Int J Clin Exp Med 2019;12(9):11141-11148
www.ijcem.com /ISSN:1940-5901/IJCEM0094521

Original Article

Regulation and mechanisms of lncRNA H19 in COPD

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Received March 28, 2019; Accepted July 11, 2019; Epub September 15, 2019; Published September 30, 2019

Abstract: Chronic obstructive pulmonary disease (COPD) is one of the most fatal diseases of the respiratory system. Moreover, lncRNA H19 is involved in the regulation of various diseases, including tumors. However, the roles of lncRNA H19 in COPD have not yet been elucidated. SD rats were randomly divided into the control group, model group, and H19 group. Real-time PCR was used to detect lncRNA H19 expression levels. H&E staining was used to analyze lung tissue pathological changes. Secretion of inflammatory factors IL-6, IL-8, and TNF-α was detected by ELISA. SOD and ROS content levels were also measured. The 16HBE cells were cultured *in vitro* and randomly divided into the control group, model group, and H19 group. This was followed by analysis of cell proliferation via MTT assays. Activity levels of caspase 3 and expression levels of Bax and Bcl-2 were analyzed by Western blotting. Compared with the control group, lncRNA H19 expression was significantly decreased in the COPD rat model group ($P<0.05$). Overexpression of lncRNA H19 improved lung tissue structure, downregulated secretion of IL-6, IL-8, and TNF-α, upregulated SOD expression, and inhibited ROS generation. Compared with the model group, differences were statistically significant ($P<0.05$). In addition, overexpression of lncRNA H19 in 16HBE cells promoted cell proliferation, decreased caspase 3 activity, decreased Bax expression, and increased Bcl-2 expression, with significant differences, compared to the control group ($P<0.05$). Expression of lncRNA H19 was decreased in COPD rats. In summary, upregulation of lncRNA H19 expression inhibits secretion of inflammatory cytokines, regulates oxidative stress, inhibits cell apoptosis, and promotes cell proliferation, alleviating COPD.

Keywords: Chronic obstructive pulmonary disease, lncRNA H19, inflammatory factor, apoptosis, oxidative stress

Introduction

Chronic obstructive pulmonary disease (COPD), or chronic obstructive pulmonary disease, is one of the most common respiratory diseases, worldwide. Incidence rates of this disease are second only to cardiovascular and cerebrovascular diseases [1]. According to statistics, deaths due to COPD are as high as 3 million people per year, ranking fourth in the world. Thus, it has been estimated to be third leading cause of deaths, worldwide, by 2020 [2, 3]. COPD can be found in all ages, but it is more common in middle-aged and elderly populations. Incidence of COPD in Chinese patients over 40 years old is as high as 13.7% [4]. Pathological changes of COPD include chronic bronchitis and/or emphysema with chronic airflow obstruction, chronic coughing, intolerance to exercise, shortness of breath when breathing, and difficulty breathing. These can further develop into pulmonary heart disease. The common chronic disease of exhaustion is one of the most prone and deadliest diseases in the respiratory system, seriously affecting the quality of life of patients and posing a serious threat to social health [5, 6]. Despite current advances in the treatment of COPD, COPD has not been completely eradicated. Incidence and mortality rates of COPD remain high [7]. The pathogenesis of COPD has not yet been elucidated. Pathological manifestations are complicated. They can accumulate in lung tissue structures and pulmonary blood vessels [8]. Therefore, finding and clarifying the exact targets of COPD is beneficial for treatment of COPD.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with a length of more than 200 nucleotides. They have been shown to play an important regulatory role in several biological

activities [9]. Moreover, lncRNAs have been thought to be the presence of transcriptional "noise" when initially recognized. Non-coding transcripts account for the vast majority of human genomes [10, 11]. A variety of biological activities and signaling pathways can be regulated by lncRNAs, including cell proliferation, differentiation, and other processes. Abnormal expression of lncRNAs has been closely related to tumors, neurological diseases, and autoimmune diseases [12, 13]. Of note, lncRNA H19 may be involved in the regulation of various diseases, including tumors [14, 15]. It has been shown to be abnormally expressed in respiratory diseases, including lung cancer [16]. However, the roles of lncRNA H19 in COPD have not been elucidated.

