MiR-4458 Regulates Autophagy and Apoptosis By Targeting P53 In Chronic Obstructive Pulmonary Disease.

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Research Article

Keywords: miR-4458, chronic obstructive pulmonary disease, autophagy, apoptosis, tumor protein53

Posted Date: November 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1089877/v1>

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a progressive chronic airway disease of which tobacco smoking is a main risk factor. Numerous studies have revealed that microRNAs participate in the pathogenesis of COPD by regulating cell autophagy and apoptosis. The goal of this study was to elucidate the potential mechanism of miR-4458 in COPD.

Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to measure the levels of miR-4458 in cigarette smoke extract (CSE)-exposed human bronchial epithelial cells (HBEs) and peripheral blood mononuclear cells (PBMCs) of patients. The effects of miR-4458 on autophagy, cell viability and apoptosis in the CSE-exposed HBEs were assessed by western blot (WB), Cell Counting Kit-8 (CCK-8) assay and flow cytometric analysis. Targetscan and miRDB databases were used to predict the downstream targets of miR-4458. Luciferase reporter assay and rescue experiments were employed to ensure the target gene.

Results: MiR-4458 was downregulated in CSE-exposed HBEs and PBMCs of COPD patients. The overexpression of miR-4458 attenuated autophagy and apoptosis in CSE-exposed HBEs, while the downregulation of miR-4458 led to opposite results. Bioinformatics analysis predicted that P53 was a target gene of miR-4458, and luciferase reporter assay further verified this prediction. Rescue experiments showed that miR-4458 regulated cell autophagy and apoptosis via the P53-mediated AKT–mTOR signaling pathway.

Conclusions: This study reveals that miR-4458 plays a protective role in CSE-exposed HBEs. MiR-4458 is a promising therapeutic target for COPD treatment.

Introduction

Chronic obstructive pulmonary disease (COPD) causes a serious disease burden due to its high prevalence and mortality all over the world[1]. In a latest epidemiological survey, the prevalence of COPD in China was 13.6%[2]. The characteristics of COPD are persistent airflow limitation and respiratory symptoms because of chronic inflammation in the airways and alveoli caused by harmful particles[3]. Tobacco smoking remains as the foremost risk factor of COPD[4]. Tobacco controlling is no doubt an effective measure to reduce the prevalence of COPD; however, early diagnosis and effective treatment can reduce its mortality[5]. Therefore, it is imperative to search for early diagnostic markers and identify effective therapeutic targets of COPD.

MicroRNAs (MiRNA) are conserved non-coding RNAs, can mediate mRNA expression by post-transcriptional regulation[6]. When bonded by miRNAs, mRNAs may be degraded or translationally inhibited[7]. Studies have reported that miRNAs play prominent roles in the pathogenesis of COPD, which suggested their potential as new biomarkers and therapeutic targets in COPD[8–10]. In addition, previous studies have indicated that miR-4458 was low expressed in human tumors, such as non-small cell lung cancer[11, 12], breast cancer[13, 14], melanoma[15], colorectal cancer[16], and hepatocellular

carcinoma[17, 18]. These studies have identified miR-4458 is a tumor suppressor. Another study also revealed that miR-4458 was upregulated in lumbar disc degeneration and that it could induce the development of lumbar disc degeneration[19]. However, the role of miR-4458 in COPD and its mechanism of action have not been identified so far.

The tumor protein 53 (P53) is a transcription factor and a well-known tumor suppressor protein[20]. When stimulated by various stress signals, P53 can promote cell apoptosis, regulate autophagy, accelerate deoxyribonucleic acid repairment and induce cell cycle arrest[21, 22]. Previous studies have revealed that the P53 expression level was elevated in COPD and accompanied by increased incidence of apoptosis[23], which indicated P53 may be a promising therapeutic target in COPD. Apart from this, a study reported that miR-150 could attenuate apoptosis by suppressing P53 expression in cigarette smoke extract (CSE)-exposed human bronchial epithelial cells (HBECS)[24]. However, the targeted relationship between miR-4458 and P53 in COPD has not yet been verified.

In this study, we evaluated the expression level of miR-4458 in peripheral blood mononuclear cells (PBMCs) of COPD patients and CSE-treated 16HBECS and its role on autophagy and apoptosis in CSE-treated 16HBECS. Moreover, we explored the molecular mechanism underlying its functions. Our research provides a new prospective for clinical therapy of COPD.

