Exosomal miR-380 derived from alveolar macrophages in COPD is involved in enhanced proliferation of human bronchial epithelial cells

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Abstract

Background

Chronic obstructive pulmonary disease (COPD) is closely related to the occurrence of lung cancer. Both diseases involve changes in the biological function and structure of bronchial epithelial cells. Recently, the role of exosomes in intercellular communication has attracted increasing attention from researchers. As an important molecule carried by exosomes, the role of exosomal miRNAs in diseases such as COPD and lung cancer has also been gradually confirmed. The aim of this study is to investigate the effect of exosomal miRNA derived from macrophages on the proliferation of bronchial epithelial cells.

Results

After co-culture with alveolar macrophages from COPD, the proliferative activity and migration capacity of 16HBE was significantly enhanced compared with those from healthy controls. Alveolar macrophages from COPD promoted the production of MUC5AC, MUC5B and MUC2 as well as TNF - α and IL-6 in 16HBE. MiR-380 was up-regulated miRNA in exosomes derived from alveolar macrophages through miRNA array analysis. Exosomal miR-380 enhanced the proliferation and migration of 16HBE, promoted the expression of mucins such as MUC5AC, MUC5B and MUC2 of 16HBE, but inhibited the expression of CFTR protein. The target gene of miR-380 was CFTR. The proliferative activity and migration ability of 16HBE was enhanced by blocking CFTR.

Conclusions

Alveolar macrophages from COPD can enhance the proliferation and migration of 16HBE and promote the expression of mucin and proinflammatory mediators such as IL-6 and TNF-α. Exosomal miR-380 derived from macrophages in COPD was significantly up-regulated. The enhancement of proliferation and migration may be related to the down regulation of CFTR by exosomes delivering miR-380 to bronchial epithelial cells, opening a new way for the therapy and prevention in COPD and its complication.

Background

Chronic obstructive pulmonary disease (COPD) is a disease characterized by incomplete reversible airflow limitation. The exact etiology and pathogenesis of COPD has not been fully elucidated, but smoking has been proven to be one of the important risk factors for COPD (1). The number of macrophages in sputum, alveolar lavage fluid and airways of patients with COPD was significantly higher than that of normal smokers, and their number was related to the severity of the disease (2). Macrophages may play a key role in COPD. When alveolar macrophages are exposed to oxidants in cigarette smoke, they are activated and further lead to the production of inflammatory mediator such as IL-8, TNF – α and the infiltration of inflammatory cells in lung tissue (3). The inflammation of COPD is not only related to immune cells such as macrophages, neutrophils, it also involves the participation of

structural cells including airway epithelial cells and endothelial cells (4). Airway epithelial cells can be activated by cigarette smoke to produce inflammatory mediators, such as TNF - α, IL-1 β, IL-6, IL-8 and GM-CSF (5). High secretion of mucus is also an important feature of patients with COPD. Oxidative stress caused by cigarette smoke can activate epidermal growth factor receptor (EGFR), and further activation of mitogen-activated protein kinase by EGFR leads to increased expression of mucin genes MUC5AC, MUC5B (6).

In patients with COPD, the damage of airway epithelium is usually more obvious than that of normal people and smokers without COPD (7). Epithelial metaplasia may be caused by increased proliferation of basal airway epithelial cells in the cellular mechanism. Studies have shown that increased expression of EGFR in airway epithelial cells in patients with COPD may promote the proliferation of airway epithelium and further cause its metaplasia, resulting in a higher risk of bronchial lung cancer in patients with COPD (7). In addition, changes in the structure and function of airway epithelial cells may also be caused by the release of inflammatory factors by other inflammatory cells or some unknown effect mechanisms. The specific molecular mechanism needs to be further clarified.

It has been reported that exosomes are involved in the regulation of inflammation and immune processes in COPD through intercellular communication (8). Exosomes are extracellular vesicles of about 50–150 nm size surrounded by phospholipid membranes, which can be secreted by epithelial cells, endothelial cells, macrophages and other cells, and exist in various biological fluids such as blood, urine, sputum, bronchoalveolar lavage fluid, synovial fluid, pleural fluid, ascites and so on (9). Moon et al. found that lung epithelial cells produce CCN1 rich exosomes induced by cigarette smoke extract. CCN1 in exosomes promotes the production of IL-8 through the Wnt signaling pathway, which leads to neutrophil infiltration and promotes the development of COPD (10). Li et al. showed that cigarette smoke induced the increased release of macrophage derived exosomes with matrix metalloproteinase MMP14 mediated collagen lysis activity, which involved in the formation of emphysema (11). The protease / anti-protease imbalance theory is one of the classic doctrines of the pathogenesis of COPD. When a large amount of protease is released, the normal balance between protease and anti-protease is destroyed, leading to increased destruction of the extracellular matrix, and then promotes the development of COPD. There are exosomes derived from neutrophil with neutrophil elastase (NE) activity in the BALF of patients with COPD, which can lead to COPD like phenotype in mice (12). Fujita et al. demonstrated that the exosomes containing miR-210 derived from epithelial cells increased the expression of type I collagen and α - smooth muscle actin in pulmonary fibroblasts by blocking ATG7, thus promoting the differentiation of pulmonary fibroblasts into myofibroblasts and inhibiting autophagy, leading to remodeling in airway of COPD (13).

COPD is considered to be an important high-risk factor for the development of lung cancer. However, the mechanism of the association between COPD and lung cancer is not fully clarified. The two diseases share a common causative factor for smoking. Recently, it has been found that the exosomes released from epithelial cells of lung after tobacco exposure can cause increased proliferation, decreased apoptosis and epithelial mesenchymal transformation in epithelial cells, which increases the risk of

malignant transformation of bronchial epithelial cells. Alveolar macrophages can release a large amount of exosomes after tobacco exposure. It has not been reported whether exosomes derived from alveolar macrophages of COPD affect biological behaviors of bronchial epithelial cell such as proliferation, apoptosis and epithelial-mesenchymal transition.

Therefore, we aimed to observe the effect of macrophages on the proliferation and secretion of bronchial epithelial cells, and investigated the specific mechanism of intercellular communication mediated by exosomes derived from macrophages in this study.

Results

Alveolar macrophages of COPD can promote the proliferation and migration of bronchial epithelial cells

To evaluate the effect of alveolar macrophages on the proliferation of bronchial epithelial cells, we first compared the differences in proliferative ability of 16HBE in each group by CCK8 kit. The results were shown in Fig. 1A. Regardless of the presence of COPD, the proliferative activity of 16HBE in the co-culture group with alveolar macrophages was time-dependent. The proliferative activity of 16HBE co cultured with alveolar macrophages from COPD group was significantly increased compared with the normal group(24 h $P = 0.0054$, 48 h $P = 0.0391$, 72 h $P = 0.0014$). No significant change in proliferative ability of bronchial epithelial cells after co-culture with macrophages in the normal group（24 h $P = 0.3608$, 48 h $P = 0.2623$, 72 h $P = 0.093$）. We next performed clone formation experiments. The results showed that colony forming ability of 16HBE was significantly enhanced after co-cultured with alveolar macrophages of COPD ($P = 0.0062$) (Fig. 1B).

The migration ability of bronchial epithelial cells was detected by transwell invasion experiment. The results were shown in Fig. 1C. After co culture with macrophages of normal control group, the migration ability of bronchial epithelial cells did not change significantly($P = 0.3613$). COPD-derived alveolar macrophages significantly enhanced the migration ability of bronchial epithelial cells compared with macrophages from normal control group ($P = 0.0014$). These data demonstrated that alveolar macrophages from COPD have a significant effect on the proliferation and migration of bronchial epithelial cells.

