COPD patients

Six clinical samples of peripheral blood from each non-smoking individual with normal pulmonary function and each smoker with COPD were collected. By high-throughput sequencing, miRNAs with large differential expression were identified. As shown in Fig. 1A, miR-1299, miR-1910-3p, miR-4454, miR-199b-5p, miR-21-3p, miR-4785 and miR-6718-5p expression was upregulated, while miR-3690 and miR-548ar-3p expression was downregulated. Among the downregulated miRNAs, the downregulated expression of miR-548ar-3p was most obvious. As shown in Fig. 1B, analysis of peripheral blood samples by qPCR revealed that the expression level of miR-548ar-3p in the COPD group was significantly lower than in the Control group (P<0.05). In addition, as shown in Fig. 1C and D, measurement by ELISA established that two inflammatory factors, IL-17α and INF-γ, in the peripheral blood of the COPD group were significantly higher than those in the Control group (P<0.05). These two inflammatory factors play a role in COPD and are considered systemic markers in COPD [27].

Transfection efficiency of miR-548ar-3p mimic in HBE cells as verified by qPCR

As shown in Fig. 2, a mimic miRNA sequence of miR-548ar-3p was constructed and successfully transfected into HBE cells. The transfection efficiency was verified by qPCR. Compared with the Control group, the expression of miR-548ar-3p in HBE cells of the miR-548ar-3p group was significantly increased (P<0.05), indicating successful transfection of the miRNA sequence into HBE cells.

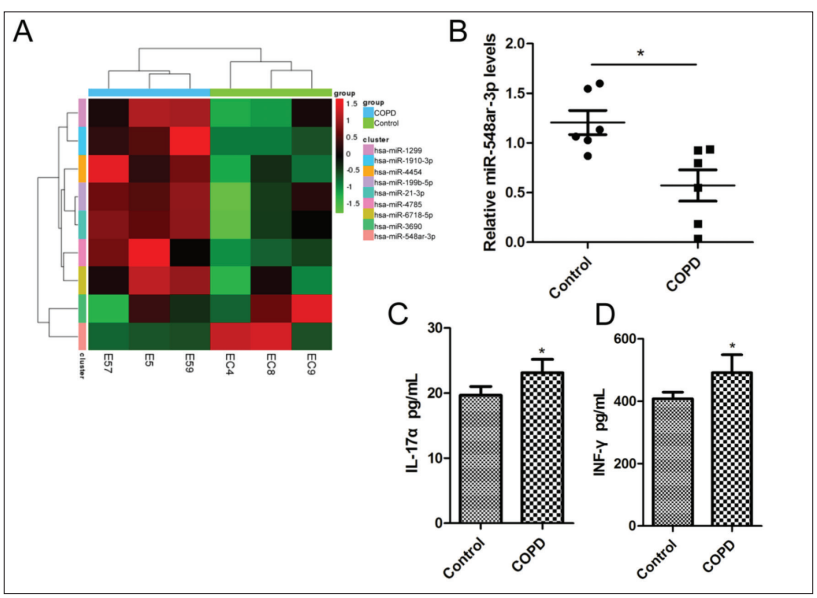

Fig. 1. High-throughput sequencing of miRNAs. miR-1299, miR-1910-3p, miR-4454, miR-199b-5p, miR-21-3p, miR-4785 and miR-6718-5p expression was upregulated, while miR-3690 and miR-548ar-3p expression was downregulated (**A**). Relative expression of miR-548ar-3p; the expression of miR-548ar-3p in the COPD group was significantly lower than in the Control group (**B**). IL-17α and INF-γ protein levels in peripheral blood (**C**). The contents of IL-17α and INF-γ in the peripheral blood of the COPD group were significantly higher than in the Control group (**D**). *P<0.05.

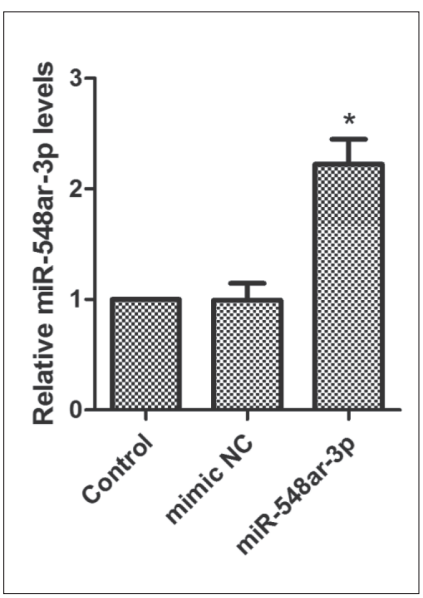

Fig. 2. Transfection efficiency of miR-548ar-3p mimic (miRNA sequence) in HBE cells as verified by qPCR. The expression of miR-548ar-3p in HBE cells of the miR-548ar-3p group was significantly increased, indicating successful transfection of the miRNA sequence into HBE cells. *P<0.05.