Materials and methods

Animals

Healthy SD male rats, 6 weeks old, SPF grade, with body weights of 200 ± 20 g, were purchased from the Experimental Animal Center of Shandong University. They were fed in the SPF Animal Experiment Center with a temperature of 21 ± 1°C, relative humidity of 50-70%, and a 12/day cycle every 12 hours.

Experimental procedures were approved by the Animal Ethics Committee of Shandong Qingdao Hospital of Integrated Traditional Chinese and Western Medicine (Qingdao, Shandong, China).

Reagents and equipment

Primary normal human bronchial epithelial cells (16HBE) (product number: ATCC® CRL-2741TM) were purchased from ATCC, USA. TRIzol Reagent was purchased from Sigma (USA). Moreover, lncRNA H19 plasmids and lncRNA H19 adenoviruses were designed and synthesized by Shanghai Gene Co., Ltd. The RNA extraction kit, RT-PCR primer, reverse transcription (RT) kit, and Real-time PCR reagents were purchased from Axygen (USA). PVDF membranes were purchased from Pall Life Sciences. Western blotting related chemical reagent was purchased from Shanghai Biyuntian Biotechnology Co., Ltd. ECL reagent was purchased from Amersham Biosciences. Rabbit anti-human Bax monoclonal antibodies, rabbit anti-human Bcl-2 antibodies, and goat anti-rabbit Horseradish peroxidase (HRP)-labeled IgG secondary antibodies were purchased from Cell Signal, Inc. (USA). DMEM medium, fetal bovine serum (FBS), and cyan chain double antibodies were purchased from Hyclone (USA). Dimethyl sulfoxide (DMSO) MTT powder was purchased from Gibco (USA). Trypsin digest was purchased from Sigma. IL-6, IL-8, and TNF-α ELISA kits were purchased from R&D (USA). Caspase 3 activity assay kit was purchased from Cell Signal, Inc. (USA). SOD activity detection kit and ROS activity kit were purchased from Wuhan Boster Biotechnology Co., Ltd. ABI7900 HT Real-time PCR was purchased from ABI (USA). LabSystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation of the United States. The CK2 fluorescence microscope was purchased from Olympus Corporation of Japan.

Rat grouping and model preparation

Healthy male SD rats were randomly divided into 3 groups, with n=10 in each group. In the control group, SD rats were normally reared. Rats in the model group were used to prepare a COPD rat model using the cigarette smoke method. H19 group was transfected with H19 plasmid adenoviruses in COPD rats.

According to a literature report [17], the rats were placed in a self-made sealed fumigation box (80 × 60 × 58 cm). They were exposed to the smoke of 20 cigarettes (yellow fruit tree, each 15 mg tar, and 1.25 mg nicotine). They were ignited twice a day, burning up to 50 minutes, twice a day for 16 weeks. Based on the preparation of rat COPD model, the H19 group received injections of lncRNA H19 plasmid adenovirus (108 pfu/500 μl) through the oral cavity.

Sample collection

A total of 5 mL of blood was collected through the tail veins. Blood was centrifuged at 3,000 rpm for 15 minutes to obtain serum, which was stored at -20°C for ELISA analysis. After the rats were sacrificed, the right middle lobes were frozen and fixed in liquid nitrogen or neutral paraformaldehyde.

H&E staining

The right middle lobes were fixed in 10% neutral paraformaldehyde fixative at room temperature for 24 hours. After dehydration, transparency, waxing, embedding, and dewaxing, hematoxylin-eosin (HE) staining, dehydration, and transparency were conducted before seal-

Table 1. Primer sequences for real-time PCR

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACTCTGTCAGTGG	TAATCGAATGTACGTGGT
lncRNA H19	CATCTTGTCTTGGATGACTT	CCTCACGCCAGGCTTTCACTG

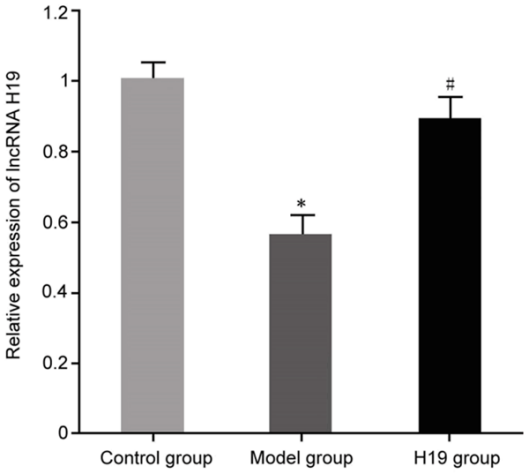

Figure 1. Expression of lncRNA H19 in a rat model of COPD. Compared with control group, *P<0.05; compared with model group, #P<0.05.

ing. The sealing film was observed under an optical microscope.