Alveolar macrophages of COPD affect the secretion function of bronchial epithelial cells

We performed western blot to observe the expression of mucin in bronchial epithelial cells after co-culture with alveolar macrophages. The results are shown in Fig. 2A and 2B. The expression of MUC5AC, MUC5B and MUC2 in bronchial epithelial cells co-cultured with macrophages of COPD was higher than that co-cultured with macrophages of normal control group. (Relative expression level: MUC5AC 0.26 vs 0.17, MUC5B 0.3 vs 0.21, MUC2 0.48 vs 0.32). By contrast, the expression level of CFTR in bronchial epithelial cells co cultured with macrophages of COPD group was lower than that in control group (COPD vs

control: 0.27 vs 0.36). In addition to the increase expression of mucins, we also observed marked increased pro-inflammatory cytokines such as TNF-αand IL-6 in 16HBE after co-culture with macrophages of COPD (TNF-α: 384.6 ± 37.7 ng/μL vs 140.3 ± 29.0 ng/μL;IL-6: 426.3 ± 38.7 ng/μL vs 130.5 ± 20.4 ng/μL) (Fig. 2C). Collectively, these results imply that macrophages of COPD play an important role in the expression of mucins and the production of proinflammatory factors in bronchial epithelial cells.

Identification Of Alveolar Macrophage-derived Exosomes

Western blot showed that CD9, CD63 and CD81 were positive in exosomes (Fig. 3A). Exosomes were analyzed by nanoparticle tracking analysis (NTA) with ZetaView PARTICAL METRIX (detection range: 10-1000 nm, detection duration: 1 min). The exosomes with vesicle-like structures were scattered in the visual field through NTA (Fig. 3B). In the COPD group, the median size of exosomes was 139.6 nm, the concentration was 2.8×10^7 / ml, the 138 nm particles were 95%, the 54 nm particles were 1.2%, the 29 nm particles were 0.9%, and the 15.9 nm particles were 0.9%. In the control group, the median size of exosomes was 139.8 nm, concentration was 2.1×10^7 / ml, 141 nm particles were 98.3%, 20.2 nm particles were 0.5%, 11.4 nm particles were 0.3%, 28.2 nm particles were 0.1%. There was no significant difference in the size and amount of macrophage derived exosomes between the COPD group and the control group (Fig. 3C). These results demonstrated that SBI kit could extract the exosomes from alveolar macrophages successfully.

miR-380 is the most significantly expressed miRNA in macrophage-derived exosomes of COPD

Differentially expression levels of miRNA were determined by analyzing GEO database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE61741(including 27 normal and 29 COPD). Four miRNAs with the most significant difference were found through comparison analysis, among which miR-380 and miR-374b were up-regulated, while the other two miRNAs (miR-1228 and miR-345) were down regulated (Fig. 4A). MiRNA array analysis of macrophage-derived exosomes in COPD group and healthy control group showed that the change of miR-380 was the most significant. Consistent with the data of the peripheral blood miRNA based on GEO, miR-380 was significantly up-regulated in macrophage-derived exosomes in COPD group (Fig. 4B).

Exosomal miR-380 enhanced the proliferative activity and migration capacity of bronchial epithelial cells

To observe the effect of exosomal miRNA derived from macrophages on bronchial epithelial cells, miR-380 was overexpressed in 16HBE by construction of miR-380 mimic. The proliferative activity of 16HBE was detected by CCK-8. The result was shown in Fig. 5A. The proliferative activity of the cells in two groups was time-dependent, and the proliferative activity of 16HBE in the miR-380 mimic group was significantly enhanced in comparison to that in the control group (48 h $P = 0.0044$, 72 h $P = 0.0018$). The migration ability of bronchial epithelial cells was observed by transwell cell invasion test. As was shown in Fig. 5B, the over expression of miR-380 enhanced the migration of 16HBE compared with that in the control group ($P = 0.0021$). These results demonstrated that exosomal miR-380 derived from alveolar

macrophages of COPD significantly enhanced the proliferative activity and migration capacity of bronchial epithelial cells.

Exosomal miR-380 Increased Mucin Expression In Bronchial Epithelial Cells

The expression level of mucins such as MUC5AC, MUC5B, MUC2 and CFTR in miR-380 overexpressed bronchial epithelial cells was assessed by Western Blot. As was shown in Fig. 6, the expression level of MUC5AC, MUC5B and MUC2 in the miR-380 overexpression group was higher than those in the control group, while CFTR expression level was down-regulated in miR-380 mimic group. (Relative expression level: MUC5AC 0.45 vs 0.23, MUC5B 0.66 vs 0.41, MUC2 0.53 vs 0.25, CFTR 0.21 vs 0.63)

Cystic fibrosis transmembrane conductance regulator(CFTR)is the regulatory gene of exosomal miR-380

The target gene of miR-380 in exosomes derived from macrophages was screened by bioinformatics in microRNA database (https://www.mirbase.org). According to the screening result (Fig. 7), cystic fibrosis transmembrane conductance regulator (CFTR) is the target gene of miR-380.

The siRNA Reduced Cftr Expression In Bronchial Epithelial Cells

MiR-380 in exosomes derived from alveolar macrophages can regulate CFTR. Over expression of miR-380 resulted in lower CFTR level in 16HBE. To evaluate the effect of CFTR on bronchial epithelial cells, the low expression of CFTR was achieved by constructing CFTR siRNA in 16HBE. The CFTR expression in 16HBE constructed by CFTR siRNA was detected by Western Blot. The CFTR expression was significantly decreased in 16HBE by the introduction of CFTR siRNA (Fig. 8).

Blockade of CFTR enhanced the proliferative activity and migration capacity of bronchial epithelial cells

CCK-8 was used to assess the proliferation of bronchial epithelial cells introduced with siCFTR. The proliferation of cells in three groups was time-dependent. As was suggested in Fig. 9A, the proliferative activity of bronchial epithelial cells in the siCFTR group was significantly enhanced compared with the siControl group (48 h $P = 0.0034$, 72 h $P = 0.0021$). In addition, the migration of 16HBE was assessed by transwell cell invasion test. The knockdown of CFTR significantly enhanced the migration of 16HBE ($P = 0.005$) (Fig. 9B). These results confirmed that the down-regulation of CFTR can enhance the proliferative activity and migration ability of bronchial epithelial cells.

Discussion

COPD and lung cancer are usually closely related to cigarette smoking which cause serious disease burden to people. The development of lung cancer is usually associated with the malignant proliferation of epithelial cells, while the progression of COPD is associated with the increase in the amount of activated neutrophils and macrophages as well as increased apoptosis of epithelial cells and endothelial cells(14). We hypothesized that alveolar macrophages from COPD may affected the function and phenotype of bronchial epithelial cells, and may be involved in the pathogenesis of COPD and lung cancer. To verify this hypothesis, macrophages were obtained by bronchoalveolar lavage from the rat model of COPD and co cultured with bronchial epithelial cells for subsequent experiments. The bronchial epithelium of patients with chronic bronchitis and COPD is often characterized by squamous metaplasia. This pathological change may be related to increased proliferation of basal airway epithelium(6). We found that the proliferation and migration of bronchial epithelial cells were significantly enhanced after co culture with alveolar macrophages from COPD (Fig. 1). The increased proliferation may be related to metaplasia, which may link COPD to carcinogenesis, but further experiments are needed to confirm. High secretion of mucus is a common pathological feature of COPD. Consistent with the trait, the expression of mucin in 16HBE was increased under the influence of macrophages (Fig. 2A,2B). Airway epithelial cells are important part of the defense barrier of the respiratory tract. The high secretion of mucus may be related to the increase of goblet cells, mucous gland metaplasia, and reduced mucus clearance. A variety of inflammatory mediators and signaling pathways regulate the expression of mucin genes. Such as IL-1β, IL-17A and TNF-α induce mucus production through NF-κB activation and nuclear translocation(15). However, the specific mechanism leading to high secretion of mucus remains to be further clarified. Macrophages and epithelial cells can be activated by irritants such as cigarette smoke to release inflammatory mediators. Previous studies have demonstrated that IL-1β and TNF-α secreted by macrophages can promote the expression of IL-6 in alveolar epithelial cells(16). Our study showed that IL-6 and TNF-α production in 16HBE significantly increased under the action of alveolar macrophages of COPD (Fig. 2C), which indicated that the production of inflammatory mediators in structural cells was not only affected by external environmental stimuli, but also the interaction between cells could not be ignored in COPD.