Materials & Methods

Sample collecting and preparation

5 ml peripheral blood samples were obtained from 65 patients (20 non-smokers, 20 smokers without COPD and 25 smokers with COPD) at Qilu Hospital of Shandong University. Thereafter, PBMCs were separated using isolation fluids (TBD sciences, Tianjin, China). All patients underwent lung function tests. The inclusion criteria of COPD were: forced expiratory volume in 1 second / forced vital capacity (FEV1/FVC) < 0.7 after bronchodilator treatment, 40 to 80 years old, and smoking for at least 20 pack-years. Non-smokers and smokers without COPD had matched age and gender with COPD patients. The procedure of study was approved by the Medical Ethics Committee of Qilu Hospital of Shandong University and consisted with the Declaration of Helsinki (as revised in 2013). All patients signed informed consent forms.

Preparation of the CSE

CSE was prepared according to a previous method[25]. One cigarette without filter was prepared, and a vacuum pump was used to draw the smoke into a glass container filled with 10 ml serum-free RPMI-1640 medium (Gibco, USA). Then, the CSE was filtered, and the pH was titrated to 7.4. The absorbance of the CSE at 320-nm wavelength was standardized to optical density (OD) of 0.74 ± 0.05 . The obtained solution was referred to as 100% CSE.

Cell treatment

16HBECS and HEK293T cells were purchased from PRO-Cell Technology (Wuhan, China). The cells were cultured in 10% fetal bovine serum (Biological Industries, Israel) supplemented RPMI-1640 medium and incubated in 5% CO₂ environment at 37°C. 16HBECS were exposed to varying concentrations of CSE for 24 h. After explore the best concentrations (10%) of CSE, the cells were stimulated with 10% CSE for different times. Before exposure to CSE, miR-4458 mimics (sense5'-AGAGGUAGGUGUGGAAGAA-3', antisense5'-CUUCCACACCUACCUCUUU-3'), NC-mimics (sense5'-UUCUCCGAACGUGUCACGUTT-3', antisense5'-ACGUGACACGUUCGGAGAATT-3'), miR-4458 inhibitor (5'-AGAGGUAGGUGUGGAAGAA-3'), NC-inhibitor (5'-CAUUACUUUUGUGUAGUACAA-3') (GenePharma, Shanghai, China), pcDNA3.1(+)-P53 overexpression plasmid (Biosune Biotechnology, Shanghai, China) were transfected into 16HBECS using Lipofectamine 2000(Invitrogen, USA).

Cell Counting Kit 8 (CCK8) assay

In this study, a total of 2×10^3 cells/ml was planted into 96-well plates. Each group had four duplicate wells. Then, 10 μ l CCK8 reagents were added into each well. Subsequently, the OD of each well was detected at 450-nmwavelength 2 h later.

Apoptosis assessment

After application of different treatments, 16HBECS were harvested to measure the apoptosis rate using Annexin V-FITC/PI apoptosis detection kit (Elabscience, Wuhan, China). Then, a FACSCalibur flow cytometer (BD Biosciences) was employed to analyze the treated cells.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA of 16HBECS and PBMCs was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Then, cDNA synthesis was conducted with the PrimeScript RT reagent kit (TakaraBio, Japan). RT-qPCR was performed using the TB Green Premix Ex Taq II (TaKaRa) on LightCycler 480II (Roche, Basle, Switzerland). GAPDH and U6 were considered as endogenous controls. The gene specific primers sequences were as follows: GAPDH (F: 5'-GCACCGTCAAGGCTGAGAAC-3', R: 5'-TGGTGAAGACGCCAGTGG-3'), LC3B (F: 5'-CAGCATCCAACCAAATCCCG-3', R: 5'-TTGAGCTGTAAGCGCCTTCTAAT-3'), BECN (F: 5'-AACCGCAAGATAGTGGCAGA-3', R: 5'-CTCTCTGATACTGAGCTTCCTCC-3'), P53 (F: 5'-AGTCACAGCACATGACGGAG-3', R: 5'-GCCAGACCATCGCTATCTGA-3'), U6 (F: 5'-CAGCACATATACTAAAATTGGAACG-3', R: 5'-ACGAATTTGCGTGTCTATCC-3') and miR-4458 (F: 5'-CAAAACCAACCAGAGGTAGGTG-3', R: 5'-TATGCTTGTTCTCGTCTCTGTGTC-3'). The $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$ method were employed to quantified the mRNA levels of the genes.

