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Original Article

CircABCB10 Promotes the Apoptosis and Inflammatory Response of 16HBE Cells by Cigarette Smoke Extract by Targeting miR-130a/PTEN Axis

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) has become a global public health problem due to its high mortality. So there is an urgent need to find an effective treatment.

Methods: The targeting relationship among circABCB10, miR-130a and PTEN was predicted by the targetscan database (TargetScanHuman 8.0, https://www.targetscan.org/vert_80/). A total of 60 patients which were from the second affiliated hospital of Qiqihar Medical University from 2022 to 2023 were enrolled. The lung condition was detected by CT (Computed Tomography). The expression levels of circABCB10, miR-130a and PTEN in lung tissues were determined by qRT-PCR. The COPD model was established by stimulating normal and silenced 16HBE cells in circABCB10 genes with cigarette smoke extract (CSE) at different concentrations. qRT-PCR was conducted for the expression levels of circABCB10, miR-130a and PTEN, WB for the expression levels of apoptotic proteins, ELISA for the content of inflammatory factors, and CCK8 for the effect of CSE on the proliferation of cells.

Results: CircABCB10 expression increased in lung tissues from patients with COPD and in 16HBE cells treated with CSE. The stimulation on cells with CSE increased the expression of inflammatory factors, while knocking down circABCB10 could reverse this response. The inflammatory response to the knockdown of circABCB10 was reversed by miR-130a inhibitor, which increased the expression of c-caspase 3. The targetscan database predicted the target factor downstream miR-130a was PTEN. Transfecting OE-PTEN reversed the inflammation of knocking down circABCB10, and increased the apoptosis and inflammation.

Conclusion: CircABCB10 can cause the inflammatory response by targeting miR-130a/PTEN axis, which is a mechanism that may lead to the occurrence and development of COPD.

Keywords: Apoptosis; Immunology; Cells; Chronic obstructive pulmonary disease

Introduction

Human bronchial epithelioid (HBE) cells are representative cells in respiratory tract. The growing burden in China has made chronic obstructive pulmonary disease (COPD) one of the biggest

challenges in medical issues (1). The pathogenesis of COPD has been associated with multiple factors, such as excessive mucus secretion, oxidative stress, and inflammation of airway and lung (2). Cigarette smoke (CS) is essential for COPD, causing oxidative stress and inflammatory responses and the pathogenesis of COPD (3). Currently, effective treatments for preventing the development of COPD have not been fully discovered, and new treatments must be focused. In this study, 16HBE cells were selected as a model, providing a scientific in vitro model for exploring the mechanisms of CSE-induced apoptosis and inflammation of 16HBE cells.

Circular RNA (circRNA) is a particular type of non-coding RNA that forms through reverse splicing events of exon or intron circularization (4,5). CircRNA acts as a miRNA sponge to regulate gene transcription and interact with the RNA binding protein (RBP) involved in tumorigenesis (6). These reports suggest circRNAs gradually provides potential perspectives for diagnosis and treatment of cancers.

PTEN has been known to induce the apoptosis by being activated, and its expression can promote the development in inflammatory bowel disease (IBD), as well as the apoptosis and production of inflammation. microRNA-374a (miR-374a) has been effectively inhibit the activation of PTEN and induce the apoptosis with its abnormal expression in IBD (7). Overall, miR-130a/PTEN was also relevant in reducing the inflammatory response and apoptosis of cells (7), so this study focused on the mechanism of circABCB10 targeting miR-130a/PTEN axis in the inflammatory response and apoptosis of CSE-induced 16HBE cells.

Methods

Clinical characteristics of patients with lung cancer

Twenty of patients were non-COPD non-smokers, 20 were non-smokers with COPD, and other 20 were smokers with COPD. The study was completed at the Second Affiliated Hospital of Qiqihar Medical University, China from September 2022 to February 2023. The enrolled patients with COPD were diagnosed according to the Global Chronic Obstructive Pulmonary Disease Initiative (GOLD) criteria (1). Non-COPD non-smokers, 2 non-COPD smokers and 2 COPD smokers were randomly selected for lung CT examination. Lung tissue at least 5 cm from the edge of cancer tissues was immediately cryopreserved in the liquid nitrogen at -80 ℃ after surgical resection.

All the patients enrolled volunteered in this study and signed the informed consent in accordance with the Declaration of Helsinki. This study has been approved by the Ethics Committee of The Second Affiliated Hospital of Qiqihar Medical University (NO.20220907-01).

Cell culture and CSE processing

The cells used in this study were 16HBE human normal bronchial epithelial cell line (purchased from ATCC, USA). The cells were incubated in a thermostatic incubator at 37℃, containing 95% oxygen and 5% CO_2. The medium used for the cells was DMEM complete medium. When the growth density of 16HBE cells reached 80-90%, CSE (purchased from Enshi Jinhua Bioengineering Co., Ltd., CAS: 98072-40-3) at different concentrations (0%, 0.5%, 1%, 1%, 2% and 4%) were diluted with distilled water, to treat the cells for 0h, 12h, 24h, 36h and 48h. CSE was prepared as previously described (2).

