RETRACTED ARTICLE: Formaldehyde inhibits proliferation of bronchial epithelial cells by down-regulating miR-375

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ABSTRACT
Objective: To study the effect of formaldehyde on the proliferation of human bronchial epithelial cells 16HBE and to explore its mechanism.
Methods: MTT assay was used to detect the inhibition rate of formaldehyde-treated 16HBE cells; FCOH + miR-375 group (transfected miR-375 mimics), FCOH + miR-con group (transfected miR-con), FCOH + si-KLF4 group (transfected si-KLF4) and FCOH + si-con group (transfected si-con), were transfected into 16HBE cells by liposome method, then treated with formaldehyde 200 μmol/L for 24 h; qRT-PCR was used to detect the expression of miR-375 in each group; the protein expression of KLF4 in each group was detected by Western blot. The fluorescence activity of each group was detected by dual-fluorescein gene detection assay.
Results: Compared with 16HBE cells in Control group, the expression of miR-375 was significantly decreased in FCOH group, cell proliferation was significantly decreased, and KLF4 expression was significantly increased (p < .05). Overexpression of miR-375 and KLF4 knockdown could reverse the inhibition effect of formaldehyde on proliferation of 16HBE cells; KLF4 is a target of miR-375. KLF4 could reverse the promotion of miR-375 on the proliferation of formaldehyde-treated 16HBE cells.
Conclusion: Formaldehyde can inhibit the proliferation of human bronchial epithelial cells. The mechanism may be related to the down-regulation of miR-375 targeting KLF4, which will provide support for the treatment of chronic respiratory diseases.

Introduction
MicroRNA is a small non-coding RNA with a length of about 20–25 nucleotides. It partially or completely complements the target gene 3’UTR by base complementation and regulates the target gene expression after transcription [1]. In recent years, more and more studies have confirmed that miRNA is involved in a variety of diseases and pathophysiological processes of tumors. Studies have reported that miR-375 plays an important role in myocardial infarction repair and testicular cell proliferation [2,3], but its role in bronchial epithelial cells is not yet clear. Krüppel-like transcription factors (KLFs) form a subclass of ~17 zinc fingers, which contain a DNA-binding transcription factor expressed in humans and belong to the specific protein 1 (Sp1)/KLF zinc finger-binding transcription factor family [4–6]. KLF4 is expressed in different cell types, such as endothelial cells, epithelial cells and bone marrow cells [7–10]. KLF4 contains activation domain and inhibition domain, so it can induce and inhibit the transcription of different genes, including molecules involved in regulating immune response [11]. However, the mechanism of KLF4 in bronchial epithelial cells has not been studied. In this study, human bronchial epithelial cells 16HBE were used as the research object to detect the effects of formaldehyde, overexpression of miR-375 and knockdown of KLF4 on the proliferation of 16HBE cells, revealing that the mechanism may be related to the inhibition of miR-375 expression targeting KLF4 by formaldehyde, which will provide a basis for the application of formaldehyde in respiratory diseases.

Materials and methods
Materials
Human bronchial epithelial cells 16HBE were purchased from ATCC; DMEM medium, fetal bovine serum, MTT reagent, trypsin, LipofectamineTM2000, BCA protein quantification kit and reverse transcription kit were purchased from Takara Company (Dalian, China); The SDS-PAGE kit, ECL luminescent solution and RIPA protein lysate were purchased from Beyotime Biotechnology Co., Ltd.; the dual luciferase reporter assay kit was purchased from Promega, USA.

Methods
Cell culture
Human bronchial epithelial cells 16HBE were cultured in DMEM medium containing 10% fetal bovine serum, and cultured in an incubator at 37°C, 5% CO₂.
Cell processing and grouping
16HBE cells were treated with formaldehyde (0, 50, 100, 150, 200, 250, 300, 350 μmol/L) for 24 h, and then used for MTT assay; 16HBE cells treated with formaldehyde at 200 μmol/L were labelled as FCOH group, while untreated 16HBE cells were labelled as Control group. MiR-375 mimics, miR-con, si-KLF4 and si-con were transfected into 16HBE cells according to the instructions of LipofectamineTM2000 kit. After treatment with formaldehyde at 200 μmol/L, they were labelled as FCOH + miR-375 group, FCOH + miR-con group, FCOH + si-KLF4 group and FCOH + si-con group. After 48 h of transfection, the transfection efficiency was detected by qRT-PCR. After successful transfection, it was used for subsequent experiments.

