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MicroRNA-378 inhibits the development of smoking-induced COPD by targeting TNF-α

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Abstract. – OBJECTIVE: To explore the mechanism microRNA-378 in smoking-induced airway inflammation and mucus hypersecretion.

MATERIALS AND METHODS: Human bronchial epithelial (HBE) cells were treated with cigarette smoke extract (CSE) to construct the *in vitro* COPD model. Expression levels of microRNA-378, inflammatory factors and MUC5AC in CSE-treated HBE cells were determined by quantitative real-time polymerase chain reaction (qRT-PCR), Western blot and enzyme-linked immunosorbent assay (ELISA). The regulatory effects of microRNA-378 on expressions of inflammatory factors and mucin 5AC (MUC5AC) were observed in CSE-treated HBE cells overexpressing microRNA-378. We verified whether tumor necrosis factor-α (TNF-α) was the target gene of microRNA-378 through dual-luciferase reporter gene assay. Expression levels of TNF-α and p-p65 in CSE-treated HBE cells were examined. Finally, CSE-treated HBE cells were co-overexpressed with microRNA-378 and TNF-α to elucidate whether microRNA-378 exerted its function in regulating the development of COPD through targeting TNF-α.

RESULTS: CSE treatment downregulated microRNA-378 expression, but upregulated expressions of inflammatory factors and MUC5AC in HBE cells. MicroRNA-378 overexpression markedly inhibited the elevated levels of inflammatory factors and MUC5AC in CSE-treated HBE cells. Dual-luciferase reporter gene assay verified that TNF-α was the target gene of microRNA-378. Moreover, TNF-α expression in CSE-treated HBE cells was time-dependently elevated. TNF-α overexpression partially reversed the decreased levels of inflammatory factors and MUC5AC in HBE cells overexpressing microRNA-378.

CONCLUSIONS: MicroRNA-378 inhibits the inflammatory response by targeting TNF-α, which may be a potential therapeutic target for COPD.

Key Words:
COPD, MicroRNA-378, TNF-α, Inflammatory response.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic bronchitis and/or emphysema, which impairs the airway and lung tissues. COPD is characterized by persistent airflow limitation, and is closely associated with abnormal inflammatory reactions resulted from toxic gases or harmful particles[1,2]. Inflammatory response is one of the important mechanisms responsible for the pathogenesis of COPD. The imbalanced apoptosis of inflammatory cells and lung parenchymal cells is also a vital reason for the pathogenesis of COPD[3,4]. The high rates of morbidity, disability and mortality of COPD force us to elucidate its pathogenesis, so as to develop effective therapeutic approach. Under normal circumstances, mucus secreted by airway epithelial cells and submucosal glands protects the airway epithelium from air pollutants and pathogens. Pathological conditions of chronic airway inflammatory diseases, such as COPD and asthma, will lead to excessive secretion of mucus[5]. Cigarette smoke (CS) stimulates the airway epithelium and releases a variety of inflammatory mediators (such as IL-6 and IL-8). As a result, abundantly accumulated inflammatory cells in the airway cause hypertrophy of the airway epithelial goblet cells and mucous hypersecretion[6,7]. Mucin 5AC (MUC5AC) is the vital gene encoding mucins in airway, which is overexpressed under pathological conditions[8,9]. CS exposure activates airway epithelial cells to

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release inflammatory factors and activate MUC5AC, but the molecular mechanisms are still not fully elucidated. Friedman et al[10] have found the crucial functions of microRNAs in regulating cell proliferation, apoptosis, and inflammatory responses by complementarily pairing with target mRNAs. It is indicated that miRNAs can serve as biomarkers for certain diseases, providing a new direction for disease diagnosis, prevention, and treatment[11]. At present, a great number of microRNAs have been confirmed to participate in pulmonary diseases, further revealing the disease pathogenesis[12]. We believed that these certain microRNAs could be utilized as novel diagnostic and therapeutic targets for COPD. As a tumor-suppressor gene, microRNA-378 is reported to be capable of inhibiting cell proliferation and inflammation[13]. Its specific function in COPD, however, is rarely reported. In this study, the regulatory effects of microRNA-378 on inflammatory factor release and MUC5AC activation in CSE-treated HBE cells were explored. We aim to elucidate the mechanism and intervention effect of microRNA-378 on smoking-induced airway inflammation and mucus hypersecretion.