Cell transfection

All of sh-NC, sh-circABCB10, OE-PTEN, OE-vector, mimics control, miR-130a mimics, inhibitor control and miR-130a inhibitor required for cell transfection were purchased from Shanghai Genechem Co., Ltd. The above plasmids was transfected into 16HBE human normal bronchial epithelial cell line with Lipofectamine 3000 (Invitrogen, USA). Subsequent experiments were performed after 72h of transfection.

Proliferation assay

Treated 16HBE cells were inoculated into the 96-well plate containing DMEM medium at 1×10^4 cells/well. After adhesion, the cells were cultured for 0h, 24h, 48h and 72h, respectively. Then, 20μL of CCK8 solution (Sigma, USA) was added to each well for another 4h of incubation. Finally, the 96-well plate was removed and the corresponding OD was measured at A450nm with MTT. All the experiments were conducted in triplicate.

ELISA

Treated 16HBE cells were inoculated into the 24-well plate containing DMEM medium, and cultured for 72h after adhesion. Subsequently, the levels of interleukin 1β (IL-1β, Art. No.: PI305), tumor necrosis factor α (TNF-α, Art. No.: PT518) and interleukin 6 (IL-6, Art. No.: PI330) were measured in the supernatant with ELISA kit (Boster, Wuhan, China).

RNA extraction and qRT-PCR analysis for mRNA expression

Total RNA was extracted from 16HBE human normal bronchial epithelial cell line with TRIzol reagent (GoScript, USA, Art. No.: A2790), which was reversely transcribed into cDNA with the reverse transcription kit (Promega, USA). Real-time quantification was performed with the ABI PRISM 7500 RT-PCR system and SYBR Green Master Mix (Takara, Japan, Art. No.: RR820Q/A/B). The reaction conditions were: 10 min at 5℃ for 40 cycles, 15s at 94℃, 1 min at 60℃ and stored at 4℃. All the primers were purchased from Sangon Biotech (Shanghai) Co., Ltd (4). See Table 1 for primer sequences.

Table 1: Primer sequences

Primer	*Forward*	*Reverse*
circ-ABCB10	5'-CTAAGGAGTCACAGGAAGACATC-3'	5'-GTAGAATCTCTCAGACTCAAGGTTG-3'
miR-130a	5'-ACACTCCAGCTGGGGCTCTTTTCACATTGT-3'	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAG TTGAGAGTAGCAC-3'
PTEN	GTGCA GATAATGACAAG	GATTTGACGGCTCCTCT

Western blot for protein expression

Treated 16HBE cells were inoculated into the 6-well plate at 1×10^5 cells/well. After 72h of culture, total protein was extracted and subjected to gel electrophoresis and electrotransfer membrane, which was then blocked with 1% BSA for 1h at the room temperature. Primary antibodies, including total-caspase 3 (t-caspase 3) (1:2000, ab13847, Abcam, USA), cleaved-caspase 3 (c-caspase 3) (1:2000, ab2302, Abcam, USA) and GAPDH (1:2000, ab8254, Abcam, USA) were incubated overnight (at 4℃). The membrane was washed with phosphate buffer solution (PBS) for 3 times, and then incubated with a secondary antibody (1:5000, Jackson ImmunoResearch, USA) at the room temperature (at 37℃) for 2h. The protein expression was detected by ECL chemiluminescence solution (Millipore, USA). All the experiments were conducted in triplicate.

Dual-luciferase reporter assay

Dual-luciferase reporter genes of circABCB1 and PTEN containing miR-130a predicted loci. HEK-293T cells were transfected with circABCB10-WT + mimic control, circABCB10-WT + miR-130a mimic, circABCB10-MUT + mimic control and circABCB10-MUT + miR-130a mimic, respectively, as well as PTEN-WT + mimic control, PTEN-WT + miR-130a mimic, PTEN-MUT + mimic control and PTEN-MUT + miR-130a mimic, respectively, at the same time. After 72h of transfection, luciferase activity was measured with the dual-luciferase reporter assay system (Promega, USA).

Statistical analysis

All experiments were conducted in triplicate. Data were expressed as mean ± standard deviation after data analysis with GraphPad Prism 7.0 software. Data from the two sets of samples were analyzed with t-test. Multiple sets of sample data were analyzed with ANOVA. $P<0.05$ was considered as the difference with statistical significance.

Results

CT examination of lung

Compared with the blank group (Fig. 1A, D), the lungs of non-COPD smokers (Fig. 1B, E) and COPD smokers (Fig. 1C, F) had a significant ($P < 0.05$) blackening trend, and toxic substances were adsorbed and precipitated on the lung mucosa, and inflammatory lesions occur as time goes on. There were more and more glands secreting mucus from smoking for a long time, which led to the thickening of respiratory mucosa, the increase of sputum secretion, the narrowing of airway, and the increasing area of white lung.