Western blot assay

Total proteins were extracted using RIPA buffer (Beyotime, Shanghai, China) and separated by SDS-PAGE gels (Beyotime), then transferred onto PVDF membranes. Thereafter, the membranes underwent blocking and then incubated in specific primary antibody. The primary antibodies used were as follows: LC3B

(ab48394, Abcam, Cambridge, UK), BECN (3495, Cell Signaling Technologies, MA, USA), P53 (9282, Cell Signaling Technologies), Bax (89477, Cell Signaling Technologies), Bcl-2 (4223, Cell Signaling Technologies), p-AKT (4060, Cell Signaling Technologies), AKT (4691, Cell Signaling Technologies), p-mTOR (5536, Cell Signaling Technologies), mTOR (2983, Cell Signaling Technologies). Then, the membranes were incubated in secondary antibodies (Beyotime Biotechnology). Finally, the proteins were visualized by ECL solution using a Chemiluminescence Imager (Tanon, China).

Bioinformatics analysis and luciferase reporter assay

The potential target genes of miR-4458 were predicted by miRDB (<http://www.mirdb.org/>) and Targetscan (http://www.targetscan.org/vert_72/). A total of 1×10^4 cells/well HEK-293T cells were planted into 96-well plates. 24 h later, miR-4458 mimics or its negative control were co-transfected into the cells with pmirGLO-P53-wt or pmirGLO-P53-mut plasmid (Atagenix, Wuhan, China). Then, a Dual-Luciferase Reporter kit (Promega, WI, USA) was employed to detect the luciferase activities.

Statistical analysis

Statistical analysis was performed using GraphPad 7.0 (Graphpad Software, CA, USA). Data are presented as mean $\pm$ standard deviation (SD). In this study, Student's t-test was used for pairwise comparison, and one-way analysis of variance and post-hoc Dunnett t-test were used for multiple comparisons. Spearman correlation analysis was employed for correlation analysis between miR-4458 and FEV1% pred. $P < 0.05$ was considered to have significant difference.

Results

The miR-4458 level was downregulated in PBMCs of COPD patients associated with pulmonary function

Among the clinical characteristics of the three groups, only lung function including FEV1% pred and FEV1/FVC showed statistical difference ($p < 0.001$, Table1). Compared with the non-smokers, the level of miR-4458 in PBMCs of smokers were reduced ($p < 0.05$, Figure1A), the level of miR-4458 in PBMCs of COPD patients was further decreased ($p < 0.001$, Figure1A). Thereafter, we analyze the correlation between FEV1% pred and miR-4458. The result showed that the level of miR-4458 was positively correlated with FEV1% pred ($p < 0.001$, Figure1B). These results indicated that down-regulation of miR-4458 was associated with poor pulmonary function in COPD.

The expression of miR-4458 was reduced in CSE-treated 16HBEs accompanied by increased autophagy

The levels of miR-4458 in 16HBEs exposed to 10% CSE at different times were also examined. Figure 1C showed that the mRNA level of miR-4458 was downregulated after exposure to CSE ($p < 0.001$). Autophagy plays a significant role in many inflammatory diseases. In order to explore whether CSE treatment could influence the autophagy level in 16HBEs, the levels of LC3B and BECN were tested in

16HBECS treated with varying concentrations of CSE (0, 2.5%, 5%, 10%, and 15%) for 24 h. Both RT-qPCR and western blot test results showed that the level of LC3B was the highest under 15% CSE, while the level of BECN was the highest in cells treated with 10% CSE ($p < 0.001$, Figure 1D–F). The expression levels of LC3B and BECN in cells stimulated with 10% CSE at 12 h were higher than those at 24 h and 48 h (Figure 1G–I). These results suggested that miR-4458 was downregulated and that autophagy was increased in CSE-treated 16HBECS.