Materials and Methods

CSE Preparation

A 1R4F standard cigarette (provided by University of Kentucky Tobacco Research Institute) was attached to a suction device and ignited. CS was extracted by a suction device and dissolved in 37°C serum-free minimum Eagle's medium (MEM) (Gibco, Rockville, MD, USA). The solution pH was adjusted to 7.4 and filtered through a 0.22 μm filter for removing the bacteria and impurities. The finally obtained CSE solution was utilized as the mother liquor at the dose of 1 mg/mL, prepacked and stored in a -80°C refrigerator in dark.

Cell Culture and CSE Exposure

HBE cells were provided by Cell Bank, Chinses Academy of Science (Shanghai, China). After HBE cells were fused to 90%, they were washed with phosphate-buffered (PBS), digested with trypsin (Beyotime, Shanghai, China), passaged at a ratio of 1:3 and placed at 5% CO_2, 37°C. Medium was replaced every other day. Cells were treated with 20 μg/mL CSE for the appointed time points and harvested for the subsequent experiments.

RNA Extraction

5×10^6 cells were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), incubated with 0.2 mL of chloroform for 5 min, and centrifuged at 4°C, 12,000 rpm for 10 minutes. The supernatant was incubated with isodose isopropanol, and centrifuged at 4°C, 12,000 rpm for another 10 minutes. The precipitate was washed with 1 mL of 75% ethanol, centrifuged and air-dried. Finally, the extracted RNA was diluted in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China), quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored in a -80°C refrigerator.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted, quantified, purified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) with U6 as an internal reference. PCR reaction system was prepared with SYBR Green (Applied Biosystems, Foster City, CA, USA) master mix, cDNA template, upstream/downstream primer and DEPC water for qRT-PCR. The primer sequences were as follows: IL-6, F: 5'-AGTAGTGAGGAACAAGCCAGA-3', R: 5'-TACATTTGCCGAAGAGCC-3'; IL-8, F: 5'-ACTTCTCCACAACCCTCTG-3', R: 5'-ACTCCAAACCTTTCCACC-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: 5'-AAATCCCATCACCATCTTCCAG-3', R: 5'-AAATGAGCCCCAGCCTTCTC-3'; MicroRNA-378, F: 5'-TGCGGACTGGACTTGGAGTCAG-3', R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACGCCTTC-3'; U6, F: 5'-TGCGGGTGCTCGCTTCGGCAGC-3', R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG-3'.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with 20 nmol/L microRNA-378 mimics or control and 600 ng tumor necrosis factor-α (TNF-α) WT or TNF-α MUT for 48 h, respectively. Co-transfected cells were lysed and centrifuged. Relative luciferase unit of Firefly (RLU-1) and Renilla (RLU-2) was determined for calculating the luciferase intensity as RLU-1/RLU-2.

Western Blot

Total protein from cells or tissues was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and load-

ed for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 300 mA for 100 minutes, it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Cell Transfection

Cell transfection was performed until 70-80% of confluence. Briefly, cells were transfected with microRNA-378 mimics, pcDNA-TNF-α or NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 4 h, and incubated for another 24 h.

Enzyme-Linked Immunosorbent Assay (ELISA)

Standard curve was established according to the experimental instructions. Test samples were added to each well and incubated at 37°C for 2 h. Samples were incubated with the primary antibody at 37°C for 1 h and the luminescent substrate for 5-10 min. The reaction solution was finally added to each well. The absorbance (OD value) of each well was measured using a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data. $p<0.05$ indicated the significant difference.