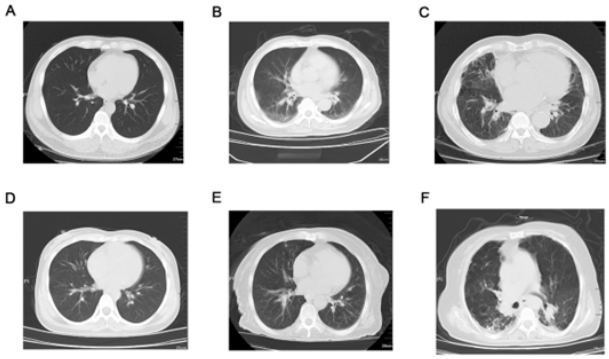

Fig. 1: Lung CT images of different groups

Notes: A. CT of healthy male lung; B. Lung CT of male non-COPD smokers (smoking age of 5 years); C. CT of male lung of COPD smokers (smoking age of 10 years); D. Lung CT of healthy women; E. Lung CT of female non-COPD smokers (smoking age 5 years); F. CT of female lung of COPD smokers (smoking age of 10 years)

circABCB10 has a targeting effect on miR-130a

According to the prediction of the targetscan database, there might be interacted loci between circABCB10 and miR-130a. The results showed that after transfecting *circABCB10* gene, the expression of *circABCB10* gene increased significantly ($P < 0.01$) in HEK-293T cells (Fig. 2A), indicating a successful transfection, while after continued transfection with miR-130a mimc, miR-130a expression in cells also increased (Fig. 2B). Fig. 2C and 2D also showed that miR-130a mimic reduced circABCB10-WT activity, as well as circABCB10 expression, with almost no effect in the circABCB10-Wut group, which initially suggested that circABCB10 had a targeting effect on miR-130a.

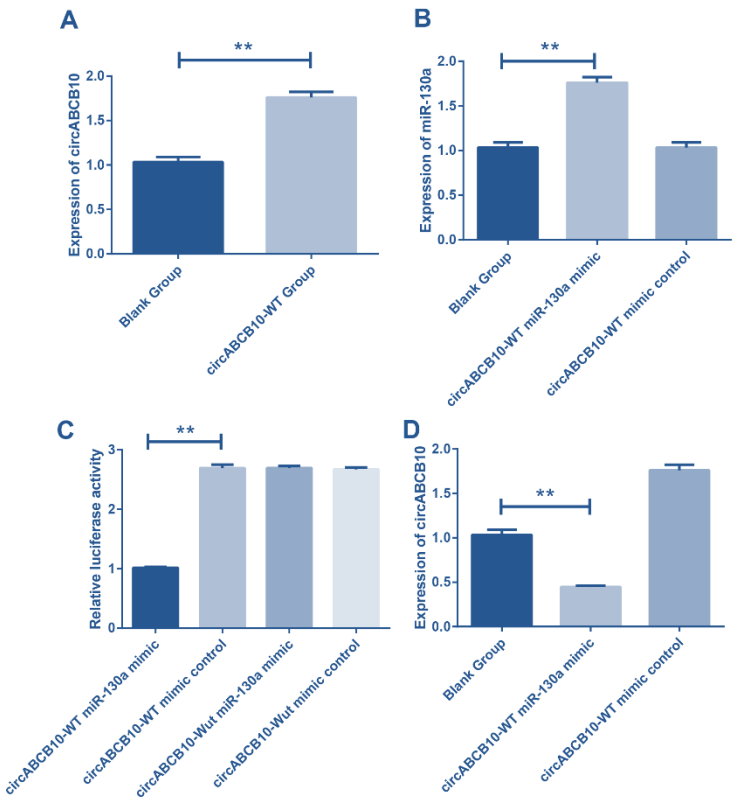

Fig. 2: circABCB10 has a targeting effect on miR-130a

Note: A, circABCB10 expression after co-transfecting circABCB10-WT and circABCB10-Mut into HEK-293T cells, respectively; B, miR-130a expression after re-transfecting miR-130a mimic; C, Effect on circABCB10 activity after transfecting miR-130a mimic; D, Effect on circABCB10 gene expression after transfecting miR-130a mimic. ** represents $P<0.01$

miR-130a can directly target PTEN gene

Through the database prediction, PTEN might be a potential target gene downstream of miR-130a. According to the results, after transfecting PTEN gene, PTEN gene expression increased significantly in HEK-293T cells (Fig. 3A), suggesting a successful transfection. After continued transfection of miR-130a mimc, miR-130a expression in cells also increased (Fig. 3B). Fig. 3C and 3D also showed that miR-130a mimic reduced PTEN activity, as well as PTEN expression, with almost no effect in the PTEN-Wut group, which initially suggested that miR-130a had a targeting effect on PTEN.

Expression levels of circABCB10, miR-130a and PTEN in lung tissues of patients with COPD

The expression levels of circABCB10, miR-130a and PTEN in lung tissues were detected by qRT-PCR, showing a higher circABCB10 expression in lung tissues of smokers than that in smokers without COPD (Fig. 4A). miR-130a expression was lower in lung tissues of smokers than that in non-smokers, and also significantly lower in smokers with COPD than that in smokers without COPD (Fig. 4B). However, PTEN expression was the opposite of miR-130a (Fig. 4C).