16HBE cell culturing, grouping, and processing

The 16HBE cells were taken out from liquid nitrogen, resuscitated, and cultured in a medium containing 10% FBS and 90% high glucose DMEM medium (containing 100 U/mL penicillin, 100 μg/mL streptomycin), at 37°C with 5% CO_2. Cells in the logarithmic growth phase were used for experiments. Cells were randomly divided into 3 groups, including the control group, model group, and H19 group. Cigarette smoke extract was continuously added to model group and H19 group, while the H19 group was transfected with H19-pcDNA3.1 using Lipo2000 Reagent. The H19-pcDNA3.1 plasmid sequence was 5'-TAACACACAGGCACCAATTCATA-3'-3'.

Real-time PCR detection of lncRNA H19 expression

Total RNA was extracted using TRIzol Reagent (American Axygen Corporation) for analysis of lncRNA H19 expression via real-time PCR. This method used primers designed by PrimerPremier6.0 (**Table 1**). GAPDH was used as a reference.

MTT assays for detection of cell proliferation

The 16HBE cells (5×10^3) were treated, as mentioned above, followed by analysis of cell proliferation by MTT assays, according to manufacturer instructions. The experiment was repeated three more times.

ELISA analysis of levels of IL-6, IL-8 and TNF-α

Serum was collected, measuring levels of IL-6, IL-8, and TNF-α using ELISA, according to kit instructions.

Caspase 3 activity assays

Changes in caspase 3 activity were examined using 2 mM Ac-DEVD-pNA, according to kit instructions.

Western blot analysis of expression of Bax and Bcl-2

Total protein was extracted using RIPA lysis buffer and quantified using the BCA method. They were stored at -20°C for Western blot analysis, examining expression levels of Bax and Bcl-2. Data were analyzed using protein image processing system software and Quantity one software. The experiment was repeated four times.

Analysis of SOD activity and ROS activity

According to kit instructions, changes in SOD activity and ROS production in lung tissues of each group were analyzed.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) and was processed using SPSS 11.5 statistical software. Mean values of the two groups were compared using Student's t-tests and analyzed with analysis of variance (ANOVA). P<0.05 indicates statistical differences.

Results

Expression of lncRNA H19 in a rat model of COPD

Compared with the control group, expression of lncRNA H19 was significantly decreased in the COPD rat model (P<0.05). After transfection of H19 plasmid adenovirus into COPD rats,

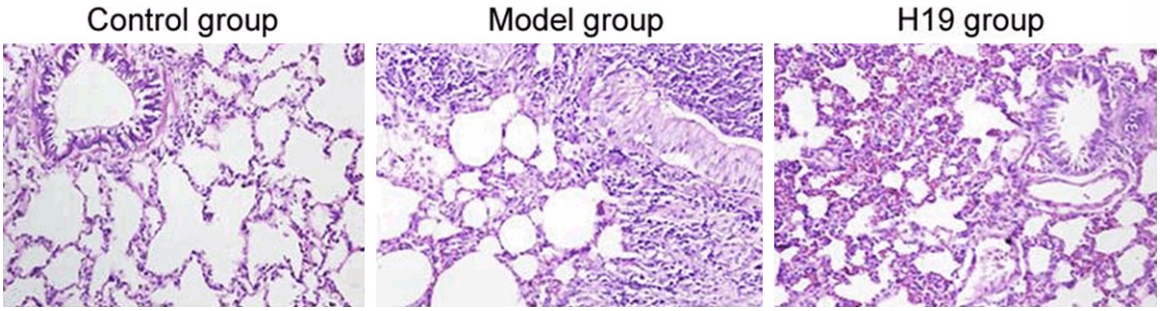

Figure 2. Changes in lung tissues of COPD rats after upregulation of lncRNA H19 expression (× 100).

