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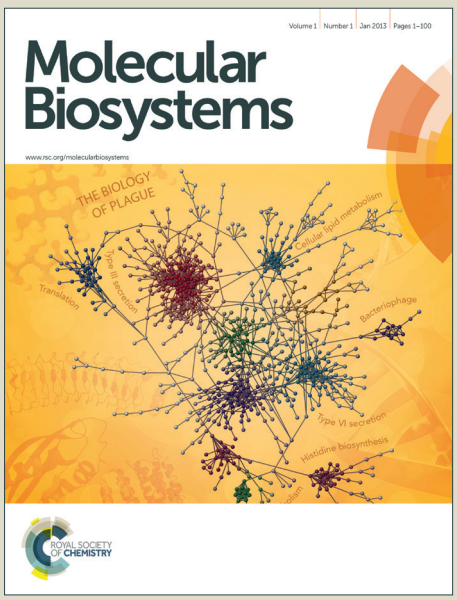

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An increased ratio of serum miR-21 to miR-181a level is associated with the early pathogenic process of chronic obstructive pulmonary disease in asymptomatic heavy smokers

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Abstract

Heavy smoking is associated with the development of chronic obstructive pulmonary disease (COPD). However, there is no valuable biomarker for evaluating COPD development in heavy smokers because they are usually asymptomatic. This study is aimed at evaluating whether the levels of serum miRNAs can serve as biomarkers for predicting the occurrence of COPD.A rat model of emphysema was induced by enforced smoking, and the dynamic miRNAs expression profile at different stages of emphysema with varying periods of smoking were analyzed by microarray and quantitative real-time polymerase chain reaction (qRT-PCR). The differentially expressing miRNAs were analyzed by Gene Ontology and KEGG PATHWAY database. The levels of three serum candidate miRNAs were measured by qRT-PCR in 41 healthy controls, 40 asymptomatic heavy smokers, and 49 COPD patients. Following smoking for varying periods, different severities of lung emphysema were observed in different groups of rats, accompanied by altered levels of some serum miRNAs associated with regulating some pathways.Furthermore, the levels of miR-21 were significantly higher in the COPD patients and asymptomatic heavy smokers than that in the HC ($P<0.001$), while the level of miR-181a was significantly lower in the COPD patients and asymptomatic heavy smokers than that in the HC ($P<0.001$). Accordingly, the levels of serum miR-21 and miR-181a as well as their ratios had a high sensitivity (0.854) and specificity (0.850) for evaluating the development of COPD.Our data suggest that the levels of serum **miR-21 and miR-181a** may be valuable for evaluating the development of COPD in heavy smokers.

Introduction

Chronic obstructive pulmonary disease (COPD) is a serious health problem and COPD is estimated to affect 600 million people worldwide [1]. Its incidence is increasing due to cigarette smoking and air pollution, particularly in developing countries, such as in China. COPD is characterized by chronic bronchitis and emphysema that progressively limit effective airflow and occasionally promote inflammatory exacerbation in the lungs [2, 3]. More importantly, patients with long term COPD and frequent exacerbation have a high rate of morbidity and mortality. Heavy cigarette smoking is the major risk factor for the development of COPD and accounts for 95% of cases [4]. However, heavy cigarette smokers usually have no COPD-related specific symptom at the early stage of the COPD process and there is little information about biomarkers for evaluating COPD development in heavy smokers. Therefore, the discovery of new biomarkers for the development of COPD in heavy smokers may help in the prevention and delay of COPD development.

MicroRNAs (miRNAs) are endogenous small (about 22 nucleotides) non-coding RNAs and bind to the 3'untranslated region (3'UTR) of targeting mRNAs to inhibit translation and promote miRNA degradation. More than thousands of miRNAs have been discovered and they can regulate many[1, 2] biological processes, such as development, apoptosis, cell proliferation, and haematopoiesis [5, 6]. Interestingly, miRNAs are remarkably stable in plasma and serum and can resist endogenous RNase, varietal storage conditions, and repeating freeze-thaw cycles [7]. Recent studies have validated that some circulating miRNAs act as biomarkers for the evaluation of different diseases, such as lung cancer, gastric cancer, nasopharyngeal carcinoma, sepsis, and pulmonary tuberculosis [8-12].