Effect of miR-548ar-3p on cell viability and apoptosis in the COPD cell model

Based on the experimental grouping, the specific groups were transfected, and treated with 2.5% CSE accordingly. After 48 h, the apoptosis rate was assessed by flow cytometry and the cell viability rate was examined by CCK-8. As shown in Fig. 3A-C, compared with the Control group, the apoptosis rate of HBE cells in the EM group was significantly increased and cell viability was significantly decreased (P<0.05), indicating that the COPD cell model was successfully established. In addition, compared with the EM group, apoptosis of HBE cells in the EM+miR-548ar-3p group was significantly increased and cell viability was significantly decreased (P<0.05, both), indicating that the extent of cell injury was elevated.

Effect of miR-548ar-3p on protein expression of SLC17A9 in the COPD cell model

According to the prediction results of miRNA targets in the "TargetScanHuman" website, *SLC17A9* may have a targeting relationship with miR-548ar-3p. The relative change in expression of SLC17A9 protein in each group of HBE cells was estimated by Western blotting. As can be seen in Fig. 4, compared to the Control and EM group, protein expression of SLC17A9 in HBE cells of the EM+miR-548ar-3p group was significantly increased (P<0.05).

DISCUSSION

COPD is a complex disease with a high incidence rate and mortality. Smoking is an important factor leading to the occurrence of COPD. Due to the complexity

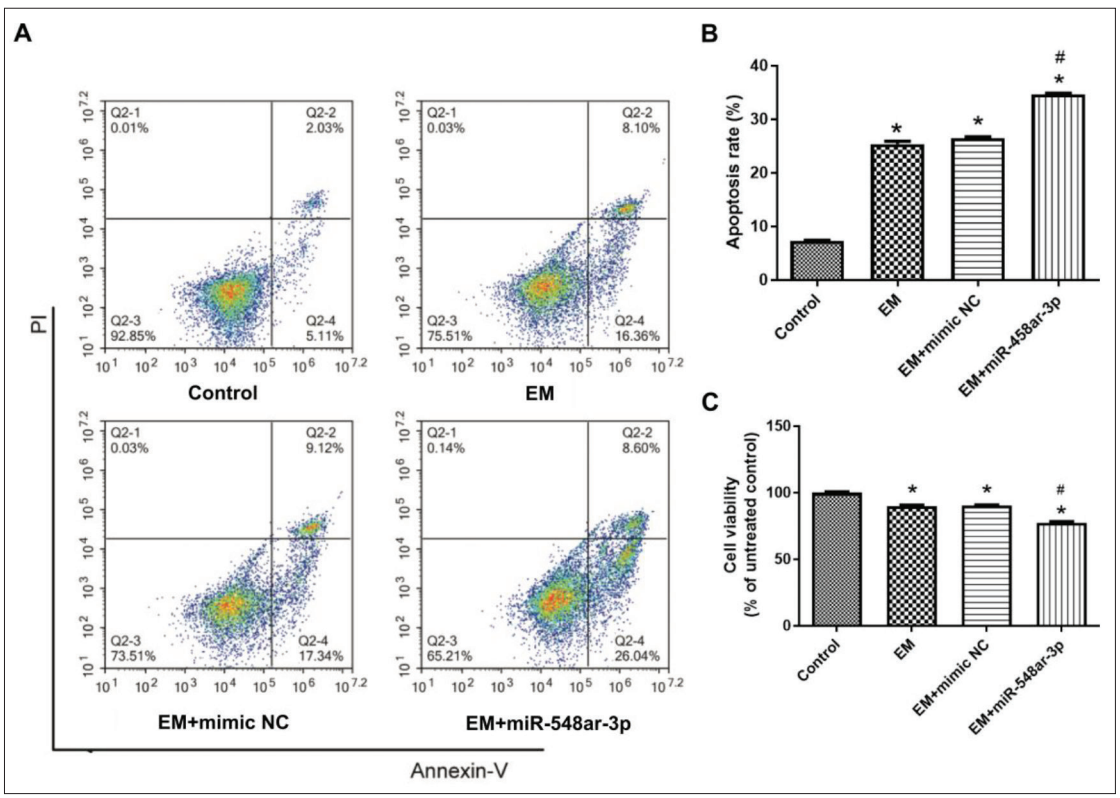

Fig. 3. Effect of miR-548ar-3p on apoptosis. Scatter plots (**A**), quantitative measurement (**B**) and cell viability (**C**). Compared with the Control group, the apoptosis rate of HBE cells in the EM group was significantly increased and cell viability was significantly decreased, indicating that the COPD cell model was successfully established. Compared with the EM group, apoptosis of HBE cells in the EM+miR-548ar-3p group was significantly increased, and cell viability was significantly decreased, indicating that the extent of cell injury was aggravated. Compared with Control group *P<0.05; EM group, #P<0.05. NC – negative control, EM – experimental model.

