



# LncRNA BMF-AS1 Exerts Anti-Apoptosis Function in COPD by Regulating BMF Expression

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## ABSTRACT

Previous studies indicate that long non-coding RNAs (lncRNAs) have crucial effect in COPD, but the exact role of most lncRNAs in COPD remains unknown. We detected differential expression of mRNAs and lncRNAs in blood samples from COPD patients (n=3) and healthy controls (n=3) with smoking history by human microarray analysis. Then, we selected lncRNAs and their associated mRNAs which were both differential expression in COPD vs healthy smokers, for quantitative reverse transcription PCR (qRT-PCR). A total of 3,359 lncRNAs revealed by microarray analysis have been differentially expressed between the two groups, 1,995 lncRNAs identified to be up-regulated in COPD, whereas 1,364 lncRNAs have been down-regulated. Meanwhile, a total of 3,283 mRNAs have been differentially expressed, 2,589 mRNAs identified to be up-regulated in COPD, whereas 694 mRNAs were down-regulated. qRT-PCR showed there was a statistically significant difference between COPD and control group for BMF ( $p=0.0121$ ), lncRNA RP11-521C20.3 ( $p<0.001$ ), CYLD ( $p=0.003$ ) and lncRNA RP1-85F18.5 ( $p=0.005$ ). Both the results of microarray analysis and qRT-PCR validation test has showed that lncRNA RP11-521C20.3 (BMF antisense RNA 1, BMF-AS1) significantly decreased, while its associated BMF mRNA increased in COPD. This study indicates that the expression of genome-wide lncRNA in COPD blood samples is different in comparison with healthy smokers by microarray analysis. LncRNA BMF-AS1 expression might potentially predict the apoptosis of COPD by regulating of BMF mRNA expression.

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## Authors' Contribution

ZZ, LF and LL were responsible for collecting samples. JZ, JS and JF made contributions to analysis of data. LH provided technical assistance.

## Key words

Apoptosis, BMF-AS1, COPD, Long non-coding RNA

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major concern in terms of health economics and is projected to become the third most prevalent cause of mortality in the world by 2020 (Hogg and Timens, 2009). The main mechanisms for pathogenesis of COPD include persistent inflammation, oxidative stress, the imbalance between proteolytic and antiproteolytic, and apoptosis (Taehong *et al.*, 2011). Excessive apoptosis of endothelial cells and alveolar epithelial cells has been postulated to lead to the destruction of lung tissue and emphysema (Liu *et al.*, 2008, Nakanishi *et al.*, 2011). The Bcl-2 modifying factor (BMF) is member of the Bcl-2 protein family. It is attached to the cytoskeleton under normal conditions. External stimulus releases BMF from the cytoskeleton to mitochondria, thereby binding to

the anti-apoptotic members such as Bcl-w, Bcl-2, Bcl-XL and Mcl-1 to induce apoptosis (Delgado and Tesfaigzi, 2014).

Smoking is the most important reason that is involved in the pathogenesis of COPD (Shakoori *et al.*, 2017). Cigarettes contain reactive oxygen species which can cause oxidative stress and play a key role in cell apoptosis (Krishnendu *et al.*, 2013). However, the probability of developing COPD among continuous smokers is about 25% (Løkke *et al.*, 2006). It suggests that genetic predisposition and epigenetic factors may be crucial to decide which smokers will develop airway obstruction. Protein-coding genes constitute approximately 1.5% of human genome, while many human genome transcripts are non-coding RNAs (Mitchell *et al.*, 2009). Long non-coding RNAs (lncRNAs) are a group of noncoding RNAs that have a length of more than 200 nucleotides (Wang and Chang, 2011) and they play significant role in cellular processes and fundamental biochemical processes such as RNA splicing, gene transcription, epigenetics and genomic rearrangement (Managadze *et al.*, 2011; E *et al.*, 2018). Previous studies have indicated that lncRNAs