MTT experiment
An appropriate amount of cells in each group were taken and 20 μL 5 g/L MTT solution was added. The solution was cultured for 3.5/4 h and then the supernatant was discarded. 150 μL DMSO was added per well with shaking and the crystal was dissolved. The cell absorbance (A) was measured at 490 nm wavelength. Cell proliferation ability was positively correlated with absorbance.

qRT-PCR experiment
An appropriate amount of cells in the logarithmic growth phase were taken following the instructions of the RNA extraction kit to extract RNA, quantify and then, cDNA was synthesized according to the instructions of the reverse transcription kit. Finally, the miR-375 detection was performed according to the instructions of the qRT-PCR kit. The expression of miR-375 was calculated using \(2^{-\Delta\Delta Ct}}\)

Dual luciferase reporter gene assay
The luciferase reporter vector (psiCHECK2-KLF4-WT, psiCHECK2-KLF4-MUT) was transfected into 16HBE cells with miR-375 mimics and miR-NC, respectively by liposome method. After 6 h of culture, fresh medium was replaced and then continued to be cultured for 48 h, then operated according to the instructions of the dual luciferase reporter assay kit. The results showed that the binding intensity of miR-375 to KLF4 was reflected by the ratio of the luminescence intensity of sea cucumber luciferase to the luminescence intensity of firefly luciferase.

Western blot experiment
The cells of each group, at the logarithmic growth phase, were taken for quantitative analysis by BCA after RIPA lysis, and the supernatant was taken for protein sampling after denaturation and centrifugation. Electrophoresis-transmembrane-blocking-I anti-incubation-II anti-incubation-development exposure was performed according to the routine procedure of Western blot experiment. Image J analyzed the grey value of the target band and expressed the expression of the target protein by the ratio of the grey value of the target band to the grey value of the beta-actin.

Statistical analysis
All data in the experiment were analyzed using SPSS 21.0 software (Armonk, NY). Measurement data were expressed as mean ± standard deviation x ± s. Data among groups were compared by one-way analysis of variance. Pairwise comparisons were performed using SNK-q test. \(p < .05\) was considered statistically significant.

Results
Effects of different concentrations of formaldehyde on the proliferation of human bronchial epithelial cells
The activity of 16HBE in human bronchial epithelial cells treated with formaldehyde (0, 50, 100, 150, 200, 250, 300, 350 μmol/L) was detected by MTT assay. Results as shown in Figure 1, the activity of 16HBE cells was negatively correlated with formaldehyde in a concentration-dependent manner, and the IC50 of formaldehyde in 16HBE cells was 172.53 ± 5.35 μmol/L. Therefore, formaldehyde with a concentration of 200 μmol/L was selected for subsequent experiments.

Effect of formaldehyde on the expression of miR-375 in 16HBE cells
The 200 HBE cells treated with formaldehyde 200 μmol/L were labelled as FCOH group, the untreated cells were labelled as Control group, and the expression of miR-375 in each group was detected by qRT-PCR. The results are shown in Figure 2. Compared with the Control group, the expression of miR-375 in the FCOH group was significantly decreased \((p < .05)\).

Expression of miR-375 reverses the inhibitory effect of formaldehyde on the proliferation of human bronchial epithelial cells
The miR-375 mimics and miR-con were transfected into 16HBE cells and treated with formaldehyde 200 μmol/L, and labelled as FCOH + miR-375 group and FCOH + miR-con group. The results are shown in Figure 3. Compared with the Control group, the miR-375 in the FCOH group was significantly decreased \((p < .05)\) and the cell viability was significantly decreased in the...
FCOH + miR-con group; compared with the FCOH + miR-con group, MiR-375 was significantly increased and cell viability was significantly increased in the FCOH + miR-375 group, both of which were statistically significant ($p < .05$).

**MiR-375 targets KLF4**

Target scan was used to predict the possible binding of SUZ12 to microRNA-195. It was found that the binding of miR-195 to SUZ12 was highly possible (Figure 4(A)). The luciferase reporter gene assay was used to detect the fluorescence activity of 16HBE cells. Compared with the miR-con group, the fluorescence activity of WT cells was significantly decreased, and the fluorescence activity of MUT cells was not affected (Figure 4(B)), which was statistically significant ($p < .05$).

**Knockdown of KLF4 affects the inhibitory effect of formaldehyde on the proliferation of human bronchial epithelial cells**

Si-KLF4 and si-con were transfected into 16HBE cells and treated with formaldehyde 200 $\mu$mol/L. They were labeled as FCOH + si-KLF4 group and FCOH + si-con group. Results as shown in Figure 5. Compared with the Control group, the expression of KLF4 protein in FCOH + si-con group was significantly increased (Figure 5(A,B)), and cell viability was significantly decreased (Figure 5(C)). Compared with the FCOH + si-con group, the expression of KLF4 protein in the FCOH + si-KLF4 group was significantly decreased (Figure 5(A,B)), and the cell viability was significantly increased (Figure 5(C)), both of which were statistically significant ($p < .05$).