Results

CSE Treatment Regulated Expressions of microRNA-378, Inflammatory Factors and Mucin in HBE Cells

HBE cells were treated with 20 μg/mL CSE for 0 h, 12 h, 24 h and 48 h, respectively. The mRNA level of microRNA-378 in CSE-treated HBE cells gradually decreased, showing a certain time-effect pattern (Figure 1A). However, inflammatory factors IL-6 and IL-8 were upregulated (Figure

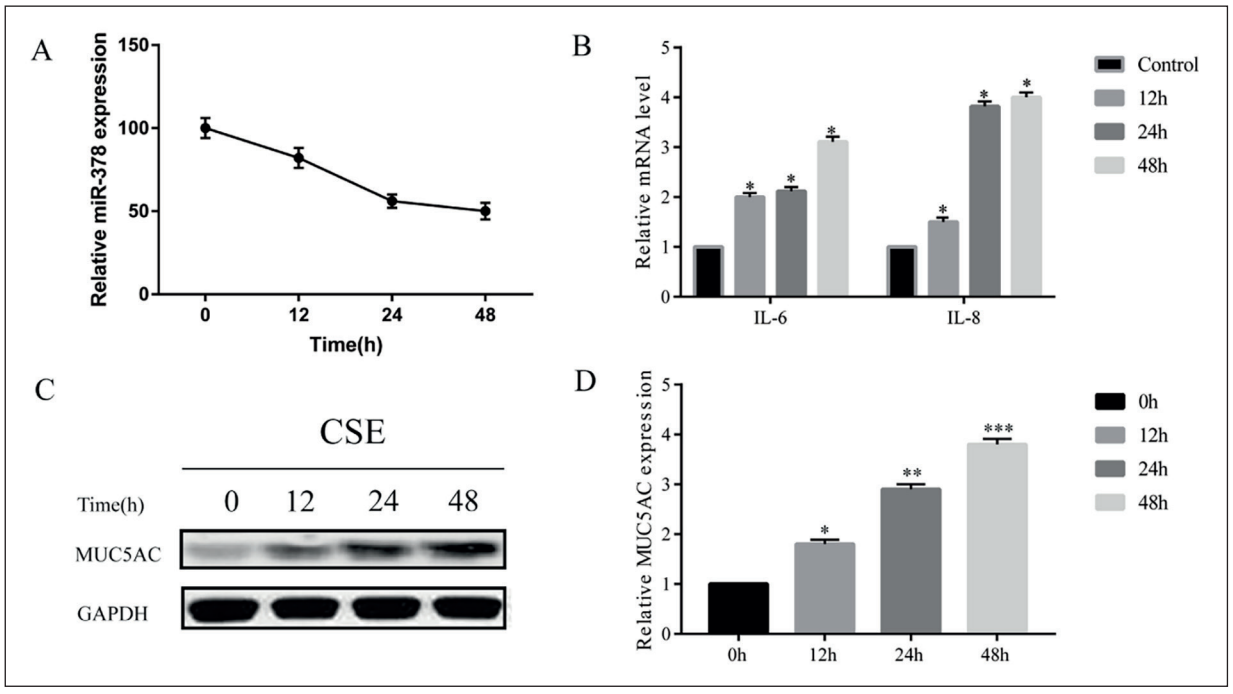

Figure 1. CSE treatment regulated expressions of miR-378, inflammatory factors and mucin in HBE cells. HBE cells were treated with 20 μg/mL CSE for 0 h, 12 h, 24 h and 48 h, respectively. **A,** The mRNA level of miR-378 in CSE-treated HBE cells gradually decreased in a time-dependent manner. **B,** The mRNA levels of IL-6 and IL-8 in CSE-treated HBE cells gradually increased in a time-dependent manner. **C,** The protein level of MUC5AC in CSE-treated HBE cells gradually increased in a time-dependent manner. **D,** The mRNA level of MUC5AC in CSE-treated HBE cells gradually increased in a time-dependent manner. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

1B). In addition, we also examined the protein and mRNA levels of MUC5AC, which was time-dependently upregulated (Figure 1C, 1D). These results suggested that CSE could induce the downregulation of microRNA-378 expression in HBE cells and upregulate expression levels of inflammatory factors and mucin.