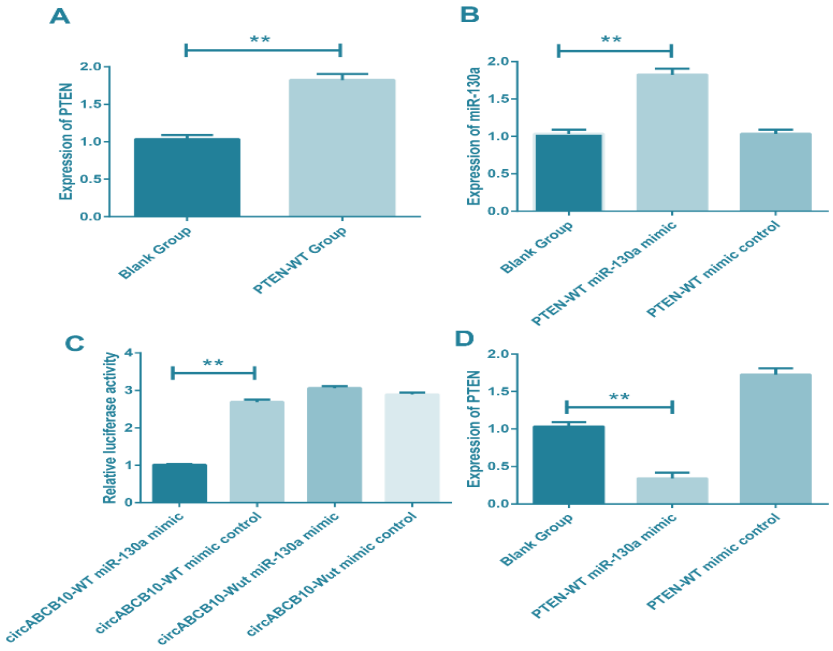

Fig. 3: miR-130a can directly target PTEN gene

Note: A, PTEN expression after co-transfecting PTEN-WT and PTEN-Mut into HEK-293T cells, respectively; B, miR-130a expression after re-transfecting miR-130a mimic; C, Effect on PTEN activity after transfecting miR-130a mimic; D, Effect on PTEN gene expression after transfecting miR-130a mimic. ** represents $P<0.01$

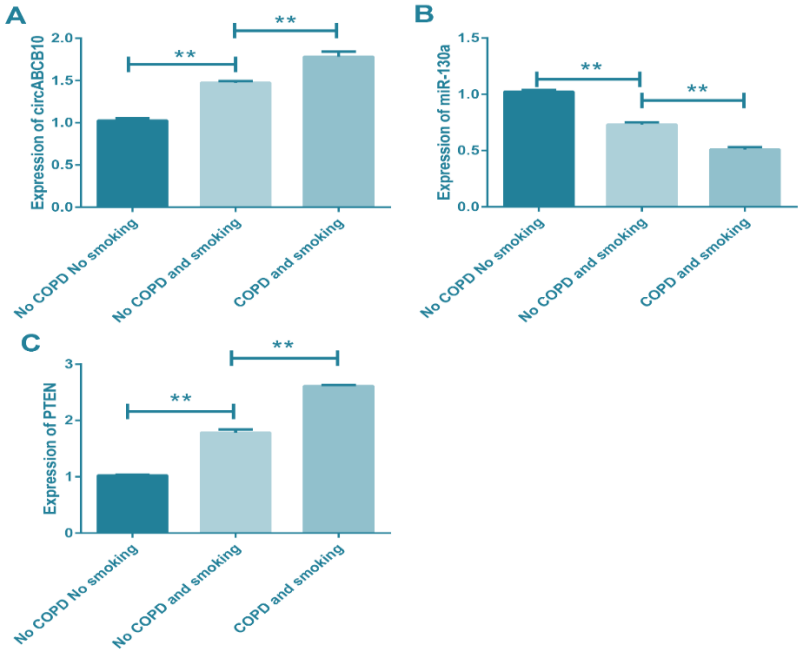

Fig. 4: Expression levels of circABCB10, miR-130a and PTEN in lung tissues of patients with COPD

Note: A, Lung tissues selected in this study were obtained from non-smokers (n=20), smokers without COPD (n=20) and smokers with COPD (n=20), and circABCB10 expression in lung tissues were determined by qRT-PCR; B, miR-130a expression in lung tissues of different groups; C, PTEN expression in lung tissues of different groups. ** represents $P<0.01$

Effects of cell expression of circABCB10, miR-130a and PTEN after CSE treatment

The CSE stimulation increased circABCB10 expression in 16HBE cells, and the increase of circABCB10 and PTEN expression was concentration-dependent (Fig. 5A and 5B), while the decrease of miR-130a expression was concentration-dependent (Fig. 5C). Subsequently, 16HBE cells were treated with 4% CSE for 0h, 12h, 24h, 36h and 48h. The results showed that the expression of circABCB10 and PTEN in 16HBE cells increased with the increase of CSE stimulation time (Fig. 5D and 5E). miR-130a expression decreased with the increase of CSE stimulation time (Fig. 5F).