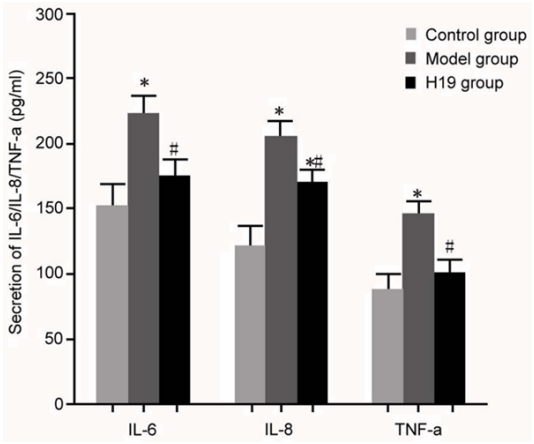

Figure 3. Effects of lncRNA H19 overexpression on inflammatory factor secretion in COPD rats. Compared with control group, *P<0.05; compared with model group, #P<0.05.

expression of lncRNA H19 in COPD rats was significantly upregulated (P<0.05) (**Figure 1**).

Upregulation of lnc RNA H19 expression in lung tissues of COPD rats

H&E staining was used to evaluate the effects of lncRNA H19 upregulation in COPD rats by transfecting H19 plasmid adenovirus on lung tissues. Results showed that lung tissues of the control group were intact, without inflammatory cell infiltration, including a normal bronchial structure and intact mucosa. However, lung tissues of COPD rats were narrowed or even broken. The blood vessel walls were thickened, with inflammatory cell infiltration and damaged bronchial mucosa, as well as a partially detached epithelium. Interestingly, overexpression of lncRNA H19 during COPD reduced inflammatory cell infiltration, improved lung tissue damage, reduced vessel wall thickness, and increased bronchial epithelial cell integrity (**Figure 2**).

Upregulation of lncRNA H19 expression on IL-6, IL-8, and TNF-α

Secretion of inflammatory factors IL-6, IL-8, and TNF-α in the serum of COPD rats was increased significantly, compared with that in the control group (P<0.05). Overexpression of lncRNA H19 in COPD rats significantly inhibited the secretion of inflammatory cytokines IL-6, IL-8, and TNF-α, compared with the model group (P<0.05) (**Figure 3**).

Upregulation of lncRNA H19 expression on oxidative stress

Changes in SOD activity and ROS production in lung tissues of each group were analyzed. The activity of SOD in lung tissues of COPD rats was significantly decreased and the production of ROS was significantly increased, compared with levels in the control group (P<0.05). Overexpression of lncRNA H19 in COPD rats significantly upregulated SOD activity and inhibited ROS production, with statistical differences, compared with the model group (P<0.05) (**Figure 4**).

Expression of lncRNA H19 in 16HBE cells

Expression of lncRNA H19 was significantly decreased in the COPD bronchial epithelial 16HBE cell injury model. It was prepared from cigarette smoke extract (P<0.05). Transfection of lncRNA H19 plasmids in the model group significantly promoted the increase of lncRNA H19 expression, compared with the model group (P<0.05) (**Figure 5**).

Upregulation of lncRNA H19 expression on proliferation of 16HBE cells

In the model of COPD bronchial epithelial 16HBE cell injury, prepared by cigarette smoke

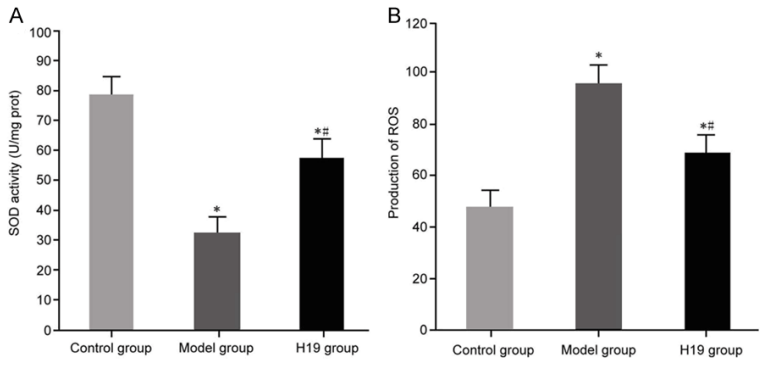

Figure 4. Upregulation of expression of lncRNA H19 on oxidative stress in COPD rats. A: SOD activity in COPD rats; B: ROS generation in COPD rats. Compared with control group, *P<0.05; compared with model group, #P<0.05.