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are associated with numerous human diseases, such as cardiovascular diseases, cancers and neurodegeneration diseases (Johnson, 2012; Cao *et al.*, 2013; Gutschner *et al.*, 2013). Moreover, lncRNAs also play an important role in COPD. Bi *et al.* (2015) reported the differential expression of lncRNAs in lung tissue of COPD patients versus healthy persons by microarray analysis. LncRNA NEAT1 promotes IL-8 expression in fibroblasts deriving from COPD subjects (Ijiri *et al.*, 2017), while lncRNA-mediated sirtuin 1/p53 and forkhead box O3a signaling pathways may regulate Type II alveolar epithelial cells senescence in the pathogenesis of COPD (Gu *et al.*, 2017). The recent study reported a novel lncRNA SCAL1, which is elevated in the airway epithelia of smokers versus non-smokers (Hackett *et al.*, 2012) and induced by cigarette smoke extract (Thai *et al.*, 2013). Knockdown of SCAL1 in bronchial epithelial cells will lead to a significant risk of CS-induced cytotoxicity, suggesting that SCAL1 may act as a protector factor against oxidative stress (Thai *et al.*, 2013). Although some studies are available on the function and expression of lncRNA in COPD, the exact role of most of the lncRNAs remain unknown in COPD. Therefore, it is imperative to search for novel lncRNAs involved in the pathophysiology of COPD.

In this study, the differentially expressed mRNAs and lncRNAs in venous blood samples from COPD and healthy individuals with smoking history were detected via human microarray analysis. Then, Gene Ontology Enrichment Analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis of the differentially expressed mRNAs were conducted. There are 3 antisense lncRNAs and their associated coding mRNAs which were differentially expressed in COPD versus smokers without COPD by quantitative reverse transcription PCR (qRT-PCR). This study aims to seek novel lncRNAs that take part in the regulation of molecular mechanisms in COPD and offers new orientation and targets for COPD therapy.

## MATERIALS AND METHODS

### *Patient samples for lncRNA microarray analysis*

In this study, venous blood samples were collected 3 from COPD patients and 3 from healthy persons with smoking history, from The Affiliated First Hospital of Kunming Medical University in 2015. Detailed information of all the participants including gender, age, smoking history, FEV<sub>1</sub>/FVC% (forced vital capacity rate of one second), medical history were collected. Exclusion criteria were as follows: patients with metabolic and immune systemic disease, with other lung benign diseases such as tuberculosis, bronchiectasis, bronchial asthma, sleep apnea hypopnea syndrome, and tumor patients were

not included in the study.

This study was approved by the Ethics Committee of the Affiliated First Hospital of Kunming Medical University.

### *LncRNA microarray*

Total RNA was extracted by Trizol (Takara, Invitrogen). We used Arraystar Human LncRNA Microarray V3.0 (Kang Chen, Shanghai, China) to analyse the lncRNAs and mRNAs.

### *Differential expression and function analyses*

Differential expression of lncRNA and mRNA should maintain at least  $\pm 2$  fold change between the two groups. Hierarchical clustering and combined analysis are performed using homemade scripts. Gene Ontology (GO) is an international standard classification system, which includes molecular function, biological processes and cellular component(s). We used top GO for function analysis which can facilitate semi-automated enrichment analysis for Gene Ontology. We analysed the pathways that differentially expressed mRNAs involved by using Kyoto Encyclopedia of Genes and Genomes (KEGG).

### *Conjoint analysis of mRNAs and lncRNAs*

We have integrated the differentially expressed antisense lncRNAs and mRNAs of COPD vs smokers without COPD. The differentially expressed antisense lncRNAs may regulate mRNAs and influence the pathophysiology of COPD.

### *Quantitative reverse transcription PCR validation*

qRT-PCR were performed on blood samples from 103 COPD patients and 101 healthy individuals with smoking history for lncRNAs (lncRNA RP11-521C20.3, RP11-327F22.4, RP1-85F18.5) and their associated mRNAs (BMF, CYLD, EP300). Total RNA was extracted from blood samples using TRIzol reagent. qRT-PCR is performed with SYBR® Premix Ex Taq™II (Tli RNaseH Plus) Takara, RR820). Data were calculated by  $2^{-\Delta\Delta Ct}$  relative to actin.

### *Statistical analysis*

SPSS software 22.0 is used to analyze the data of experiments. Significant differences of two groups were estimated using Pearson's chi-square test, Student t-test, and Wilcoxon signed-ran test, as appropriate. *P*-values < 0.05 were considered to be statistically significant.

**RESULTS**

*Study subjects for lncRNA microarray analysis*

Blood samples were obtained from 3 smokers with COPD and 3 healthy smokers without airflow limitation. Basic information is listed in Table I. There is no significant difference between the ages ( $p=0.057$ ) and smoking index ( $p=0.765$ ) of all subjects. There is a significant difference in FEV<sub>1</sub>/FVC% between the two groups ( $p<0.001$ ).