Although there is interaction between macrophages and bronchial epithelial cells, the mechanism of how the two kinds of cells communicate with each other and produce relevant effects has not been fully elucidated. The role of exosomes in mediating cell-to-cell communication in physiology and pathology has been extensively studied by researchers(17, 18). Exosomes are involved in biological processes such as immune regulation, angiogenesis, proliferation and differentiation through mediating intercellular communication(19). Cigarette smoke can induce macrophages to release exosomes with protease activity. Besides, exosomes containing miR-210 derived from bronchial epithelial cells induced by cigarette smoke can promote myofibroblast differentiation(20). Based on this, we hypothesized that exosomes derived from alveolar macrophages of COPD may affect the function of bronchial epithelial cells through intercellular communication.

We used the SBI kit to isolate and extract exosomes from alveolar macrophages in this study. CD9, CD63, and CD81 of exosomes were all positive by western blot. Enough exosomes with high purity can be

obtained by SBI kit through nanoparticle tracking analysis.

Exosomes play important role in biological process by the shuttle of a variety of molecules including proteins, lipids, DNA, mRNA and miRNA in intercellular communication(21). Among these molecules, miRNA has been widely concerned due to the function of regulating gene expression. Goldie et al. Showed that the proportion of miRNA in exosomes was higher than that in their parental cells(22). Guduric-Fuchs et al. found that a subset of miRNAs (such as miR-150, miR-142-3p, and miR-451) preferentially enter exosomes body by analysis of the expression levels of miRNAs in various cell lines and exosomes derived from them(23). It can be inferred that different types of miRNAs may be present in exosomes derived from different cells under different physiological and pathological conditions. We found that miR-380 and miR-374b were up-regulated, while miR-1228 and miR-345 were down regulated in COPD through GEO database (Fig. 4A). MiR-380 inhibits p53 to control cell survival and correlates with poor prognosis of MYCN-expanded neuroblastoma(24). MiR-374b inhibits tumor growth and promotes apoptosis in non-small cell lung cancer by targeting p38 / ERK signaling pathway(25). MiR-1228 can prevent apoptosis by targeting MOAP1 protein(26). MiR-345 induces apoptosis in pancreatic cancer by enhancing caspase-dependent and independent pathways(27). It was speculated that these miRNAs may be expressed differently in the exosomes derived from alveolar macrophages in COPD group and the normal control group. The subsequent miRNA array analysis showed that miR-380 expression level was significantly up-regulated in the exosomes of COPD-derived macrophages (Fig. 4B). The result suggests that COPD significantly affects the level of exosomal miR-380 in macrophages. We assumed that macrophages from COPD may regulate the function of 16HBE through exosomal miR-380. MiR-380 mimic was constructed by cell transfection to study the effect of macrophage-derived exosomal miRNA on 16HBE. The results showed that over expression of miR-380 significantly enhanced the proliferation and migration of 16HBE compared with control group (Fig. 5). Thus, it shows that exosomal miR-380 might play a pivotal role in the promotion of the proliferation and migration in 16HBE by macrophages in COPD. Besides, we found the expression of mucin in 16HBE of miR-380 mimic group was significantly higher than that in the control group by Western Blot (Fig. 6). It is also suggested that macrophages in COPD are likely to increase the expression of mucin in 16HBE through miR-380, which in turn helps promote high secretion of mucus and aggravate airway obstruction in COPD.

Exosomes can reach adjacent or distant cells for intercellular communication, and miRNAs of exosomes are released to recipient cells to produce corresponding biological effects. Exosomal miRNAs play a role in receptor cells by negatively regulating expression levels of target gene. For example, exosomal miR-92a derived from K562 cells significantly reduced the expression of integrin α5 in human umbilical vein endothelial cells and enhanced migration of endothelial cell(28). Our results demonstrated that exosomal miR-380 derived from macrophages of COPD can reduce the expression level of cystic fibrosis transmembrane conductance regulator (CFTR) in 16HBE. Besides, we identified CFTR as the target gene by screen of miRbase.

CFTR is a chloride channel mainly expressed in the apical membrane of bronchial epithelial cells. It plays a key role in the lung by participating in maintaining homeostasis of airway surface fluid(29). The

deficiency or dysfunction of CFTR can lead to cystic fibrosis (CF). CF is an autosomal dominant genetic disease caused by mutations in the CFTR gene, and is characterized by chronic infection and inflammation(30). Some clinical symptoms of CF are similar to COPD, suggesting that the two diseases may share common pathogenesis. Bodas et al. showed that CFTR was inhibited in the lungs of patients with COPD, and the lack of CFTR could lead to alveolar apoptosis and deficient autophagy, which indicated that the decreased expression of CFTR might be involved in the development of COPD(31). The increased miR-145, miR-223 and miR-494 in CFTR mutant patients with cystic fibrosis is related to the decreased expression of CFTR(32), while miR-223 expression is increased in patients with COPD(33). It can be speculated that the reduction of CFTR in COPD may be related to miR-223. In addition, cigarette smoke extract can induce the up-regulation of miR-101 and miR-144 in human bronchial epithelial cells and mouse lungs. These two miRNAs directly target CFTR and inhibit the expression of CFTR protein in the lungs of patients with COPD(34). Consistent with it, our data illustrated that exosomal miR-380 was significantly increased in alveolar macrophage of COPD. Exosomes containing miR-380 negatively regulate CFTR expression in bronchial epithelial cells through intercellular communication. The proliferative activity and migration capacity of bronchial epithelial cells were promoted by the block of CFTR (Fig. 9). Our study provides strong evidence to build a link between exosomal miRNA and enhanced proliferation mediated by CFTR. Mechanistically, COPD may stimulate the production of miR-380 in exosomes derived from alveolar macrophages, and thereby induce proliferation and migration of bronchial epithelial cells by negatively regulating the target gene CFTR, which may contribute to the inflammatory process of COPD. In addition to the classical role of targeting mRNA, miR-21 and miR-29a of exosomes were also found to have the ability to bind with toll like receptors (TLRs) as ligands and activate immune cells(35). Thus, the specific mechanism of exosomal miRNA derived from macrophages in the pathogenesis of COPD is worth further investigation. Of note, a recent study reported the level of TGF - β was increased in bronchial epithelial cells of patients with COPD(36). The increased TGF - β which is involved in the decreased synthesis of CFTR in bronchial epithelial cells is important for the recruitment of macrophages to bronchial epithelial cells. It is conceivable that the regulation of CFTR in bronchial epithelial cells might be mediated through exosomal miR-380 derived from macrophage via the TGF-β signaling pathway. Subsequent research could focus on the role of TGF-β signaling pathway in intercellular communication between macrophage exosomes and bronchial epithelial cells. Altogether, these results suggested that exosomal miR-380 derived from alveolar macrophages in COPD promoted proliferation of bronchial epithelial cell by negatively regulating CFTR. Our study reveals an unappreciated role of exosomes derived from alveolar macrophages in promoting proliferation of 16HBE. However, the specific signaling pathways for exosomes regulating CFTR need to be further elucidated. Our results are limited by animal models. Exosomes of bronchoalveolar lavage fluid from patients with COPD might be an ideal choice for the further research.