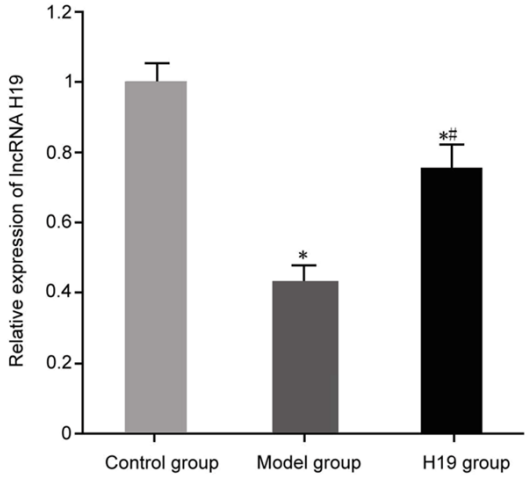

Figure 5. Expression of lncRNA H19 in 16HBE cells from COPD bronchial epithelium. Compared with control group, *P<0.05; compared with model group, #P<0.05.

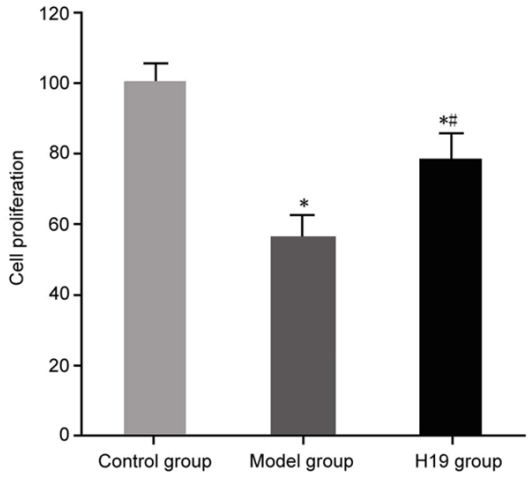

Figure 6. Upregulation of expression of lncRNA H19 on the proliferation of 16HBE cells; Compared with control group, *P<0.05; compared with model group, #P<0.05.

extract, cell proliferation was significantly reduced, compared with the control group (P<0.05). Upregulation of lncRNA H19 expression in the model group of 16HBE cells significantly promoted cell proliferation, compared with the model group (P<0.05) (**Figure 6**).

Upregulation of lncRNA H19 expression on caspase3 activity

In the model of COPD bronchial epithelial 16HBE cell injury, prepared by cigarette smoke extract, the activity of caspase3 was significantly increased, compared with the control group (P<0.05). Upregulation of lncRNA H19 expression in the model group of 16HBE cells significantly reduced caspase3 activity, compared with the model group (P<0.05) (**Figure 7**).

Upregulation of lnc RNA H19 expression on apoptosis of 16HBE cells

In the model of COPD bronchial epithelial 16HBE cell injury, prepared by cigarette smoke extract, expression of Bax-2 was increased and expression of anti-apoptotic protein Bcl-2 was decreased. Upregulation of lncRNA H19 expression in the model group of 16HBE cells inhibited Bax expression and increased expression of Bcl-2 (**Figure 8**).

Discussion

Prevalence and mortality rates of COPD are high. The high costs of treatment “three highs” poses a serious threat to public health and social economy [18]. The pathogenesis of COPD is very complicated, involving several factors, including chronic inflammation and oxidative stress. This can lead to chronic bronchial inflammation, bronchial epithelial cell damage, and destruction of lung parenchyma, ultimately leading to persistent airflow limitations and occurrence and progression of COPD [19].

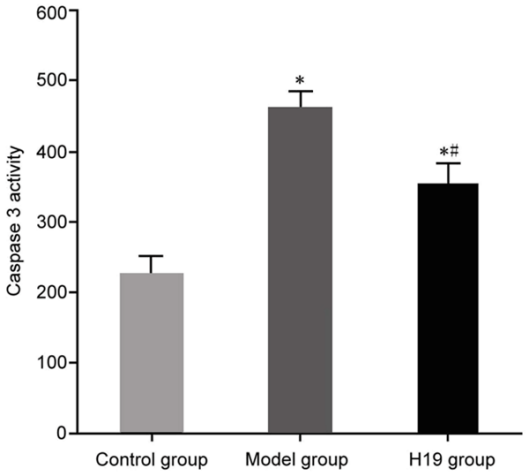

Figure 7. Upregulation of lncRNA H19 expression on caspase3 activity in 16HBE cells; Compared with control group, *P<0.05; compared with model group, #P<0.05.