**Table I. Clinical characteristics of the study patients for lncRNA microarray analysis.**

	COPD	Non-COPD	p Value
Gender (male/ female)	2/1	2/1	1
Age (Mean±SD, year)	71±5	65±4	0.057
FEV <sub>1</sub> /FVC%	82.17±5.60	38.11±1.45	<0.001
Smoking index	28.2±6.9	28.4±7.1	0.765

FEV<sub>1</sub>/FVC%, forced vital capacity rate of one second. Smoking index is calculated by pack\*years.

*Different expression of lncRNAs between COPD and healthy smoker blood*

A comparison of microarray results revealed that a total of 3,359 lncRNAs were differentially expressed between the two groups (fold change  $\geq \pm 2$ , Fig. 1A). There were 1,995 lncRNAs identified up-regulated, whereas 1,364 lncRNAs were down-regulated in COPD. lncRNA MIR155HG (fold-change 46.7352822) and AC098971.2 (fold-change 217.2122864) were the most up- and down-regulated lncRNAs in COPD compared with smokers without COPD.

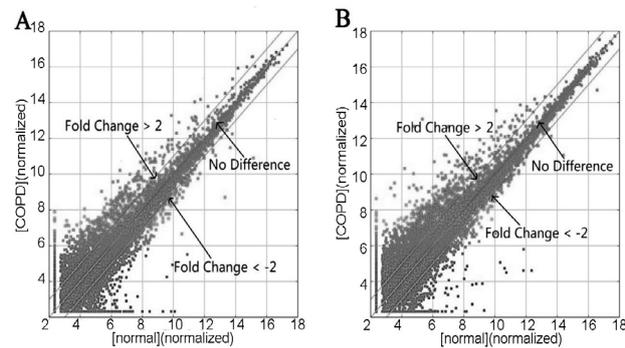


Fig. 1. Scatter plot of COPD and control group. (A) Scatter Plot shows lncRNAs are differentially expressed between COPD and control group; (B) Scatter Plot shows mRNAs are differentially expressed between COPD and control group.

*Differential expression of mRNAs between COPD and healthy smoker blood*

The results of lncRNA microarray between the two groups revealed a total of 3,283 genes which were differentially expressed (fold change  $\geq \pm 2$ , Fig. 1B). There were 2,589 mRNAs identified to be up-regulated, whereas 694 mRNAs down-regulated in COPD. CCL3L1 (fold-change 237.2944586) and ATP5J (fold-change -38.3183624) were the most up- and down-regulated mRNAs in COPD compared with smokers without COPD.

*Pathway analysis*

The differentially expressed mRNAs (DEmRNAs) were analyzed in the KEGG pathway database to find out which pathways they take part in, thus to study the pathogenesis of COPD. Top ten enrichment score of the significant enrichment pathways are presented in Figure 2. Among up-regulated pathways, the top 10 pathways are listed in Figure 2A, which include viral carcinogenesis, systemic lupus erythematosus, ribosome, spliceosome, alcoholism, Epstein-barr virus infection, non-alcoholic fatty liver disease, salmonella infection, antigen processing and presentation and alzheimer’s disease. Meanwhile, the down-regulated pathways include thyroid hormone synthesis, proximal tubule bicarbonate reclamation, bile secretion, cAMP signaling pathway, glucagon signaling pathway, protein digestion and absorption, insulin secretion, adrenergic signaling in cardiomyocytes, estrogen signaling pathway, and melanogenesis (Figure 2B).

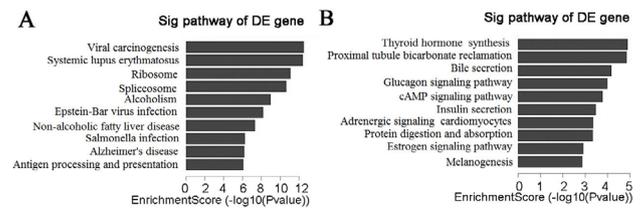


Fig. 2. KEGG pathway analysis of the DEmRNAs. (A) The pathways of significantly up-regulated mRNAs in COPD compared with control group; (B) The pathways of significantly down-regulated mRNAs in COPD compared with control group.