**Overexpression of KLF4 can reverse the overexpression of miR-375 to promote the proliferation of formaldehyde-treated 16HBE cells**

Results, as shown in Figure 6, compared with the miR-con group, the expression of KLF4 protein in the miR-375 group treated with formaldehyde, was significantly decreased (Figure 6(A)), and the cell viability was significantly increased (Figure 6(B)). Compared with the miR-375 + pcDNA group, the expression of KLF4 protein was significantly decreased in the formaldehyde-treated cells of the miR-375 + pcDNA-KLF4 group (Figure 6(A)), and the cell viability was significantly increased (Figure 6(B)), both of which were statistically significant ($p < .05$).

**Discussion**

MicroRNAs (miRNA) are small RNA molecules that play a role in gene silencing and translational inhibition by binding to target mRNAs [12]. In recent years, the regulation of miRNAs has received widespread attention due to its role in biological processes and the development of various human diseases including retinal diseases, neurodegenerative diseases, cardiovascular diseases, and cancer [13-15]. Bleck et al. [16] found in human bronchial epithelial cells that environmental particulate matter (PM) and diesel exhaust particles (DEP) upregulate thymic stromal lymphopoietin TSLP mRNA and human miR-375 in primary human bronchial epithelial cells (pHBEC), the aromatic hydrocarbon receptor (AhR) is a target of miR-375, and inhibition of miR-375 could up-regulate AhR mRNA expression in DEEP-treated pHBEC. It is suggested that both DEP and ambient PM can up-regulate TSLP in human bronchial epithelial cells through hsa-miR-375 and other
mechanisms, which have complex regulatory effects on AhR mRNA. Lu et al. [17] reported that in the differential miRNA of human esophageal squamous and bronchial columnar epithelial cells induced by interleukin-13, the expression of miR-375 was conservatively down-regulated, and the expression of miR-375 was abnormally down-regulated in human diseases characterized by overproduction of interleukin-13. In this study, the expression of miR-375 in formaldehyde-treated 16HBE cells was detected by qRT-PCR. It was found that formaldehyde could significantly inhibit the expression of miR-375 in 16HBE cells. Dual luciferase reporter gene assay confirmed that miR-375 targets KLF4. It was found that overexpression of microRNA-375 could reverse the inhibition of formaldehyde on the proliferation of 16HBE cells, and inhibit the expression of KLF4 protein and promote cell proliferation in 16HBE cells treated with formaldehyde.

KLF participates in various pathophysiological processes such as cell proliferation, differentiation and the occurrence and development of tumors in the body. KLF4 is one of them and has similar functions with KLF [18]. In the study of diabetic pulmonary fibrosis, Zou et al. [19] found that the levels of LOX-1, TGF-β1 and krüppel-like factor 6 (KLF6) in lung tissue of STZ-induced diabetic rats increased significantly. LOX-1 siRNA inhibits high glucose-induced EMT in human bronchial epithelial cells HBECS and human lung adenocarcinoma cells A549. TGF-β1 siRNA can decrease the expression of LOX-1 and KLF6, suggesting that EMT is involved in the pathological process of diabetic pulmonary fibrosis, which is related to the activation of LOX-1/TGF-β1/KLF6 signal transduction pathway. Zahlten et al. [20] reported that pneumococcus could induce KLF4 expression by activation of tyrosine kinase in lung epithelial cells BEAS-2B. Overexpression of KLF4 inhibits the activation and release of NF-κB and IL-8 reporter genes induced by Streptococcus pneumoniae, while silencing of KLF4 or yes1 kinase mediated by small interfering RNA increases IL-8 release. KLF4-dependent downregulation of NF-κB luciferase activity can be rescued by overexpression of histone acetylase p300/cAMP response element binding protein-related factors. It is revealed that KLF4 acts as a counter-regulatory transcription factor in the pneumococcal-related proinflammatory activation of lung epithelial cells, possibly preventing organ failure caused by excessive inflammation of the lung. In this study, Western blot was used to detect the protein expression of KLF4 in formaldehyde-treated 16HBE cells. It was found that formaldehyde can promote the expression of KLF4 in cells and inhibit cell

Figure 4. MiR-375 targets KLF4. (A) Complementary sequence; (B) the effect of miR-375 on the fluorescence activity of 16HBE cells *p < .05.

Figure 5. Knockdown of KLF4 affects the inhibition of formaldehyde on human bronchial epithelial cell proliferation. Compared with the Control group, *p < .05; compared with the FCOH + si-con group, #p < .05.
proliferation, while KLF4 knockdown can promote the proliferation of 16HBE cells. Further in-depth studies found that over-expression of KLF4 could reverse the promotion of miR-375 on the proliferation of formaldehyde-treated 16HBE cells.

In conclusion, formaldehyde can inhibit the proliferation of human bronchial epithelial cells, and its mechanism is related to the down-regulation of miR-375 by formaldehyde, and then to the negative regulation of KLF4, providing experimental evidence for the clinical use of formaldehyde.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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