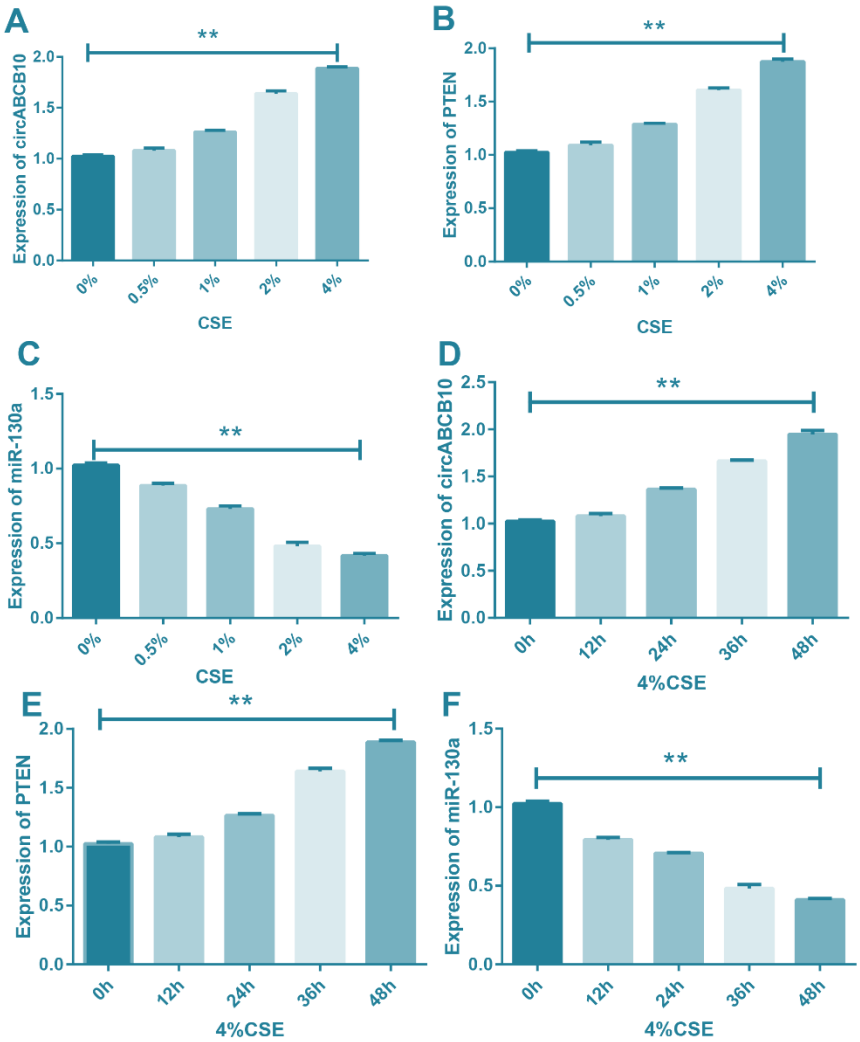

Fig. 5: Expression levels of circABCB10, miR-130a and PTEN in 16HBE cells after CSE treatment

Note: A,16HBE cells were treated with CSE at different concentrations (0%, 0.5%, 1%, 2%, 2% and 4%) for 48h, and circABCB10 expression at the mRNA level was determined by qRT-PCR; B, Detection of PTEN expression in cells; C, Detection of miR-130a expression in cells; D-F, Gene expression of circABCB10, miR-130a and PTEN in 16HBE cells treated with 4% CSE at different time, respectively. ** represents $P<0.01$

Knocking down circABCB10 can reverse CSE-induced proliferation, apoptosis and inflammatory response

The CSE stimulation increased circABCB10 expression in 16HBE cells, which, however, was significantly down-regulated after transfecting sh-

circABCB10 (Fig. 6A). It indicated that CSE stimulation inhibited the proliferative ability of 16HBE cells, while knocking down circABCB10 increased the proliferation capacity (Fig. 6B). Fig. 6C and 6D also showed that CSE stimulation increased the expression of the apoptotic protein, c-caspase 3, in cells, which were reversed by knocking down circABCB10. Finally, the expression levels of inflammatory cytokines, IL-1β, IL-6 and TNF-α, in the supernatant of the medium were detected. According to the results, CSE stimulation increased the expression levels of inflammatory factors, in the supernatant, while knocking down circABCB10 reduced the expression of the above inflammatory factors (Fig. 6E-6G).