*GO analysis*

The Gene Ontology (GO) is a community bioinformatics resource that describes gene product function by use of controlled vocabularies. The ontology include three domains: Molecular Function, Biological Process, and Cellular Component. The bar chart shows the top ten counts of the significant enrichment terms (Fig. 3). The highest enriched GOs targeted genes by the up-regulated mRNAs were mRNA metabolic process

(ontology: biological process), poly (A) RNA binding (ontology: molecular function) and intracellular part (ontology: cellular component). With regard to down-regulated mRNAs, the highest enriched GOs are regulation of multicellular organism process (ontology: biological process), cation transmembrane transport activity (ontology: molecular function) and intrinsic component of plasma membrane (ontology: cellular component).

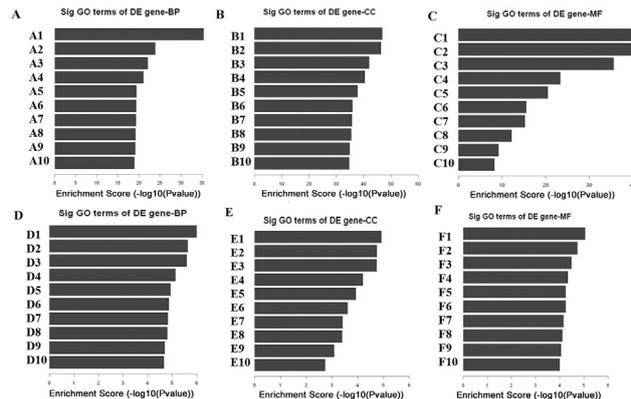


Fig. 3. Gene ontology enrichment analysis of DEMRNAs. (A) GO biological process classification of up-regulated mRNAs. A1: mRNA metabolic process, A2: gene expression, A3: immune system process, A4: cellular macromolecule metabolic process, A5: establishment of protein localization to membrane, A6: protein targeting to ER, A7: response to stress, A8: SRP-dependent cotranslational protein targeting to membrane, A9: establishment of protein localization to endoplasmic reticulum, A10: cellular component organization or biogenesis; (B) GO cellular component classification of up-regulated mRNAs. B1: intracellular part, B2: intracellular, B3: membrane-bounded organelle, B4: intracellular membrane-bounded organelle, B5: intracellular organelle part, B6: intracellular organelle, B7: organelle part, B8: nucleus, B9: macromolecular complex, B10: nuclear part; (C) GO molecular function classification of up-regulated mRNAs; C1: poly(A)RNA binding, C2: RNA binding, C3: protein binding, C4: binding, C5: nucleic acid binding, C6: organic cyclic compound binding, C7: heterocyclic compound binding, C8: structural constituent of ribosome, C9: enzyme binding, C10: protein complex binding; (D) GO biological process classification of down-regulated mRNAs; D1: regulation of multicellular organismal process, D2: regulation of cell morphogenesis involved, D3: regulation of multicellular organismal development, D4: system development, D5: nervous system development, D6: regulation of development process, D7: multicellular organismal development, D8: forebrain development, D9: positive regulation of potassium ion, D10: regulation of cell differentiation; (E) GO cellular component classification of down-regulated mRNAs; E1: intrinsic component of plasma membrane, E2: integral component

of plasma membrane, E3: plasma membrane part, E4: apical part of cell, E5: apical plasma membrane, E6: cell periphery, E7: plasma membrane, E8: sodium potassium-exchanging ATPase complex, E9: cation-transporting ATPase complex, E10: plasma membrane region; (F) GO molecular function classification of down-regulated mRNAs; F1: cation transmembrane transporter activity, F2: ion transmembrane transporter activity, F3: anion cation symporter activity, F4: transmembrane transporter activity, F5: substrate-specific transporter activity, F6: substrate-specific transmembrane transporter activity, F7: RNA polymerase II transcription regulatory, F8: monovalent inorganic cation transmembrane transporter, F9: sequence-specific DNA binding RNA polymerase, F10: metal ion transmembrane transporter activity.