MiR-4458 attenuated CSE-induced apoptosis, autophagy, and proliferation toxicity in 16HBECS

In this study, some in vitro experiments were performed in 16HBECS which exposed to 10% CSE to explore the roles of miR-4458 in CS-related COPD. First, the transfection efficiency was verified. When transfected with miR-4458 mimics, the levels of miR-4458 in CSE-exposed 16HBECS were upregulated, while the opposite results were observed in cells transfected with miR-4458 inhibitor ($p < 0.001$, Figure 2A). As shown in Figure 2B, expression levels of LC3B and BECN were increased in the cells stimulated with 10% CSE for 12 h. The aforementioned result revealed that autophagy could be activated by CSE. When miR-4458 expression was upregulated in 16HBECS, LC3B and BECN levels were decreased (Figure 2B), while opposite results were observed when miR-4458 expression was downregulated (Figure 2D). Meanwhile, CSE significantly upregulated Bax protein level and downregulated Bcl2 protein level and inhibited the viability of 16HBECS (Figure 2B–C). The overexpression of miR-4458 led to decreased Bax expression and increased both Bcl-2 expression (Figure 2B) and cell proliferation activity (Figure 2C), while the downregulation of miR-4458 aggravated the apoptosis and proliferation toxicity of 16HBECS (Figure 2D–E). Flow cytometry also showed similar results, the CSE group had more apoptotic 16HBECS than the control group, and the miR-4458 mimics group had lower apoptotic cells than the NC-mimics group ($p < 0.005$, Figure 2F), indicating that miR-4458 could relieve apoptosis of 16HBECS. The results revealed that miR-4458 plays a protective role in CSE-treated 16HBECS.

MiR-4458 directly targeted P53 and regulated the AKT–mTOR signaling pathway

The direct mechanism of miR-4458 in regulating autophagy and apoptosis in CSE-treated 16HBECS was also explored. In this study, among the genes that were predicted to be targeted by miR-4458, P53 was chosen for further investigation. The predicted binding site between P53 mRNA 3'UTR and miR-4458 was showed in Figure 3A. The dual luciferase reporter assay was then conducted to verify the direct targeted relationship between P53 and miR-4458. We observed that when co-transfected with miR-4458 mimics and pmirGLO-P53-wt, the relative luciferase activity was significantly decreased ($p < 0.01$, Figure 3B). The P53 levels in patients with COPD were higher than non-smoker and smoker without COPD ($p < 0.05$, $p < 0.001$, Figure 3C). Meanwhile, RT-qPCR ($p < 0.005$, Figure 3D) and western blot (Figure 3E) revealed that P53 was increased in CSE-treated 16HBECS. Following transfection with miR-4458 mimics or inhibitor before treatment with CSE, the level of P53 protein was negatively regulated (Figure 3E–F). Therefore, we believed that P53 was a direct target gene of miR-4458. Since the AKT–mTOR signaling pathway has

been confirmed to be involved in regulating autophagy, we also measured the protein expression of p-AKT and p-mTOR by WB assay, we found p-AKT and p-mTOR proteins were positively regulated by miR-4458 in CSE-treated 16HBEs (Figure 3E–F). The results revealed that miR-4458 could modulate the AKT–mTOR signaling pathway.

MiR-4458 exerted its biological functions via the P53-mediated AKT–mTOR signaling pathway

In order to ensure that miR-4458 exerted its biological function via the P53-AKT–mTOR signaling pathway, rescue experiments were conducted. When transfected pcDNA3.1(+)-P53 plasmids into cells, the protein level of P53 was upregulated ($p < 0.05$; Figure 4A). When upregulation of P53, the expression levels of LC3B and BECN were elevated (Figure 4B). Compared with the control group, the P53 overexpression group demonstrated higher Bax level, lower Bcl-2 level (Figure 4B) as well as the cell viability (Figure 4D). Further flow cytometry results revealed that the P53 overexpression group had more apoptosis cells ($p < 0.05$, Figure 4C). The above results revealed that P53 could increase autophagy and apoptosis and decrease cell viability. Compared with the group transfected with miR-4458 mimics alone, co-transfected miR-4458 mimics and pcDNA3.1(+)-P53 plasmids upregulated LC3B, BECN, and Bax levels, downregulated Bcl-2 level (Figure 4B), elevated the number of apoptotic cells ($p < 0.005$, Figure 4C), and reduced cell proliferation (Figure 4D). Consequently, we confirmed that miR-4458 regulated autophagy and apoptosis via P53.

Moreover, the effect of miR-4458 and P53 on p-AKT and p-mTOR was explored. When overexpression of P53, the p-AKT and p-mTOR levels were downregulated (Figure 4E). The overexpression of miR-4458 upregulated p-AKT and p-mTOR protein levels, whereas the co-transfection with miR-4458 mimics and pcDNA3.1(+)-P53 plasmids reversed such regulation (Figure 4E). All above results proved that miR-4458 protected CSE-treated 16HBEs via P53 by regulating the AKT–mTOR signaling pathway.