Conclusions

In conclusion, we demonstrated that alveolar macrophages of COPD enhanced the proliferation and migration of bronchial epithelial cells and promoted the expression of mucin and proinflammatory

mediators ILn-6 and TNF-α. Exosomal miR-380 derived from alveolar macrophages of COPD was significantly increased. The enhanced proliferative activity and migration ability of bronchial epithelial cells may be related to the exosomes delivering miR-380 to bronchial epithelial cells as well as the down-regulation of CFTR protein caused by the negative regulation of CFTR gene. Our studies suggest that targeting exosomal miR-380 may be a potential target for treatment and prevention of COPD and its complications.

Methods

Animals

Twenty SD rats, all male, weighing 250–360 g, 8 weeks of age, were purchased from Shanghai Slark Laboratory Animal Co., Ltd. All relevant animal experiments were approved by the Animal Ethics Committee of Naval Military Medical University. Animal experiments were performed in accordance with NIH (1996) Guide for the Care and Use of Laboratory Animals.

Models

Twenty SD rats were randomly distributed into two groups, namely the blank control group and the COPD group. Model rats were intranasally administered 200 ul endotoxin (1 mg / ml LPS) on the first day and fourteenth day, and cigarette smoke was inhaled on days 2–13 and 15–30. The rats in the COPD group were placed in a self-made smoke chamber. The volume of the smoke chamber is 110 cm × 86 cm × 72 cm. Using the static inhalation method, 20 cigarettes were placed in the smoking hole, and the cigarettes were ignited. Each cigarette was inhaled once a minute for 30 seconds each time. The collected main stream and side stream fumes were introduced into the smoke box through a plastic hose. Every 50 seconds, the flow fan in the box was turned on for 10 seconds to make the gas distribution in the box uniform. Turn off the smoking system after 8 minutes. The rats were exposed to the smoke for 1 hour each time, and were removed after 1 hour. The animals can eat and drink freely in the box when they were exposed to cigarette smoke. The blank control group was placed in the smoke chamber 3 times a day using the same method. Except for inhaling fresh air, other conditions were same as those in the COPD group.

Isolation And Extraction Of Alveolar Macrophages

Rats were anesthetized by intraperitoneal injection of 300 mg / kg of 10% chloral hydrate and sacrificed. Alveolar macrophages were collected by bronchoalveolar lavage. Macrophages were washed twice with RPMI-1640 medium. Trypan blue staining was used to detect cell viability and cell number, and the cells were formulated to the required concentration. The viability of macrophages can reach more than 90%, and macrophages accounted for more than 90% of the total.

Bronchial Epithelial Cell Culture

The human bronchial epithelial cell line 16HBE was purchased from ATCC (Manassas, VA, USA). 16HBE cells were cultured in RPMI-164 medium containing 100 U / mL penicillin, 100 μg / mL streptomycin and 10% (v / v) inactivated fetal calf serum, and placed in a cell incubator for routine subculture. Set the incubator temperature to 37 ° C and contain 5% CO2. Cells were exchanged every other day, depending on the growth, and passaged once every 2–3 days. In this experiment, cells in logarithmic growth phase were used.

Co Culture Of Alveolar Macrophages And 16HBE

16HBE cells were plated in the upper chamber of a 24-well transwell chamber at 5.0×10^5 and cultured in RPMI-164 containing 10% FBS. After 24 hours, the serum-free medium was changed, and co-cultured with the above-mentioned alveolar macrophages for 8 hours, 12 hours, or 24 hours. 16 HBE cells in the upper chamber were collected for subsequent experiments.

Isolation And Identification Of Exosomes Derived From Alveolar Macrophages

Exosomes were extracted from alveolar macrophages via SBI kit (Sigma,USA) according to manufacturer's instructions. The exosomes were refrigerated at − 80℃ for long-term storage. The morphology of exosomes was observed with an electron microscope. The size and concentration of exosomes was performed upon resuspension using nanotracking analysis on a ZetaView Particle Metrix (PMX, Germany). Western blot was used to detect the expression of exosomal marker protein.

Cell Transfection

The cells were inoculated in a 10 mm dish, cultured at 37 ℃ and 5% CO2 for 18–24 hours to reach 60% fusion degree. According to the transfection steps of miR-380 mimic, CFTR-siRNA and Lipofectamine 2000 TM reagent instructions. Liposomes, antibiotic-free OPTI-MEM medium and plasmids (24 μg), or negative controls was mixed and added to the cell culture medium. The medium containing the liposome complex was replaced after 4–6 hours of incubation. The cells were collected for subsequent experiments after 48 hours of transfection.

Quantitative Real-time PCR

Total RNA was obtained from exosomes stored at -80 °C with Trizol reagent according to the manufacturer’s protocol (Takara, China). Reverse transcription: The first strand of cDNA was synthesized

by using Takara's m-mlv reverse transcription kit. The first strand of cDNA was synthesized by reverse transcription using total RNA as template and random primers as reverse transcription primers. GAPDH was used as the internal reference gene. The total RNA of all samples was transcribed in the same batch, and the operation was carried out according to the instructions provided by the manufacturer.

Reaction conditions: 37 ° C, 15 min; 85 ° C, 5 sec; 4 ° C, 10 min. The reaction system is as follows:

Components	Volume (μl)
5 × PrimeScript RT Master Mix	4 μl
Total RNA	1 μL(1 μg/μl)
RNase Free ddH2O	Up to 20 μl

Real-time PCR was performed on a Stepone Plus system (Applied Biosystems, Foster city, CA). The reagent used for quantification was 2xSYBR Green real-time PCR master mix. Reaction conditions: 1.95 ℃, 10 min; 2. 95 ℃, 15 s; 3. 56 ℃, 55 s; 4. 72 ℃, 20 s. 2–4 total 35 cycles. The reaction system is as follows:

Components	Volume (μl)
2 × SYBR Green real-time PCR Master Mix	10 μl
PCR Forward Primer (10 μM)	0.8 μl
PCR Reverse Primer (10 μM)	0.8 μl
DNA template (<100 ng)	2 μl
Sterile water	Up to 20 μl

CCK8 Proliferation Experiment

Cell proliferation was analyzed by cell counting kit 8 (CCK8, Beyotime, Japan).16HBE cells were implanted into 96-well plates (the number of cells in each well was about 1×10^4), and cultured in CO2 incubators. Before each detection, the cells were added into 10 μL of CCK-8 reagent and cultured for 1.5 h. The cell activity was measured at 0 h, 24 h, 48 h, and 72 h. Finally, the optical density (OD) values were measured by microplate reader at the 450 nm wavelength.

Transwell Invasion Experiment

Cell culture: Collected the transfected cells, inoculated group cells and 300 μl of serum-free RPMI-164 suspension in the upper chamber (including Matrigel) of the transwell chamber.

Placed 500 μl of RPMI-164 medium containing 10% fetal bovine serum in the lower chamber. After the treatment was finished, the 24-well plate was placed in a cell incubator for 48 hours.