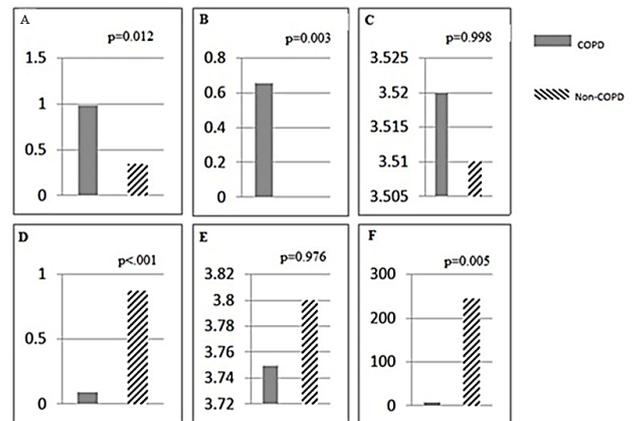


Fig. 4. Quantitative reverse transcription PCR validation of differentially expressed mRNAs and lncRNAs. Data are presented as  $2^{-\Delta\Delta Ct}$  relative to actin. (A) BMF mRNA expression (B) CYLD mRNA expression (C) EP300 mRNA expression (D) RP11-521C20.3 expression (E) RP11-327F22.4 expression (F) RP1-85F18.5 expression.

#### Conjoint analysis of lncRNAs and mRNAs

There are 65 antisense lncRNAs and their associated nearby coding mRNAs are identified differentially expressed both in COPD and smokers without COPD. Based on the previous studies, BMF, CYLD, EP300 may be related with the pathophysiology of COPD. BMF facilitates interaction to pro-survival Bcl-2 family protein thereby triggering apoptosis (Schmelzle *et al.*, 2007; Delgado and Tesfaigzi, 2014). Apoptosis is involved in the pathogenesis of COPD (Aoshiba, 2007; Zeng *et al.*, 2012). The CYLD can suppress the expression of interleukin-8 (IL-8), and as we know COPD is an overactive inflammatory response, including the up-regulation of the expression of IL-8 (Wang *et al.*, 2014). In addition, histone acetyltransferase p300 (EP300) can regulate the level of acetylation of specific lysine residues of NF- $\kappa$ B, which is a key pathway in inflammatory COPD (Van Den Bosch *et al.*, 2016).

### Quantitative reverse transcription PCR result

qRT-PCR was performed on 103 COPD patients and 101 healthy smoker blood samples for lncRNAs (lncRNA RP11-521C20.3, RP11-327F22.4, RP1-85F18.5) and their nearby mRNAs (BMF, CYLD, EP300). They were chosen for validation based on conjoint analysis results of mRNAs and lncRNAs and their potential role in the pathophysiology of COPD as mentioned above. Statistically significant difference measured by the Student's 't' test is found in COPD compared with healthy smokers in blood samples for BMF ( $p=0.0121$ ), RP11-521C20.3 ( $p<.001$ ), CYLD ( $p=0.003$ ), and RP1-85F18.5 ( $p=0.005$ ). The expression of RP11-327F22.4 and EP300 were not found to be statistically significant (Fig. 4).

## DISCUSSION

lncRNAs are important functional molecules connected with human diseases, such as cardiovascular diseases, cancer, inflammatory disease, and neurodegeneration diseases (Johnson, 2012; Cao *et al.*, 2013; Gutschner *et al.*, 2013; Klattenhoff *et al.*, 2013). However, the mechanism that lncRNAs regulate the pathogenesis of COPD are still unclear. Therefore, we investigated the differentially expressed lncRNAs and mRNAs between COPD and healthy smoker blood samples by using microarray analysis. We have found that 3,283 mRNAs and 3,359 lncRNAs were differentially expressed between COPD and healthy smokers. Other researches also echo this conclusion. Bi *et al.* (2015) detected genome-wide expression of lncRNAs in lung tissue by microarray analysis and showed that 43 lncRNAs were underexpressed and 120 overexpressed in COPD patients in comparison with healthy smokers. Tang *et al.* (2016) also found that there were 5,094 mRNAs and 8,376 lncRNAs turned out to be differentially expressed in COPD lung tissues. These investigators forecast that there are abundant differentially expressed lncRNAs and mRNAs in COPD blood or lung tissues and they might play a vital role in the pathogenesis of COPD.

Previous studies have shown that COPD is related to Epstein-barr virus infection (McManus *et al.*, 2008), cAMP signaling pathway (Basoglu *et al.*, 2015), insulin secretion (Wells *et al.*, 2016), antigen processing and presentation (Andreo *et al.*, 2010) and adrenergic signaling in cardiomyocytes (Baker and Wilcox, 2017), which are consistent with our KEGG pathway results. In addition, our study results showed that the highest enriched GOs targeted genes included mRNA metabolic process, poly (A) RNA binding, intracellular part, regulation of multicellular organism process, cation transmembrane transport activity and intrinsic component of plasma

membrane. The discovery of novel genes and signaling pathways associated with COPD in this study may provide new ideas for further elucidating the pathogenesis of COPD in the future.