Discussion

Cigarette smoking has been identified as a major risk factor of COPD; therefore, cigarette smoke exposure is commonly used in in vitro and in vivo experiments of COPD. Cigarette smoking is considered associated with inflammation, oxidative stress, autophagy, apoptosis, and pyroptosis[26, 27]. A previous study showed that apoptosis and autophagy were elevated in CSE-treated mice and Beas-2B cells[28]. Another study revealed that inhibition of autophagy could reduce the release of inflammatory mediators[26]. In the present study, we observed activated autophagy and apoptosis in CSE-treated 16HBEs. Thus, the results supported the point that autophagy and apoptosis were overactivated and that they played important roles in the pathogenesis of COPD.

MiRNAs were reported to participate in the pathogenesis and may act as novel therapeutic targets in COPD[8, 29]. Zeng et al. showed that the inhibition of miR-21 alleviated autophagy and apoptosis in mice and 16HBEs exposed to cigarette smoke[30]. MiR-4458, as a novel miRNA, has been reported to regulate apoptosis in hemangiomas[31] and breast cancer[13]. However, as far as we know, the function and

mechanism of action of miR-4458 on autophagy and apoptosis in COPD has never been investigated. Thus, in this study, we found that miR-4458 was decreased in CSE-treated 16HBEs and PBMCs of patients with COPD. Meanwhile, the expression of miR-4458 was positively related to the FEV1% pred level. The upregulation of miR-4458 reduced autophagy and apoptosis and promoted cell proliferation, while the downregulation of miR-4458 revealed opposite results. The results of the experiments revealed that miR-4458 plays a protective role in COPD.

Previous studies have revealed that P53 levels were elevated in patients with COPD and in CS-exposed mouse model and epithelial cells[23, 32]. Our results supported this conclusion. As a transcription factor, P53 regulates several biological processes including autophagy and apoptosis. When P53 accumulates in the nucleus, it can stimulate autophagy[33]. In a recent study, P53 was activated in PM2.5-exposed Beas-2B cells and P53-DRAM1 axis played an important role in autophagy induction[34]. Moreover, an in vivo study indicated that the overexpression of P53 could restore apoptosis in CSE-treated Beas-2B cells[24]. Another study noted that the induction of apoptosis is accompanied by the upregulation of P53 and Bax in CS-exposed primary alveolar type cells in mice[32]. Therefore, P53 could be a promising therapeutic target in COPD. In the present study, when P53 expression level was upregulated in 16HBEs, cell autophagy and apoptosis were further intensified, but cell viability was decreased. The luciferase reporter assay showed that miR-4458 regulates P53 expression level by directly binding to its 3' UTR mRNA. Moreover, the results of the rescue assays further confirmed that P53 is a direct target gene of miR-4458. Collectively, these results suggested that miR-4458 alleviates autophagy and apoptosis by directly targeting P53 in CSE-exposed 16HBEs.

We also explored the mechanism of action of miR-4458. When upregulation of miR-4458, the p-AKT and p-mTOR levels were increased. However, downregulation of miR-4458 produced the opposite results. The AKT–mTOR signaling pathway is a key pathway in cell metabolism[35]. AKT is a serine/threonine protein kinase, and its activation often induces apoptosis[36]. Activated by AKT, mTOR is able to regulate several processes including autophagy[37]. Meanwhile, a previous study showed that P53 can negatively regulate the AKT–mTOR signaling pathway[35, 38]. In the present study, co-transfection of miR-4458 mimics and pcDNA3.1(+)-P53 plasmids reversed the changes in the AKT–mTOR signaling pathway, indicating that miR-4458 protected HBEs by targeting P53 via the AKT–mTOR signaling pathway.

Conclusions

This is the first study confirming that miR-4458 was downregulated in CSE-treated 16HBEs and patients with COPD and that miR-4458 attenuated autophagy and apoptosis of 16HBEs. Meanwhile, miR-4458 exerted a protective effect mainly via the p53–AKT–mTOR signaling pathway. Therefore, miR-4458 may be a aussichtsreich therapeutic target for COPD treatment.

Declarations

Funding: This study was funded by the Science and Technology Foundation of Shandong Province (2015GGH318002), the Special project of Respiratory Clinical Medicine Research Center of Shandong Province (201912011), and the Shandong Province Medical and Health Technology Development Plan Project (2019WS082).