Fixation and staining: After 48 hours of culture, the 24 hole plate (transwell) was taken out and washed gently with PBS for 3 times. 4% paraformaldehyde was fixed at room temperature for 15 minutes. The fixative solution was removed by the rinse with PBS. Crystal violet staining solution was added to stain for 15 minutes and the stained cells of the upper chamber was removed with cotton swabs.

Counting statistics: Five high power visual fields were randomly selected for observation and statistical analysis of photographic counting under the microscope.

Clone Formation Test

The cells were inoculated at a density of 100 cells / 4L in a 6-well plate and were incubated for 10 days at 37 ° C in a 5% CO2 incubator. The supernatant was discarded after 3 weeks. The cells was washed twice with PBS and fixed with methanol for 15 min. The fixing solution was discarded and cells were dyed for 10–30 min with Giemsa staining solution. Wash the staining solution slowly with running water and dry it. The number of clones greater than 50 cells was counted under a microscope, and the colony formation rate was finally calculated. Colony formation rate = number of clones / number of seeded cells × 100%.

Western Blot

We assessed the expression of mucins and CFTR in 16HBE by western blot. Total protein in cells was extracted and measured by BCA analysis. Samples ran on SDS-PAGE gel. Gel was then transferred to nitrocellulose membrane using Tris-Glycine transfer buffer. Nitrocellulose membranes were then blocked with 5% milk/TBST solution for 1 hour at room temperature and incubated with primary antibody overnight at 4C. Membranes were washed 3x with TBST ant then incubated 1:5000 dilution with HRP conjugated secondary antibody. Membranes washed 3X as above, and then developed using ECL Chemiluminescent Substrate kit (Biological Engineering Co., Ltd. Shanghai, China). Antibodies used for detection are as follows: MUC5AC (Abcam, ab77576, 1: 5000), MUC5B (1: 5000; ab105460; Abcam), MUC2 (1: 10000; ab133555; Abcam), CFTR (1: 500, ab2784; Abcam), GAPDH (1: 2500, ab9485; Abcam). Band detection was performed using QUANTITY ONE software.

ELISA

Cell-free supernatants were collected and stored at -80 °C. After thawing, the expression of IL-6 and TNF-αwere analyzed with IL-6 and TNF-αELISA kit (Abcam) according to the manufacturer’s instructions.

Statistical Analysis

Software SPSS 19.0 (SPSS, Chicago, IL, USA) were used for statistical analysis, and all measurement data were expressed as the mean ± standard deviation (mean ± SD). Comparisons between two groups were analyzed by unpaired student's t-test analysis. Comparisons between multiple groups were analyzed by one-way analysis of variance. P values < 0.05 were considered significantly different. Statistical graphs were completed by using Graphpad Prism 5 (GraphPad Software Inc, San Diego, CA, USA).

Abbreviations

COPD: chronic obstructive pulmonary disease; CFTR: cystic fibrosis transmembrane conductance regulator; IL-6: interleukin-6; si-CFTR: siRNA to CFTR; TNF-α: tumor necrosis factor-α

Declarations

Ethics approval and consent to participate

Animal experiments were performed in accordance with NIH (1996) Guide for the Care and Use of Laboratory Animals. All relevant animal experiments were in compliance with the revised Animals (Scientific Procedures) Act 1986 in the UK and Directive 2010/63/EU in Europe and approved by the Animal Ethics Committee of the Navy Military Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest in this work.

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Authors' contributions

YF researched conception and drafted the manuscript; XF and CB contributed to the data interpretation; JZ designed and revised the manuscript critically; All authors approved the final manuscript.

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Figures

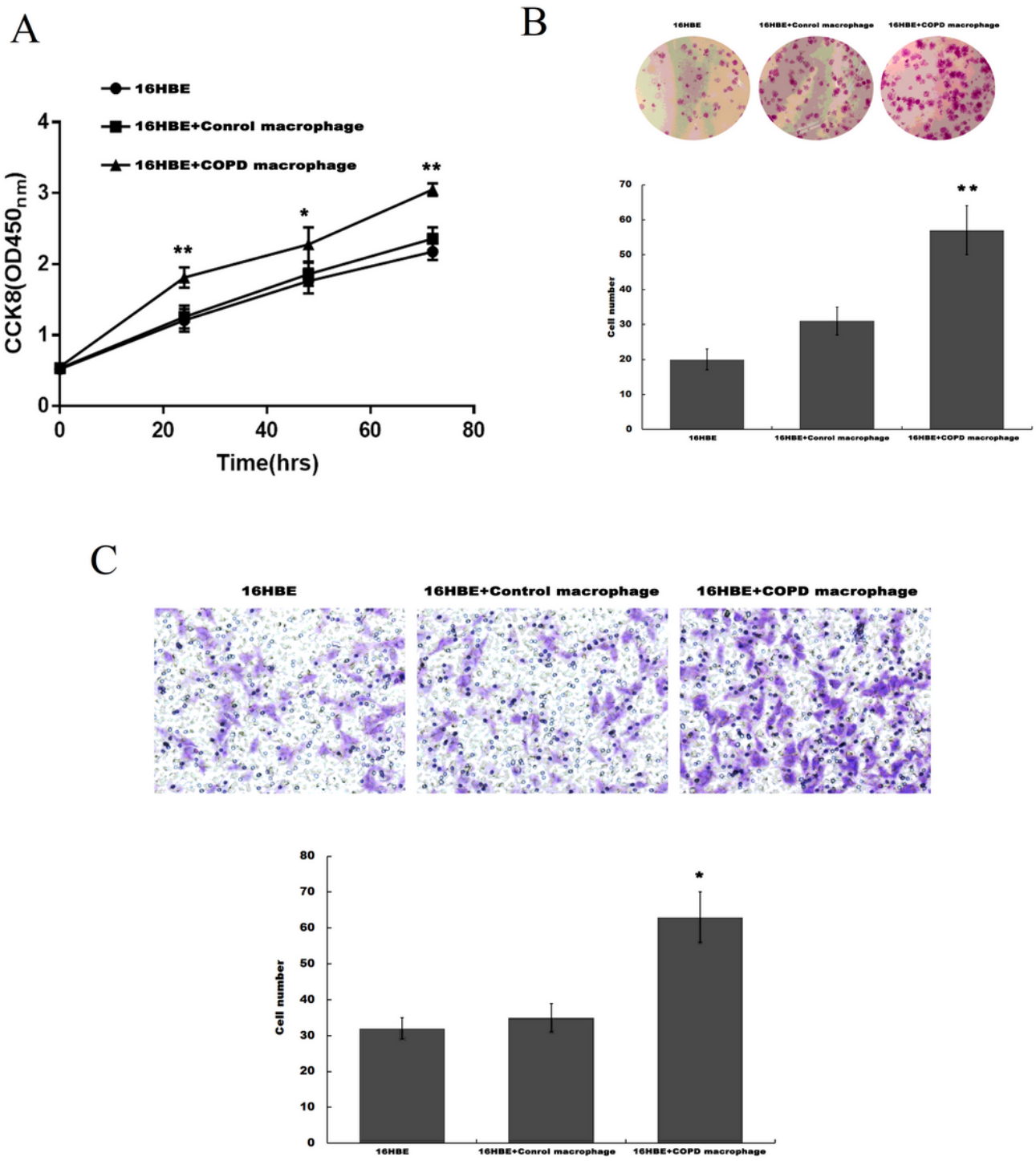


Figure 1

The proliferation and migration of 16HBE was enhanced co cultured with alveolar macrophages of COPD. (A). The proliferation of 16HBE co cultured with alveolar macrophages of COPD increased significantly. (B). The colony forming ability of 16HBE was significantly enhanced after co-cultured with alveolar macrophages of COPD. (C). The migration ability of 16HBE was enhanced by COPD-derived alveolar

macrophages significantly compared with macrophages from normal control group. *, P<0.05 in comparison to others; **, P<0.01 in comparison to others.