MicroRNA-378 Overexpression Protected CSE-Induced Inflammatory Response

HBE cells were first transfected with microRNA-378 mimics or negative control (NC) for 24 h, and then treated with CSE for another 24 h. Compared with controls, transfection of microRNA-378 mimics in CSE-treated HBE cells downregulated mRNA levels of IL-6 and IL-8 (Figure 2A, 2B). By collecting the culture medium of the treated HBE cells, ELISA data revealed higher levels of IL-6 and IL-8 in culture medium of CSE-treated HBE cells overexpressing microRNA-378 relative to controls (Figure 2C). Besides, both mRNA and protein levels of MUC5AC decreased in CSE-treated HBE cells transfected with microRNA-378 mimics (Figure 2D, 2E). Identically, ELISA also showed an inhibited level of MUC5AC in culture me-

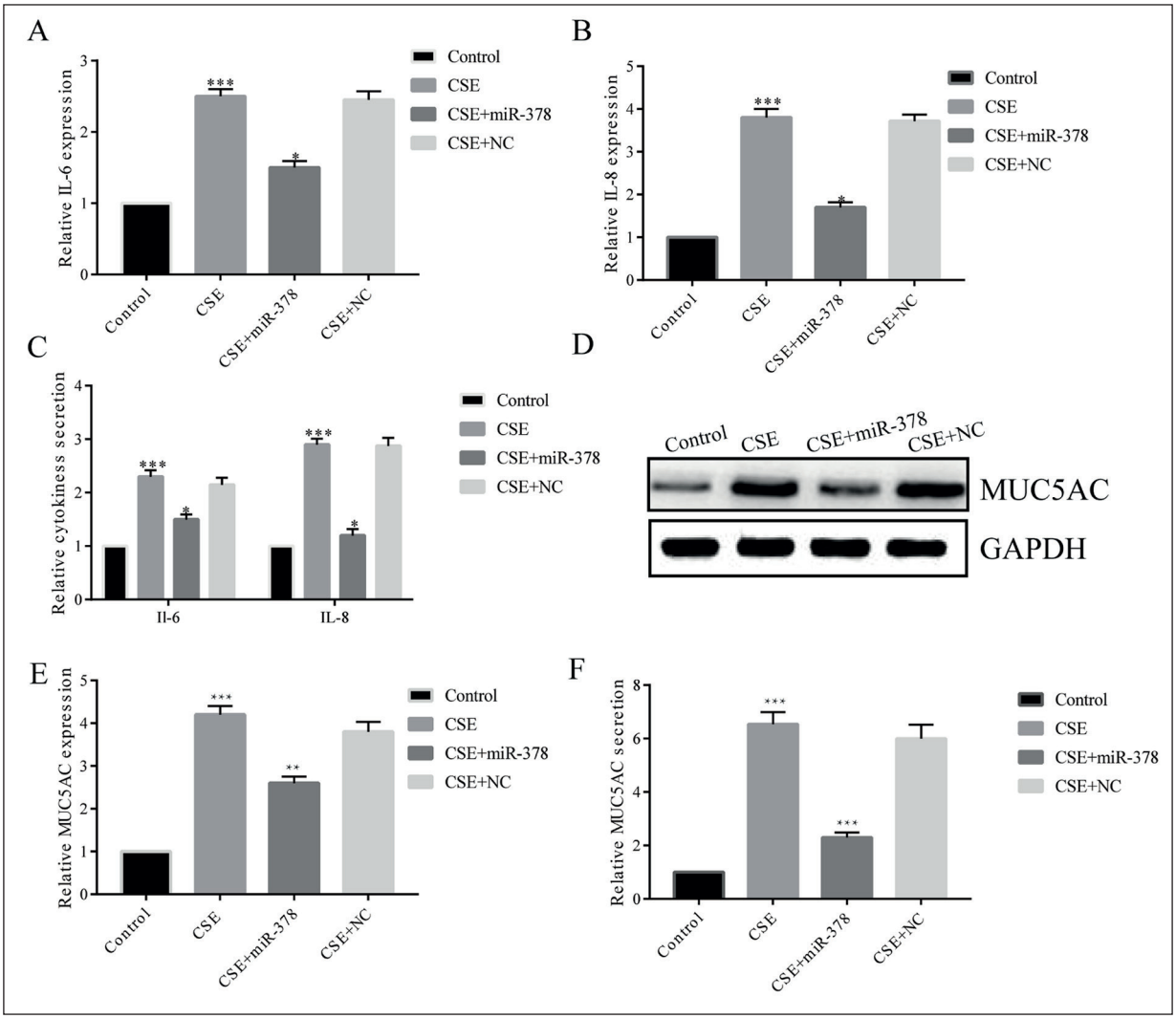

Figure 2. MiR-378 overexpression protected CSE-induced inflammatory response. HBE cells were first transfected with miR-378 mimics or negative control (NC) for 24 hours, and then treated with or without CSE for another 24 h. **A-B,** The mRNA levels of IL-6 (**A**) and IL-8 (**B**) in HBE cells. **C**. Levels of IL-6 and IL-8 in culture medium of CSE-treated HBE cells overexpressing miR-378 or controls. **D,** The protein level of MUC5AC in