The lncRNA microarray analysis results showed that lncRNAs RP11-521C20.3, RP11-327F22.4, RP1-85F18.5 and their associated mRNAs BMF, CYLD, EP300 were differentially expressed in COPD patients, respectively. Meanwhile, according to the qRT-PCR results, only lncRNA RP11-521C20.3 with its associated mRNA BMF are both statistically significant. lncRNA RP11-521C20.3 is decreased, while BMF was increased in COPD vs healthy smokers.

BMF is an apoptosis-inducing BH3-only protein and interacts with the actin-based myosin V motor complex via binding to a dynein light chain, which settles BMF to the cytoskeleton under normal circumstances (Puthalakath *et al.*, 2001). It is confirmed that apoptotic stimuli, release BMF from the myosin V motor complex allowing it to bind with anti-apoptotic member Bcl-2, Mcl-1, Bcl-w and Bcl-XL (Delgado and Tesfaigzi, 2013, 2014). Apoptosis mechanisms lead to the pathogenesis of COPD. Apoptosis of small capillaries and alveolar capillary endothelial cells can contribute to the destruction of alveolar septal cells, ultimately leading to emphysema (Liebow, 1959; Kasahara *et al.*, 2000). Several researches have shown that the apoptosis of endothelia and alveolar epithelial cells increased in lung tissues from COPD or emphysema subjects (Yokohori *et al.*, 2004; Imai *et al.*, 2005; Liu *et al.*, 2009). Makris *et al.* (2009) presented an increase in both neutrophils and T cells apoptosis in induced sputum from COPD patients. Consistent with increased T cells apoptosis, a significantly increased apoptotic rate of airway epithelial cells was found in BALF and brushing from COPD patients (Hodge *et al.*, 2005). Moreover, it's becoming evident that the pro-apoptotic Bcl-2 Family proteins own the ability to adjust the level of apoptosis. Yokohori *et al.* (2004) and Liu *et al.* (2009) reported that Bax index increased in alveolar wall cells from emphysema subjects. Another study showed that expression of Bax and Bad were detected in emphysematous lungs with higher apoptotic rate (Imai *et al.*, 2005). CS exposure caused an increase of pro-apoptotic proteins, t-Bid and Bax in lung tissues from rats (Wu *et al.*, 2006). In vitro human airway smooth muscle cells culture also showed that the expression of Bad and Bax was increased with CSE-induced apoptosis (Hu *et al.*, 2009). These studies implied the involvement of pro-apoptotic proteins in the apoptosis of COPD. BMF is a member of pro-apoptotic Bcl-2 Family proteins, but there is no study showing the role of BMF in the apoptosis of COPD.

lncRNA RP11-521C20.3 is antisense to the protein

coding BMF gene. Following standard HGNC guidelines (<http://www.genenames.org/>), we name this as BMF-AS1 for BMF antisense RNA 1. Antisense lncRNAs are transcribed from the antisense strand, and more than 30% of the annotated human transcripts own corresponding antisense lncRNAs. These antisense lncRNAs play a key role in biological functions by regulating the level of corresponding sense mRNA at the transcriptional or post-transcriptional network (Faghihi and Wahlestedt, 2009). Both the results of microarray analysis and qRT-PCR validation test shown that BMF-AS1 was significantly decreased, while its associated BMF mRNA was increased in COPD compared with healthy smokers blood samples. We presume that the decrease of lncRNA BMF-AS1 may release more BMF from the cytoskeleton and bind to anti-apoptotic member to activate apoptosis pathway, which may exert anti-apoptosis function in COPD.

## CONCLUSION

To sum up, our study indicates differential expression of genome-wide lncRNA in COPD blood samples compared with healthy smokers by microarray analysis. Our qRT-PCR analysis reveals that lncRNA BMF-AS1 expression may potentially predict the apoptosis of COPD patients by regulating BMF mRNA expression, and this possibility must be confirmed in future studies. We have recognized the limitation of this study in that the number of subjects should have been larger for validation PCR test. Further studies are needed to look into the molecular mechanisms of lncRNAs in COPD.

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### Statement of conflicts of interest

The authors declare no conflict of interest.

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