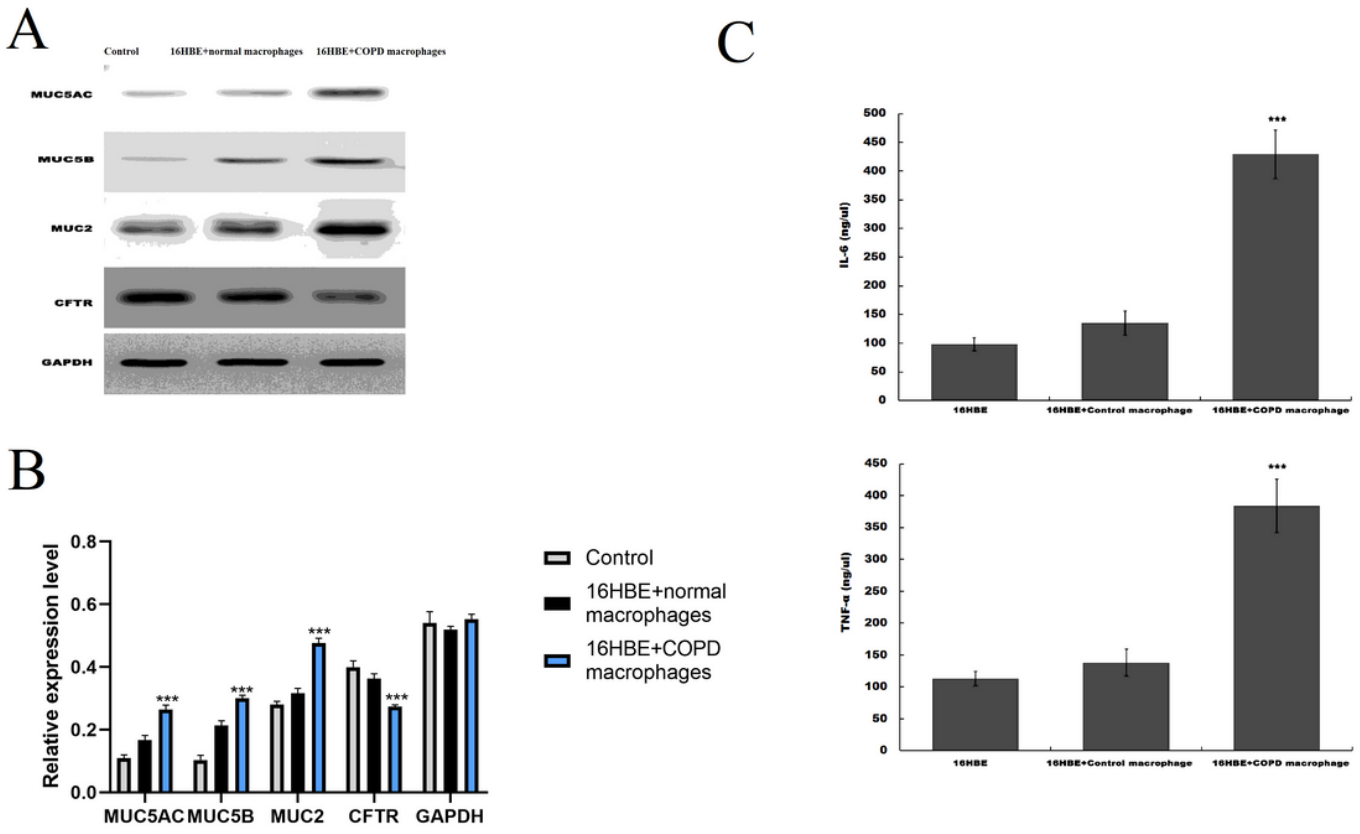


Figure 2

The expression of mucin and inflammatory mediator in 16HBE. (A). The expression of mucin in 16HBE was increased after co-culture with macrophages from COPD. (B). The statistics of gray value of protein bands. (C). The expression of TNF-αand IL-6 in 16HBE was marked increased after co-culture with macrophages from COPD. ***, P<0.001 in comparison to others.

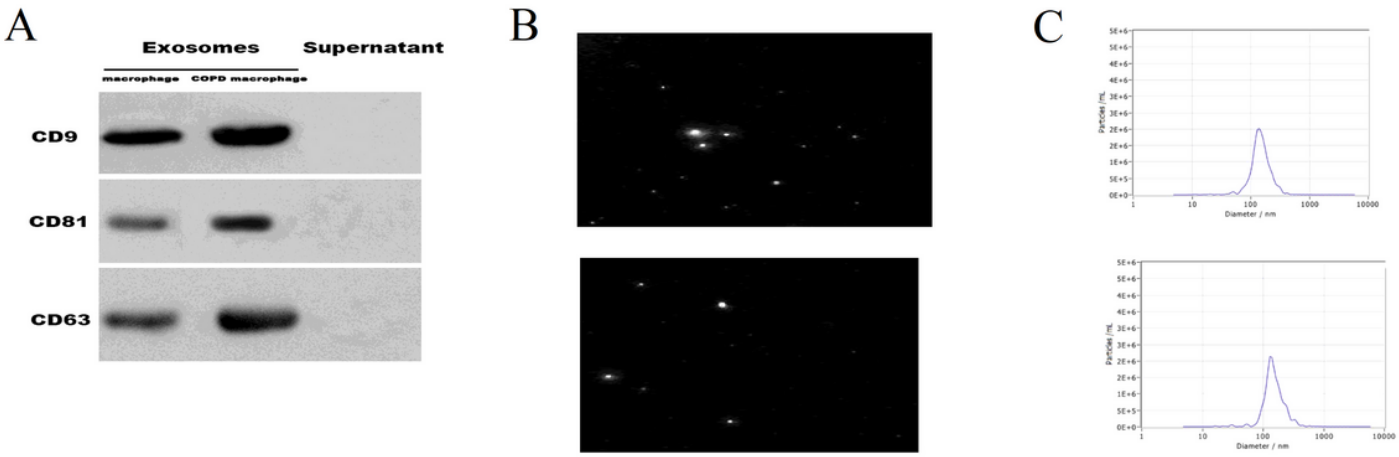


Figure 3

The exosomes were identified by western blot and NTA. (A). Western Blot bands for specific markers on the surface of exosomes. (B). Dynamic imaging pictures of exosomes. (C). The particle size and concentration of exosomes were analyzed by NTA.