However, there is no available information about which serum miRNA(s) are valuable for the evaluation the development of COPD in heavy smokers.

Currently, the role of miRNAs in regulating the development of COPD remains unclear. Previous studies have suggested that miRNAs may play a vital role in the development and progression of COPD [13, 14]. Significantly lower levels of sputum let-7c and miR-125b were detected in patients with COPD [13], but elevated levels of miR-15b, miR-223, miR-1274a, and miR-424 were detected in the lung tissues from COPD patients [14]. In addition, few studies were focused on the circulating miRNAs of COPD. A recent study of 72 miRNAs by qRT-PCR revealed that the levels of serum miR-20a, miR-28-3p, miR-34c-5p, and miR-100 were elevated, but the levels of serum miR-7 decreased in COPD patients, as compared with that in the healthy controls in a Turkey population [15]. However, the role of miRNAs in regulating the development of COPD remains largely unknown. Furthermore, there is little information about the dynamic changes in the levels of miRNAs in the lungs during the development of heavy smoking-related COPD and whether altered levels of miRNAs in the lungs can be detected in the serum samples.

The purpose of our study are to explore the miRNA expression profiles in different stages of COPD and seek miRNA biomarkers in serum of COPD patients and high risk group(heavy smokers).We employed a rat model of heavy smoking-related COPD [16] to test the miRNA expressing profile in the lungs during the development of COPD by microarray using the mouse & rat miRNA OneArray (MRmiOA) that contained a total of 676 mature miRNA sequence probes. After validating the differentially expressing miRNAs by qRT-PCR, we validated the differential expression of some candidate miRNAs in the serum samples

from healthy controls, heavy asymptomatic cigarette smokers, and COPD patients. Our data suggest that an increased ratio of serum miR-21 to miR-181a may be a risk factor for heavy smokers to develop COPD.

Experimental

Animals

Male Wistar rats at 8 weeks of age and weighing at 200±10 g were purchased from the Laboratory Animal Center of Agricultural University of Hunan, China (license No.: SCXK(Xiang) 2009-0012). All animals were housed in a specific pathogen free facility with a 12-hour light-dark cycle at a room temperature of 22± 2°C and allowed to free access of normal chow and water. The experimental protocol was approved by the Animal Ethics Committee of Central South University, China.

Cigarette smoking-induced emphysema animal model

Thirty male Wistar rats were randomly divided into six groups：1. the healthy control group; 2. the cigarette smoking (CS) for two weeks; 3. the CS for 4 weeks; 4. the CS for 6 weeks; 5. the CS for 8 weeks; and 6. the CS for 15 weeks plus elastase-treated group. The rats (group 1) were exposed to filtered air and intratracheally administrated with the same volume (as the group 6) of physiologic saline on the same day of the animals receiving elastase in group 6. The rats in groups 2-5 were forced to smoke 8-10 commercial unfiltered cigarettes (Huangfurong, Hunan, China) per day for 2, 4, 6, or 8 weeks, respectively. The rats (group 6) were forced to smoke cigarette for 15 weeks and treated intratracheally with 18 U/100 g body weight of elastase (porcine pancreatic elastase, Sigma Aldrich, St Louis, USA) on day 15 post

the last day of smoking, as previously described [17].

One day post the elastase administration, the rats in the different groups were anesthetized intraperitoneally with chloral hydrate and subjected to thoractomy and laparotomy. Subsequently, the lungs of individual rats were quickly removed. One part of the lung tissues from individual rats was quick-frozen in liquid nitrogen and stored at -80°C for later biochemical analyses. The left lung tissues were immediately fixed in 4% paraformaldehyde overnight and paraffin-embedded. The paraffin-embedded tissue sections (5 μm) were stained with Hematoxylin-Eosin for histological examination under a light microscope in a blinded manner. Histopathologic inflammatory score was performed by blinded pathologist as described previously[18].