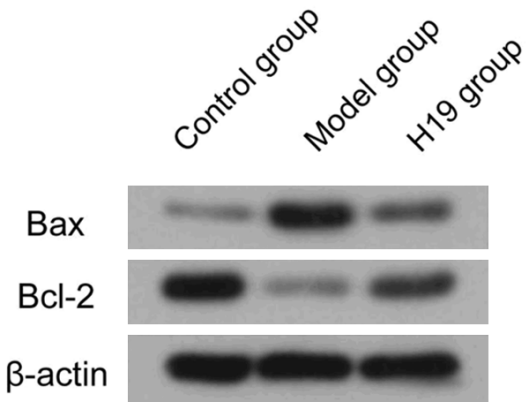

Figure 8. Upregulation of lncRNA H19 expression on apoptosis of 16HBE cells.

Smoking can lead to the development of chronic lung diseases, such as COPD [20]. Therefore, the purpose of this study was to investigate the effects of cigarette smoke exposure on the lungs, aiming to evaluate possible therapeutic targets for treatment of chronic obstructive pulmonary disease. Moreover, lncRNAs do not encode proteins, have no biological function, and are called dark substances in gene transcription. However, they have been found to be involved in the regulation of protein coding, before and after transcriptional regulation, at epigenetic levels [21]. Additionally, lncRNA H19 has been confirmed to be abnormally expressed in respiratory diseases [16]. In this study, it was confirmed that expression of lncRNA H19 was decreased in the COPD rat model. Expression in COPD rats was upregulated after transfection with the H19 plasmid adenovirus. Upregulation of lncRNA H19 expression in COPD rats inhibited secretion of IL-6, IL-8, and TNF-α inflammatory factors and effectively improved the lung tissue structure of COPD. Results suggest that lncRNA H19 might be involved in the regulation of the development and progression of COPD and can be used as a target for prevention and treatment of COPD, as well as a biomarker.

A previous study confirmed that all COPD patients have elevated oxidative stress and chronic inflammation [22]. In COPD patients, free radical production increases. Thus, the balance between reactive oxygen species production and antioxidant defense functions is impaired. This, in turn, aggravates the inflammatory process [23]. Numerous studies have confirmed that increased oxidative damage in smokers leads to lung injuries through various biological effects. Oxidative stress index ROS is increased and SOD activity is decreased, which further aggravates inflammation [24]. The present study found that overexpression of lncRNA H19 in COPD rats improved lung tissue structure, downregulated secretion of inflammatory factors IL-6, IL-8, and TNF-α, upregulated SOD activity, and inhibited ROS generation, suggesting that lncRNA H19 upregulation can inhibit COPD by regulating oxidative stress and inhibiting inflammation. Apoptotic protein Bax and anti-apoptotic protein Bcl-2 are important members of the apoptotic protein family. Abnormal expression of both can lead to abnormal expression of caspase3 activity and apoptosis, which leads to changes in cell biological processes [20]. Present results confirmed that Bax expression was decreased, Bcl-2 expression was increased, caspase3 activity was increased, bronchial epithelial cell apoptosis was promoted, and cell proliferation was inhibited. By upregulating lncRNA H19 expression in COPD bronchial epithelial cells, Bax expression was significantly inhibited, Bcl-2 expression was increased, and caspase3 activity was decreased. These factors protected bronchial epithelial cells from apoptosis, promoting cell proliferation and preventing cell damage. Further investigation is required, analyzing expression of lncRNA H19 in clinical samples and examining possible mechanisms. This

will provide reference for diagnosis and treatment of COPD.

In conclusion, expression of lncRNA H19 is decreased in COPD rats. Upregulation of lncRNA H19 expression in bronchial epithelial cells in COPD rats or COPD can inhibit oxidative stress, regulate oxidative stress, inhibit apoptosis, and promote cell proliferation, alleviating COPD.

Disclosure of conflict of interest

None.

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