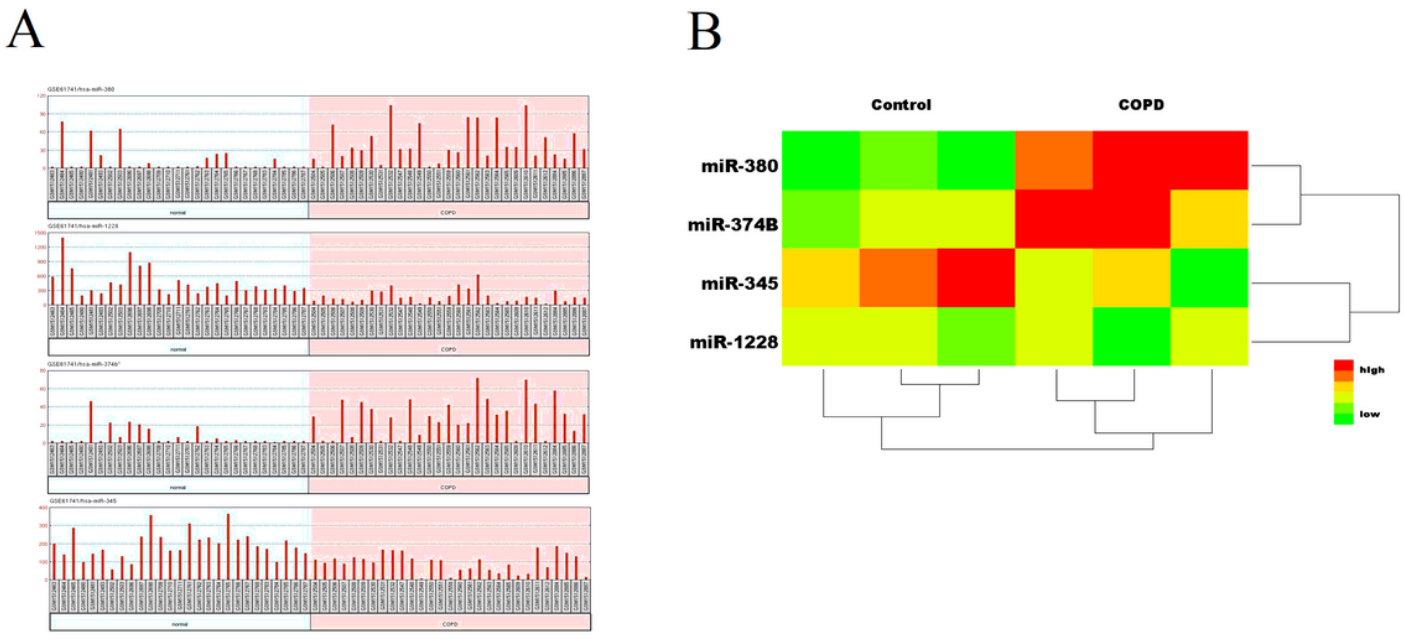


Figure 4

Bioinformatics and PCR analysis of miRNA. (A). Bioinformatics analysis of differentially expressed miRNAs. (B). Analysis of microRNA array of exosomes derived from alveolar macrophages.

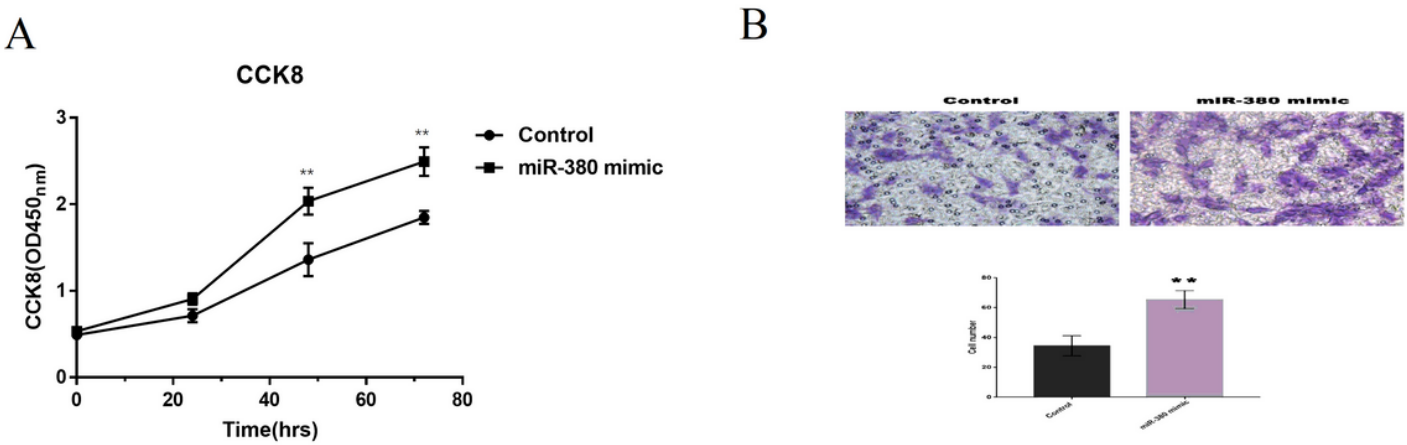


Figure 5

The proliferation and migration of 16HBE was enhanced by miR-380. (A). The proliferative activity of 16HBE in miR-380 mimic group was significantly enhanced compared with that in the control group. (B).

The migration capacity of 16HBE in miR-380 mimic group was significantly enhanced compared with that in the control group. **, P<0.01 in comparison to others.

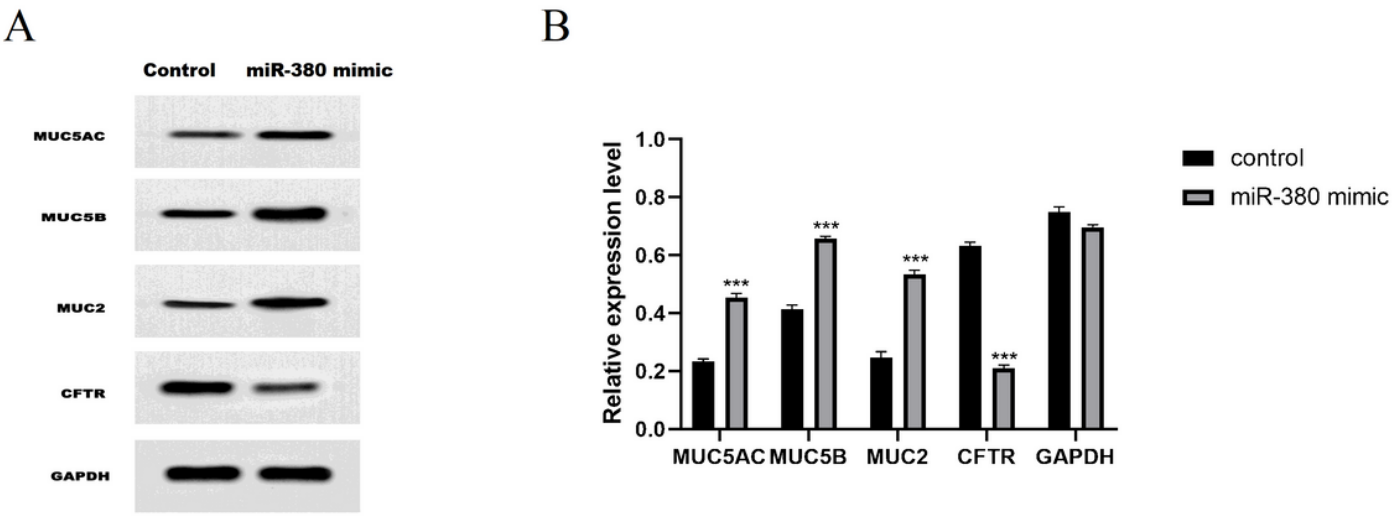
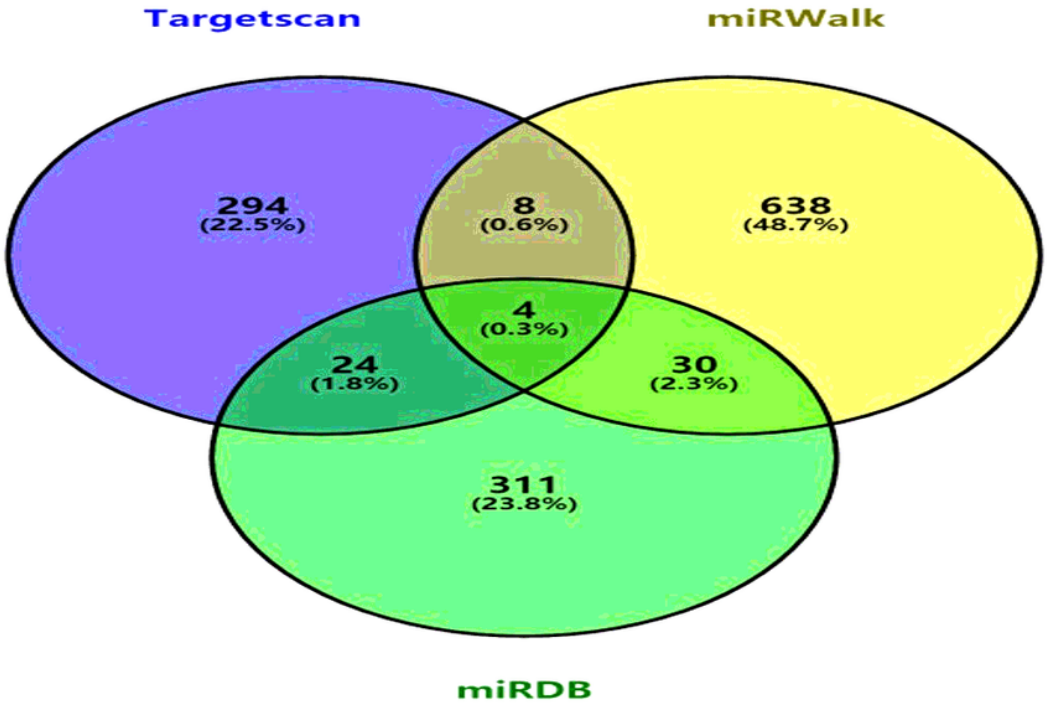