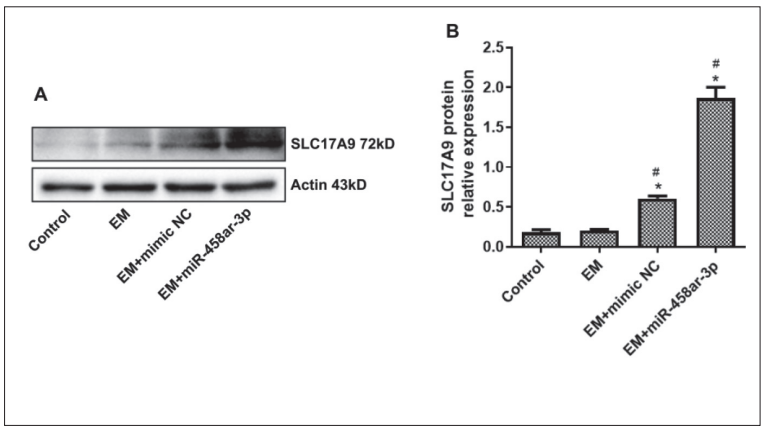

Fig. 4. Effect of miR-548ar-3p on SLC17A9 protein expression. Western blot image (**A**) quantitative measurement (**B**). Compared with the Control group and the EM group, the expression level of SLC17A9 in HBE cells of the EM+miR-548ar-3p group was significantly increased; Compared with Control group *P<0.05; EM group, #P<0.05. NC – negative control, EM – experimental model. The formula for the calculation of relative protein expression was: [IOD/Area (ratio of gray value to area) of target protein (SLC17A9)]/[IOD/Area (ratio of gray value to area) of internal reference protein (actin)].

of CSE and pathogenesis of various chemical components such as toxic oxidants, the mechanism of COPD induced by smoking is complex. In this study, miRNAs exhibiting large differential expression, and assumed to be key genes of the EM, were screened in peripheral blood samples of non-smoking individuals with normal pulmonary function and from smokers with COPD by miRNA high-throughput sequencing. Seven upregulated and 2 downregulated miRNAs were identified. Among the downregulated miRNAs, mir-548ar-3p was most significantly downregulated and was therefore selected.

miR-548 belongs to a unique miRNA gene family of primates with a high nucleotide divergence level and low conservation and plays a role in different biological processes [28]. At present, miR-548 is mainly studied in cancers. miR-548 has been shown to be strongly expressed in different tumor tissues and to participate in the regulation of tumor progression. miR-548ar can downregulate the expression of nuclear enriched abundant transcript 1 (NEAT1) in breast cancer cells and promote apoptosis [29]. miR-548c-3p is highly expressed in prostate cancer and inhibits phosphatase and tensin homolog (PTEN) expression [30]. miR-548a-5p can negatively regulate Tg737 in hepatocellular carcinoma and promote hepatoma cell proliferation [31]. miR-548b is expressed at a low level in lung cancer tissues and was shown to inhibit cell proliferation and to promote apoptosis through the phosphatidylinositol-3-kinase and protein kinase B (PI3K/AKT) pathway [32]. The role of miR-548 in COPD has not been reported yet. Herein we observed that the expression of miR-548ar-3p was downregulated in COPD patients. HBE cells were treated with CSE to establish the COPD cell model, and after transfection with mimic NC and miR-548ar-3p, respectively, the viability of HBE cells in the EM+miR-548ar-3p group was decreased and apoptosis was increased as compared to the EM group, suggesting that miR-548ar-3p could inhibit cell proliferation and promote apoptosis and cause cell injury.

Using predictive analysis, we determined that miR-548ar-3p establishes a targeting relationship with the *SLC17A9* gene, and that *SLC17A9* can regulate lysosomal proteolysis through the accumulation of ATP, promoting cell injury. We observed protein expression of SLC17A9 in the CSE-induced COPD model. In the COPD cell model with considerably enhanced expression of miR-548ar-3p, protein expression of SLC17A9 was increased. Therefore, in COPD, miR-548ar-3p establishes a targeting relationship with *SLC17A9*, influencing its expression and causing cell injury.

There are some limitations in this study. Due to the difficulty in obtaining suitable clinical samples, the number of samples tested for verification after high-throughput sequencing might not circumvent the existence of individual differences. At the cellular level, this study was mainly focused on the effect on apoptosis rate and cell viability. At present, studies of COPD and miR-548ar-3p mechanisms are scarce, and research into miR-548ar-3p and *SLC17A9* gene targeting needs to be expanded.

CONCLUSION

The expression of miR-548ar-3p in the peripheral blood of COPD patients was lower, and inflammatory factors IL-17α and INF-γ were increased. In the CSE-induced COPD cell HBE cell model, upregulated

expression of miR-548ar-3p led to increased apoptosis and decreased cell viability. The expression of *SLC17A9* gene was consistent with miR-548ar-3p. We report for the first time the role of miR-548ar-3p in COPD, while the potential function of SLC17A9 in COPD was expounded, which could provide a novel target for the prevention and treatment of COPD.

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Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: Data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Zhang%20et%20al_7465_Data%20Report.pdf

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