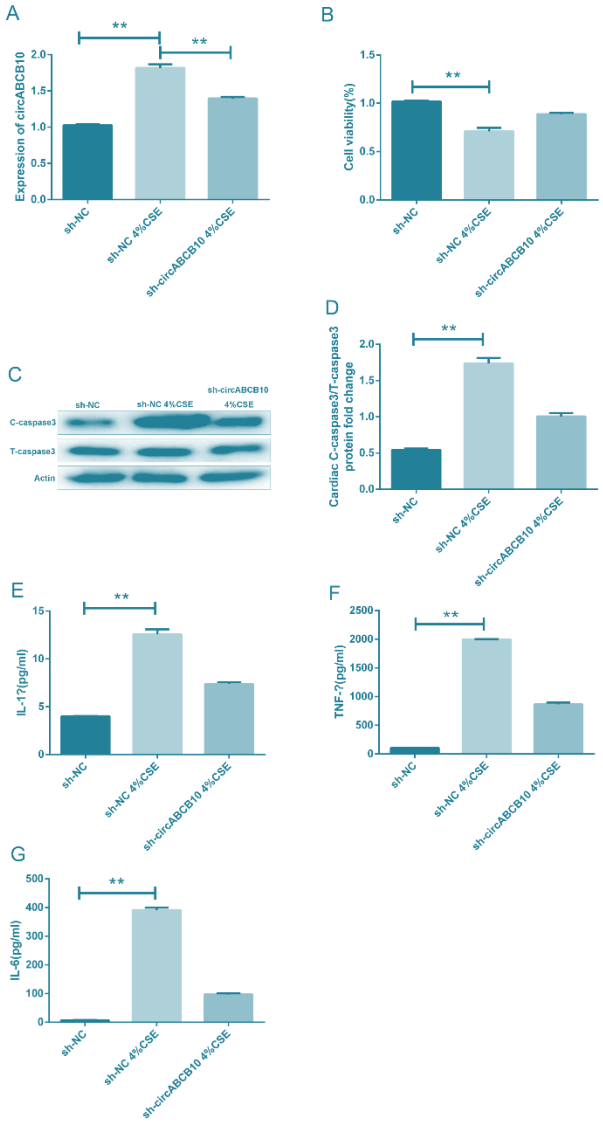

Fig. 6: Knocking down circABCB10 can reverse CSE-induced proliferation, apoptosis and the inflammatory response

Note: A, circABCB10 expression was determined by qRT-PCR; B, The proliferation was determined by CCK8; C-D, The expression of the apoptotic protein, c-caspase 3, and the protein ratio of c-caspase 3/T-caspase 3 was determined by Western blot; E-G, The changes of expression levels of cytokines IL-1β, IL-6 and TNF-α in the supernatant were determined by ELISA. ** represents $P<0.01$

miR-130a inhibitor can reverse the biological effect of knocking down circABCB10

The miR-130a was significantly (P<0.01) down-regulated in cells transfected with miR-130a inhibitor (Fig. 7A). CCK-8 proliferation assay indicated that CSE stimulation can inhibit the proliferation capacity of 16HBE cells, which can be enhanced after knocking down circABCB10. However, transfecting miR-130a inhibitor can inhibit the effect of enhancing the proliferation capacity after knocking down circABCB10 (Fig. 7B). The CSE stimulation increased the expression of the apoptotic protein, c-caspase 3, in cells and the expression levels of these proteins decreased after knocking down circABCB10, which could be reversed after transfecting miR-130a inhibitor (Fig. 7C-7D). Finally, we also found that CSE stimulation could increase their expression levels, the expression levels of the above inflammatory factors could be decreased after knocking down circABCB10, however, the expression levels of inflammatory factors in the supernatant of the medium could be increased after transfecting miR-130a inhibitor (Fig. 7E-7G).