Figure 6

The expression of mucin and CFTR was enhanced in 16HBE with miR-380 mimic. (A). The expression levels of MUC5AC, MUC5B and MUC2 of 16HBE in miR-380 mimic group were significantly higher than those in the control group. (B). The statistics of gray value of protein bands. ***, P<0.001 in comparison to others.



	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P_{CT}
Position 1189-1195 of CFTR 3' UTR	5' ..... AAAGAAUGAUUAUGAAUUACAUU...	7mer-m8	-0.08	87	-0.08	0.013	N/A
hsa-miR-380-3p	3' UUCUACACCUGGUAUAAUGUAU						

Figure 7

Screening the target genes of miR-380 by the microRNA database.

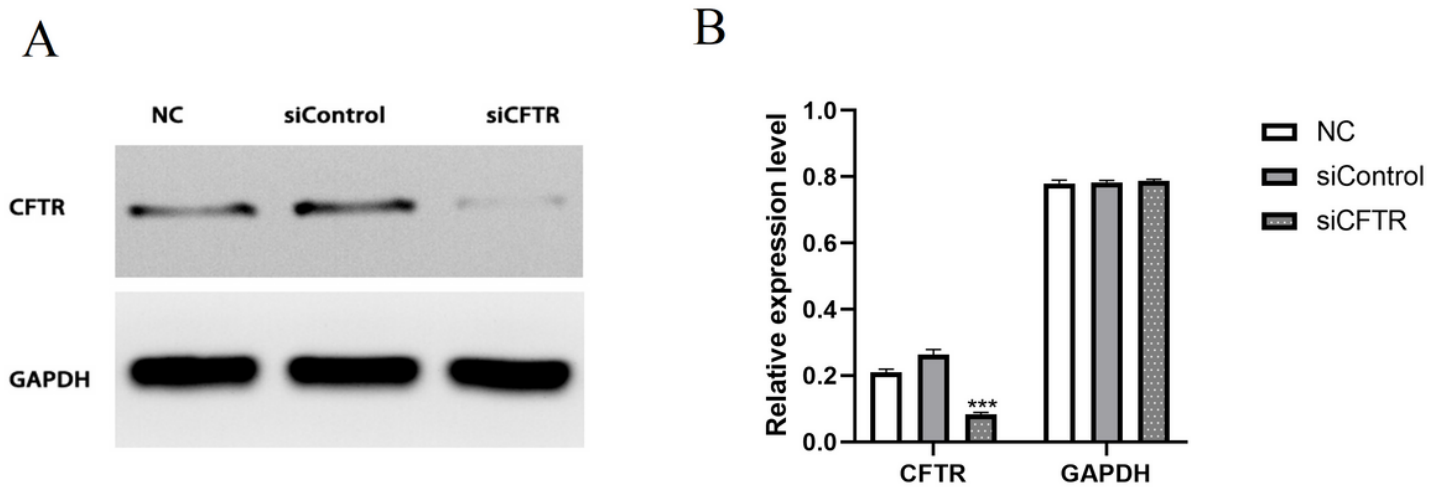


Figure 8

The CFTR expression was decreased in 16HBE with siCFTR. (A). CFTR of 16HBE was significantly decreased by siRNA in 16HBE. (B). The statistics of gray value of protein bands. ***, P<0.001 in comparison to others.

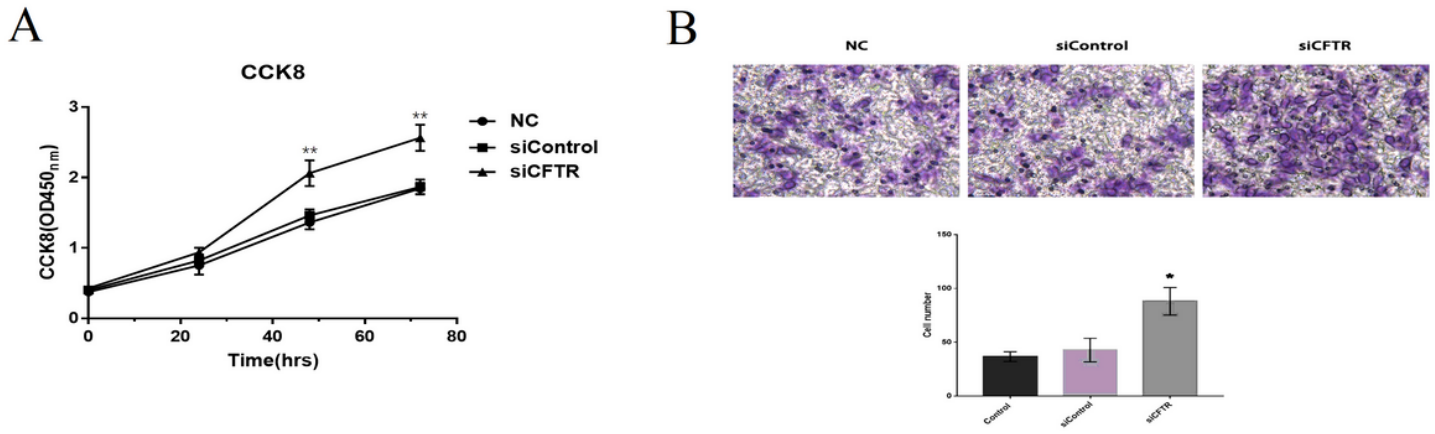


Figure 9

The proliferation and migration of 16HBE was enhanced by siCFTR. (A). The proliferative activity of 16HBE in siCFTR group was significantly enhanced compared with that in the control group. **, P<0.01 in comparison to others. (B). The migration capacity of 16HBE in siCFTR group was significantly enhanced compared with that in the control group. *, P<0.05 in comparison to others.

Supplementary Files

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