HBE cells. **E,** The mRNA level of MUC5AC in HBE cells. **F,** The level of MUC5AC in culture medium of CSE-treated HBE cells overexpressing miR-378 or controls. *p<0.05, **p<0.01, ***p<0.001.

dium of CSE-treated HBE cells overexpressing microRNA-378 relative to controls (Figure 2F). We may conclude that microRNA-378 inhibited CSE-induced increase of inflammatory factors and mucin levels.

TNF-α Was the Target Gene of microRNA-378

Through bioinformatics analysis, we found that TNF-α was a potential target gene for microRNA-378, exerting binding sequences in 3'UTR (Figure 3A). Luciferase reporter vectors of TNF-α WT and TNF-α MUT were constructed, and we verified the binding between microRNA-378 to TNF-α through dual-luciferase reporter gene assay (Figure 3B). Protein level of TNF-α was determined in CSE-treated HBE cells transfected with microRNA-378 mimics or NC. As Western blot analysis indicated, microRNA-378 overexpression upregulated microRNA-378 level in HBE cells (Figure 3C). Moreover, protein levels of TNF-α and p-p65 gradually upregulated with the prolongation of CSE exposure, while p65 level did not change (Figure 3D). The mRNA levels of TNF-α and p-p65 obtained the similar increased trends in a time-dependent pattern (Figure 3E, 3F).