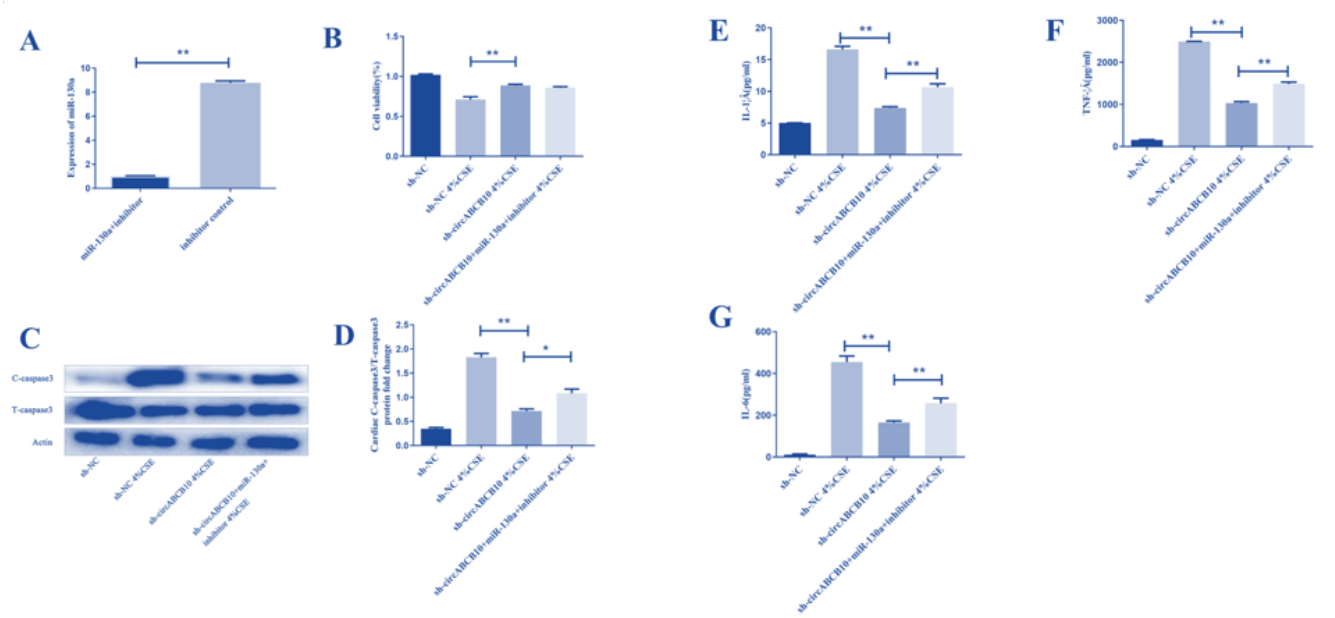

Fig. 7: miR-130a inhibitor can reverse the biological effect of knocking down circABCB10

Note: A, The expression of miR-130a was determined by qRT-PCR; B, The proliferation of 16HBE cells was determined by CCK8; C-D, The expression level of apoptosis protein c-caspase 3 and the protein ratio of c-caspase 3/ t-caspase 3 were detected by Western blot; E-G, The changes of expression levels of cytokines, IL-1β, IL-6 and TNF-α in the supernatant were determined by ELISA. ** represents *P*<0.01

circABCB10 was involved in the pathogenesis of COPD by regulating miR-130a/PTEN axis

OE-PTEN and OE-vector lentiviral plasmids were transfected into 16HBE cells. Then, it found that mRNA expression of PTEN was up-regulated after transfecting OE-PTEN (Fig. 8A). The CSE stimulation could inhibit the proliferation capacity of 16HBE cells, which could be enhanced after knocking down circABCB10. However, transfecting OE-PTEN could inhibit the effect of enhancing the proliferation after knocking down circABCB10 (Fig. 8B). According to Western blot for expression levels of apoptotic proteins, CSE stimulation could increase the expression of c-caspase 3 in cells, which could decrease after knocking down circABCB10. However, this effect could be reversed by transfecting OE-PTEN (Fig. 8C and 8D). Finally, with the detection of ELISA on the expression levels of inflammatory factors IL-1β, IL-6 and TNF-α in the supernatant of the medium, it was also found that CSE stimulation could increase their expres-

sion levels after knocking down circABCB10. However, the expression levels of inflammatory factors in the supernatant of the medium could be increased after transfecting OE-PTEN (Fig. 8E-8G). These findings suggested that circABCB10 can be involved in the pathogenesis of COPD by regulating miR-130a/PTEN axis.

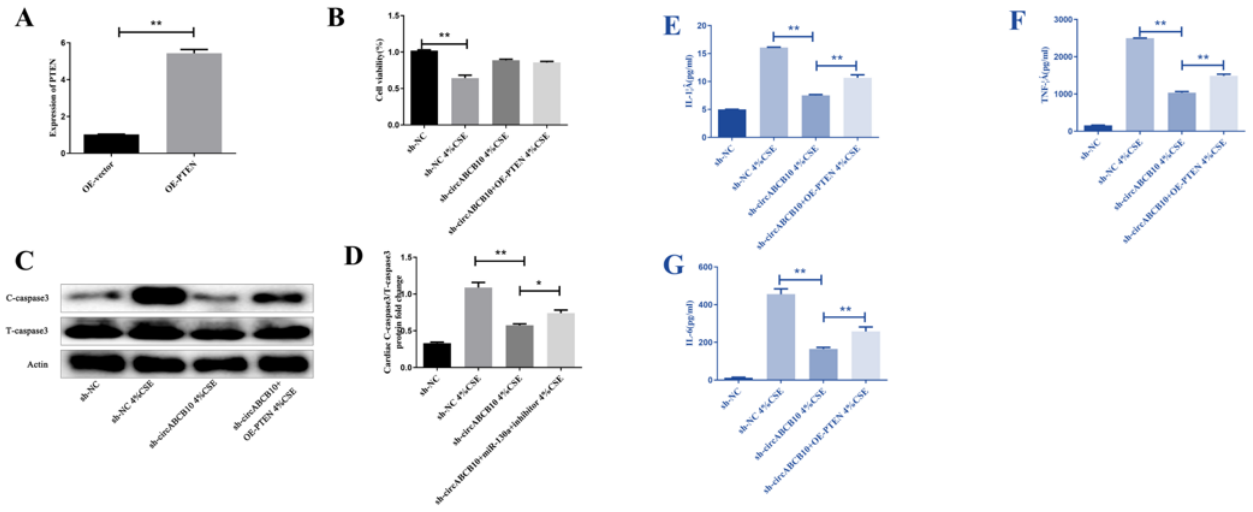

Fig. 8: circABCB10 was involved in the pathogenesis of COPD by regulating miR-130a/PTEN axis.
Note: A, The mRNA level of PTEN were determined by qRT-PCR; B, The proliferation of 16HBE cells was determined by CCK8; C-D, The expression levels of the apoptotic protein c-caspase 3 and the protein ratio levels of c-caspase 3/T-caspase 3 were determined by Western blot; E-G, The changes of expression levels of cytokines, IL-1β, IL-6 and TNF-α in the supernatant of the medium were determined by ELISA. ** represents $P<0.01$