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Author Contributions: Jie Yang and Yi-Qing Qu conceived and designed the project; Xiu-Li Ji and Yi-Qing Qu provided materials and patients; Meng-Yu Zhang and Jian-Yu Liu acquired, analyzed, and interpreted the data; all authors wrote and final approved the manuscript.

Ethics approval: The trial was conformed to the Declaration of Helsinki (as revised in 2013). The procedure of study was approved by the Medical Ethics Committee of Qilu Hospital of Shandong University. All patients signed informed consent forms.

Data Availability: All data analysed in this study are included in this published article.

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Tables

Table1 The clinical characteristics of patients.

Variables	Non-smokers	Smokers	COPD
Number	20	20	25
Gender(Male/Female)	18/2	20/0	23/2
Age (Years)	62.2 ± 2.03	62.95 ± 1.82	64.56 ± 1.89
Pack-years	0	29.75 ± 1.64	34.8 ± 3.85
FEV1% pred	107.6 ± 4.15	101 ± 3.57	81.64 ± 3.83***
FEV1/FVC (%)	79.48 ± 1.21	77.53 ± 1.05	60.97 ± 1.76***

***p < 0.001

COPD, chronic obstructive pulmonary disease; FEV1% pred, forced expiratory volume in the first second of expiration for predicted values; FEV1/FVC, forced expiratory volume in 1second / forced vital capacity

Figures

Figure 1

MiR-4458 was downregulated in CSE-treated 16HBEs and patients with COPD, and autophagy was increased A. The miR-4458 levels in patients of COPD. B. The pearson correlation analysis between miR-4458 and FEV1% pred. C. The miR-4458 level of 16HBEs in 10% CSE at 12 h, 24 h and 48 h was evaluated by RT-qPCR. D–F. The levels of LC3B and BECN in 16HBEs exposed to varying concentrations of CSE were tested by WB and RT-qPCR. H–I. The levels of LC3B and BECN in 10% CSE-treated 16HBEs at different times were examined by WB and RT-qPCR. *p < 0.05, **p < 0.01, ***p < 0.001. CSE, cigarette

smoke extract; HBECS, human bronchial epithelial cells; COPD, chronic obstructive pulmonary disease; FEV1% pred, forced expiratory volume in the first second of expiration for predicted values; RT-qPCR, real-time quantitative polymerase chain reaction; WB, western blot

Figure 2

MiR-4458 decreased autophagy and apoptosis in CSE-treated 16HBECS A. mRNA levels of miR-4458 in 16HBECS tranfected with miR-4458 mimics or inhibitor. Protein levels of LC3B, BECN, Bax, and Bcl2 in

16HBECS tranfected with miR-4458 mimics (B) or inhibitor (D). Cell viability of HBECS tranfected with miR-4458 mimics (C) or inhibitor (E). F. Apoptosis rate of 16HBECS measured by flow cytometry. **p < 0.01, ***p < 0.001. CSE, cigarette smoke extract; HBECS, human bronchial epithelial cells

Figure 3

P53 was a direct target gene of miR-4458 A. Predicted binding site between miR-4458 and TP53 mRNA. B. Targeted relationship between miR-4458 and TP53 validated by the dual luciferase reporter assay. C-D. The mRNA levels of P53 in PBMCs of COPD patients and 10% CSE-treated 16HBECS. P53, mTOR, p-mTOR, AKT, and p-AKT levels in 16HBECS tranfected with miR-4458 mimics (D) or inhibitor (E) tested by WB. *p < 0.05, **p < 0.01, ***p < 0.001. PBMCs, peripheral blood mononuclear cells; CSE, cigarette smoke extract; HBECS, human bronchial epithelial cells; WB, western blot

Figure 4

MiR-4458 protected 16HBECS via the AKT-mTOR signaling pathway A. The mRNA levels of P53 in 16HBECS transfected with P53 overexpression plasmid. B. TP53 overexpression reversed the changes of LC3B, BECN, Bax, and Bcl2 protein levels induced by miR-4458 mimics in 16HBECS. Changes of apoptosis rate (C) and cell viability (D) in 16HBECS caused by miR-4458 mimics were reversed by TP53. E. Protein

levels of mTOR, p-mTOR, AKT, and p-AKT in 16HBEs evaluated by rescue assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CSE, cigarette smoke extract; HBEs, human bronchial epithelial cells