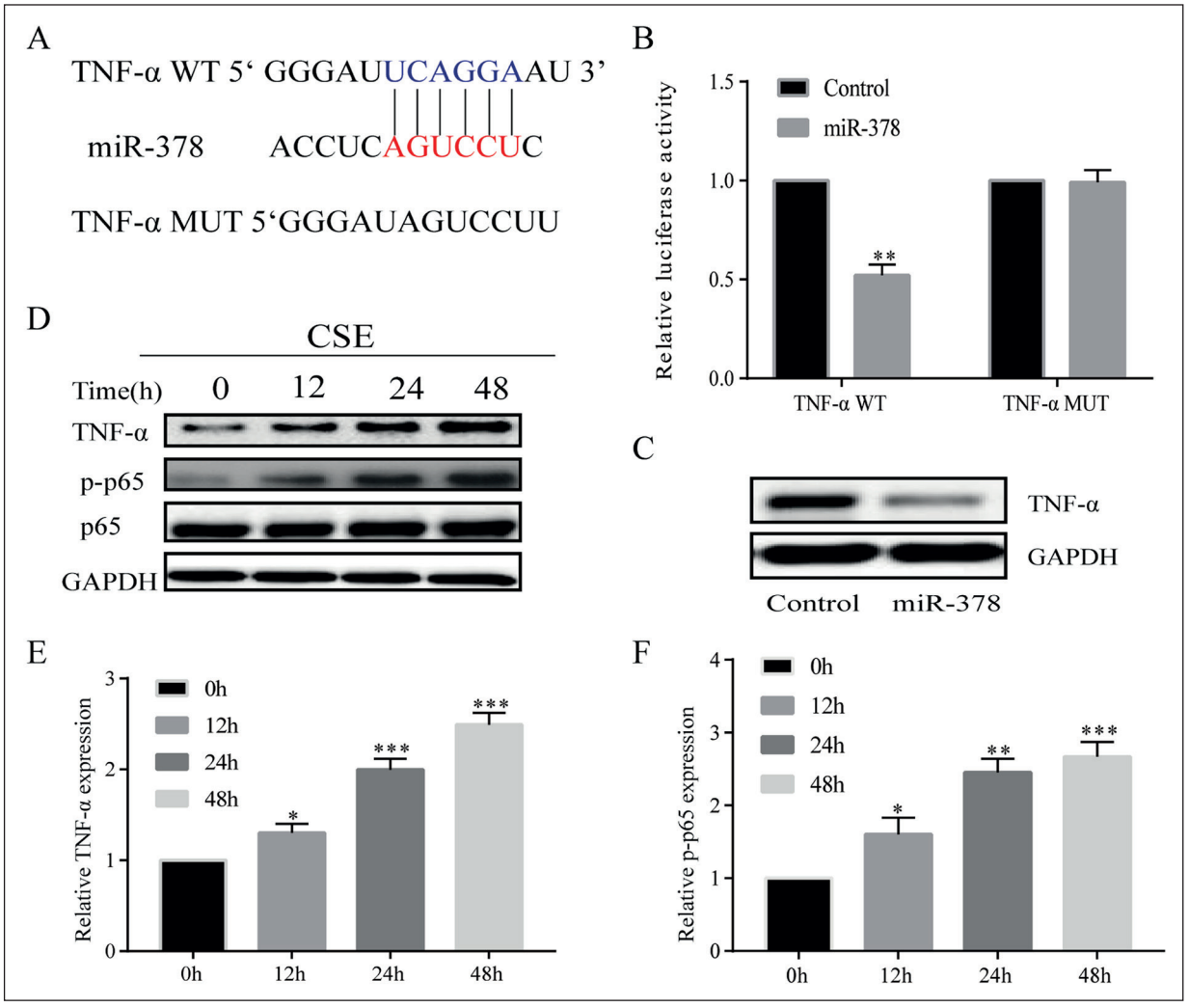

Figure 3. TNF-α was the target gene of miR-378. **A,** Potential binding sites between TNF-α and miR-378. **B,** Dual-luciferase reporter gene assay verified the binding between miR-378 to TNF-α. **C,** Western blot analysis of TNF-α in CSE-treated HBE cells transfected with miR-378 mimics or NC. **D,** Western blot analyses of TNF-α, p-p65 and p65 in HBE cells with CSE treatment for 0, 12, 24 and 48 h. **E-F,** The mRNA levels of TNF-α (**E**) and p-p65 (**F**) in HBE cells with CSE treatment for 0, 12, 24 and 48 h. *p<0.05, **p<0.01, ***p<0.001.