Discussion

COPD is usually caused by smoking, which can also be caused by long-term exposure to other lung irritants (e.g. second-hand smoke). Up to as many as a quarter of Americans with COPD never smoke (8,9).

In emphysema, stimuli (e.g. smoking) can cause an inflammatory response. Neutrophils and macrophages are recruited and release multiple inflammatory mediators. Oxidizing agents and excessive protease lead to the destruction of air sacs. Protease-mediated destruction of elastin results in loss of elastic retraction and airway collapse on exhalation (10,11).

miR-130a/PTEN axis signaling plays a role in the occurrence of branch morphology and formation of airways during lung development (12,13). In the mature lung, miR-130a/PTEN axis signaling is frequently activated during damage repair and tissue regeneration. Several evidence suggested that miR-130a/PTEN axis signaling molecules are suppressed in lung biopsies and murine models of patients with COPD (14).

MicroRNA (miRNA) is a family of small non-coding RNA, 21-25 nucleotides in length, which can promote mRNA degradation or translational inhibition by binding to downstream targets in its 3'UTR (15). Dysregulation of miRNA is associated with many pathological conditions, including COPD (16-18). As Molina-Pinelo reported (19), there is significant expression of 15 miRNAs in COPD compared with that in normal controls ($P<0.05$). By targeting different downstream transcription, miRNA exerts different functions in COPD progression. Alternatively, miR-130a mediated post-transcriptional regulation of PTEN in promoting tumorigenesis. A large proportion of human cancers exhibit the loss of only one PTEN allele. Given the importance of miRNAs in neogenesis (20) and the fact that PTEN is a direct target of miR-130a, it is possi-

ble to assess whether miR-130a may promote the transfer of cigarette smoke to promote the development of chronic obstructive pulmonary disease, in particular, whether it is highly correlated with cellular inflammation and elevation of circABCB10 cause by CSE, causing the pulmonary tissue disease process (21,22).

Increasing evidence suggest that different types of RNAs have become important regulators of tumor development and progression by acting in a variety of ways (23,24). Recent study has shown that circRNA is dysfunctional in multiple human cancers, and dysregulated circRNA plays an important role in tumorigenesis and tumor progression of multiple cancers, including CRC (25). Li et al. found that circRNA is significantly down-regulated in samples of esophageal squamous cell carcinoma (ESCC), and circITCH possesses multiple miRNA binding loci, which may function as a sponge, transporter, and/or regulator for these mirnas. Another study demonstrated the same role of circRNA in lung cancer (26), which found lower circITCH levels in lung cancer tissues compared with that in normal tissues. Low expression may be caused by low transcription of genes, and ITCH mRNA was also significantly down-regulated in patients with lung cancer. The authors found that over-expression of circITCH plays a specific role in Wnt/β-catenin mediated lung cancer models, and circITCH may inhibit cancer progression by regulating the activity of oncogenic Mir-7 and Mir-214 factors. However, the function and mechanism of the role of circRNA are still unclear. CircABCB10 is a newly identified circRNA, which has been shown to have a neoplastic function that promotes proliferation and progression of breast cancer, clear cell renal cell carcinoma and epithelial ovarian cancer cells (27). There is also increasing evidence that circRNA plays a critical role in cell development and is involved in different pathological and physiological processes (28). Qiao et al. demonstrated that knocking down circRBMS1 attenuates CSE-induced apoptosis, oxidative stress and lung inflammation process by up-regulating miR-197-3p in FBXO11 or 16HBE cells (29). ccircFOXO3 has been reported to promote cardiac senescence and knocking down circFOXO3 can reduce myocardial ischemia-reperfusion injury (MIRI) (30,31). However, the role of circABCB10 in COPD remains unknown. In this study, 16HBE was used as a model and found that CSE can cause cell inflammation and further exacerbate apoptosis. miR-130a can reduce the inflammatory response and apoptosis by targeting PTEN. However, after cigarette extract treatment, circABCB10 induces an inflammatory response by targeting the new activation of mir-130A /PTEN axis, which may lead to the occurrence and development of COPD.

Conclusion

CircABCB10 could cause the inflammatory response, increase the apoptosis and inflammation, and lead to the occurrence and development of COPD by targeting miR-130a/PTEN axis under the induction of CS factors, thus this conclusion is expected to provide the basis for further scientific prevention and treatment of COPD.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest.

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