MicroRNA-378 Protected CSE-Induced Inflammatory Response by Targeting TNF-α

To verify whether microRNA-378 exerted anti-inflammatory effects through targeting TNF-α, we co-transfected with microRNA-378 mimics and TNF-α overexpression plasmid in CSE-treated HBE cells. After CSE treatment, downregulated mRNA and protein levels of TNF-α and MUC5AC induced by microRNA-378 overexpression were partially reversed by TNF-α overexpression (Figure 4A-4C). Identically, expression levels of IL-6 and IL-8 decreased by microRNA-378 overexpression in CSE-treated HBE cells, which were partially reversed by TNF-α overexpression (Figure 4D, 4E).

Discussion

Smoking is an important factor in the occurrence and progression of COPD. It induces the release of inflammatory cytokines such as IL-6 and IL-8 from airway epithelial cells. IL-6 is capable of mediating the acute onset and exacerbations of COPD, while IL-8 is a key chemokine that regulates the recruitment of neutrophils. Release of inflammatory factors recruits inflammatory cells to cause inflammatory infiltration in the airway and induces mucin overexpression. More seriously, excessive mucus production can block the airway and aggravate the inflammatory response during COPD deterioration[14,15]. Airway mucus hypersecretion has become an independent risk factor for increased mortality in patients with acute exacerbations of COPD[16]. Smoking has a pronounced effect on pulmonary expressions of microRNAs. It is reported that CS markedly affects microRNA expressions in mice lung tissues, mostly of which are dose-dependently or time-dependently downregulated[17]. An *in vivo* smoking model showed that there are 16 and 23 kinds of microRNAs are upregulated in the plasma at 4 weeks and 15 weeks, respectively, among which miR-21 expression is the mostly upregulated. Meanwhile, 14 kinds of microRNAs are downregulated and miR-181 is the most pronounced[18]. In a smoking-induced

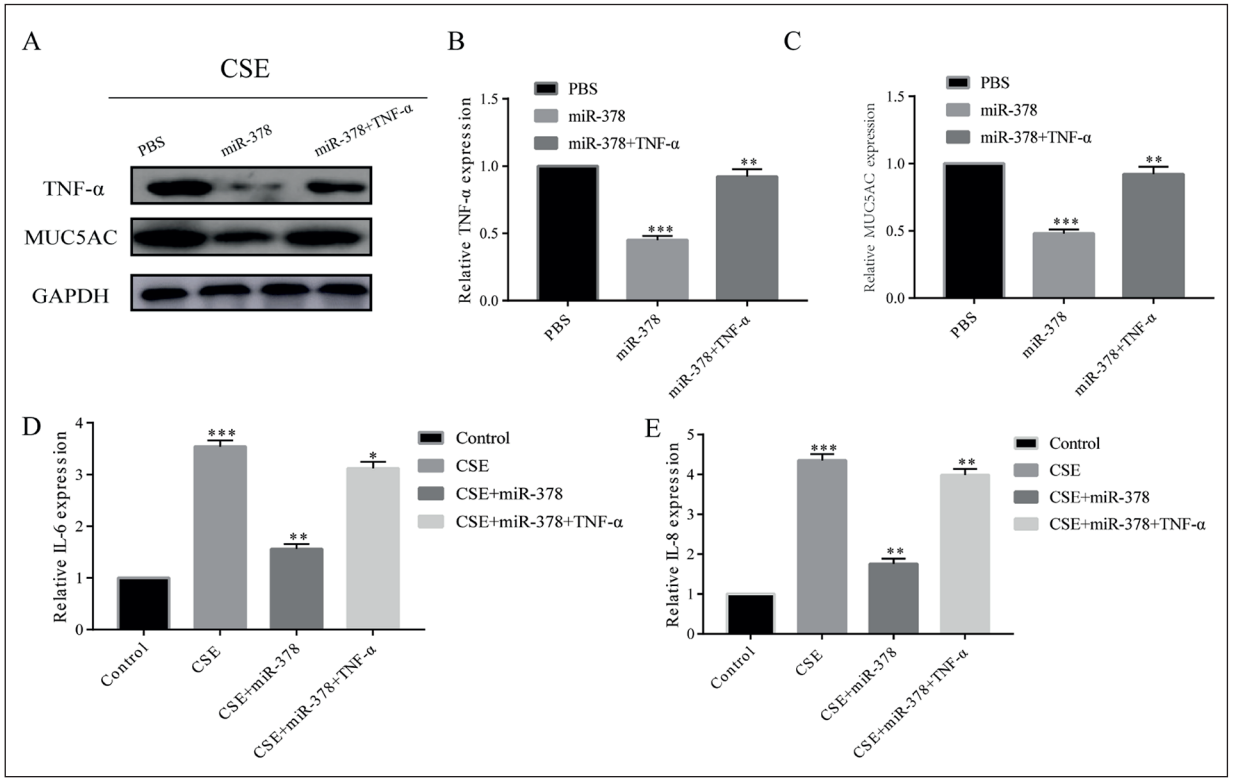

Figure 4. MiR-378 protected CSE-induced inflammatory response by targeting TNF-α. CSE-treated HBE cells were transfected with miR-378 mimics or co-transfected with miR-378 mimics and TNF-α overexpression plasmid. **A,** Western blot analyses of TNF-α and MUC5AC in HBE cells. **B-C,** The mRNA levels of TNF-α **(B)** and MUC5AC **(*C*)** in HBE cells. **D-E,** The mRNA levels of IL-6 **(D)** and IL-8 **(E)** in HBE cells. **p*<0.05, ***p*<0.01, ****p*<0.001.

COPD model, expressions of miR-20a, miR-28-3p, miR-34c-5p, and miR-100 are downregulated, while miR-7 is upregulated[19]. In this report, we established an *in vitro* COPD model by CSE treatment in HBE cells. MicroRNA-378 was markedly downregulated, while levels of IL-6, IL-8 and MUC5AC were upregulated in CSE-treated HBE cells, indicating the successful construction. Moreover, transfection of microRNA-378 mimics markedly inhibited levels of IL-6, IL-8 and MUC5AC in CSE-treated HBE cells. TNF-α exerted an important role in tumor suppression. Chen et al[20] have confirmed that TNF-α promotes the release of inflammatory factors such as IL-6 and IL-8 by activating NF-κB. NF-κB is a ubiquitous transcription factor composed of five distinct subunits, namely p65 (Rel A), Rel B, c Rel, p50 and p52. Activation of NF-κB can be detected in sputum, macrophages, and airway epithelial cells of COPD patients. It is also related to the production of various inflammatory factors and chemokines, including IL-1, IL-6, and IL-8[21]. Song et al[22] have found that TNF-α promotes the development of COPD by activating NF-κB to induce the synthesis of MUC5AC in airway epithelial cells. In this study, TNF-α was identified to be the target gene of microRNA-378 through online prediction and functional analysis. Our study further verified that microRNA-378 could bind to TNF-α and regulate its expression. In the CSE-induced COPD cell model, we examined the expressions of TNF-α and p-p65. TNF-α expression showed a time-dependent elevation with the prolongation of CSE induction. Similarly, p-p65 was gradually upregulated in a time-dependent manner, indicating that TNF-α may promote the activation of p-p65. To test whether microRNA-378 inhibited inflammatory response by degrading TNF-α expression, rescue experiments were conducted by co-overexpression of microRNA-378 and TNF-α in CSE-treated HBE cells. TNF-α overexpression partially reversed the decreased levels of inflammatory factors and MUC5AC in HBE cells overexpressing microRNA-378, which confirmed our speculation.

Conclusions

MicroRNA-378 inhibits the inflammatory response by targeting TNF-α, which may be a potential therapeutic target for COPD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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