RNA extraction

Total RNA was extracted from the frozen rat lung tissues using TRIzol, according to the manufacturers' instruction (Invitrogen, USA). Individual RNA samples were qualified by spectrometry at 260 and 280 nm, agarose electrophoresis and Agilent RNA 6000 Nano/Pico Assay (Agilent Bioanalyzer 2100 system), and quantified by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, USA). Individual RNA samples with a value of OD 260/280 between 1.8-2.0 were quantified and stored at -80°C for further analysis.

MiRNA microarray and bioinformatics analyses

The relative levels of miRNA transcripts were subjected to miRNA microarray using the Mouse & Rat miRNA OneArray (MRmiOA) 3.0, according to the manufacturers' instruction (Phalanx Biotech Group, Taiwan, China). The MRmiOA contains a total of 676 mature

miRNA sequence probes from mouse miRBase database release 18.0. Briefly, the enriched RNA was labeled with the miRNA ULS™ Labeling Kit (Kreatech Diagnostics, Amsterdam, Netherlands) and hybridized to the chips at 37°C for 14-16 hours, followed by scanning on a Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) with appropriate PMT setting and resolution. The raw data were extracted using GenePix™ 4 software and standard selection criteria to identify differentially expressed genes (Log 2 ≥ 0.8 and P<0.05). The dysregulated expression of miRNAs was further demonstrated by unsupervised hierarchical clustering analysis using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/). The potential targeting genes of differentially expressing miRNAs were predicted using five databases(miRanda,miRDB,miRNAWALK,RNA22,Targetscan),the candidate target gene were selected if it was predicted by three databases. Their classification and functions were analyzed by Go Ontology analysis using miRNA target prediction software (Gene Ontology Enrichment Analysis Software Toolkit，GOEAST). The biological processes and signaling pathways associated with dysregulated genes in each stage of COPD were analyzed using the online databases KEGG (Kyoto Encyclopedia of Genes and Genomes).

Patients and samples

A total of 40 asymptomatic heavy smokers and 49 COPD patients were recruited at the Third Xiangya Hospital of Central South University, China. Another 41 age- and gender-matched healthy subjects were recruited at the Health Management Center of the Third Xiangya Hospital. The sample size was estimated by $n = 2[\frac{(Z_\alpha + Z_\beta) \times s}{\delta}]^2$，（α=0.05，β=0.1） and the power was 90%. S and δ was estimated by preliminary experiment. The sample size should larger than 25 samples.Asymptomatic heavy smokers were defined if individual smokers had a smoking index greater than 20 pack-years without any smoking-

related clinical symptom. The COPD patients were diagnosed according to the GOLD guidelines[19]. –All COPD patients were in stable phase without acute exacerbation during the past three months. Exclusion criteria included the presence of other chronic lung diseases, such as asthma, pulmonary tuberculosis, and pulmonary interstitial fibrosis, and other illnesses, including unstable cardiovascular diseases, uncontrolled diabetes, malignant neoplasm, nervous system diseases, serious liver and kidney diseases. The demographic and clinical characteristics of all subjects are summarized in Table 1. Written informed consent was obtained from individual subjects, and the experimental protocol was approved by the Medical Ethics Committee of the Third Xiangya hospital of Central South University.

Serum preparation and RNA extraction

Five milliliters of venous blood samples were collected in the serum tubes from individual subjects, and after coagulation at room temperature for 30 min, the blood samples were centrifuged at 1000 x g for 15 min. The sera were collected in 1.5 milliliter Eppendorf tubes and stored at -80°C for further analyses [9]. Total RNA was isolated from 250 μL of serum samples using the UNIQ-10 total RNA extraction Kit, according to the manufacturers' instruction (Sangon Biotech, Shanghai, China). Because there was not an abundant and stable endogenous miRNA reference in the serum, individual serum samples were mixed with an exogenous reference of cel-miR-39 at the same volume in Trizol [20]. The purified RNA was eluted with 40 μL of RNase-free water and stored at -80°C until analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The purified RNA samples were reversely transcribed into cDNA using the PrimeScript RT reagent Kit (TaKaRa,Japan) and miRNA specific stem-loop primers (RiboBio ,Guangzhou, China), according to the manufacturers' instructions. Briefly, a

reaction mixture (10 µl) containing 2 µl of 5* PrimeScript buffer, 0.5 µl of PrimeScript RT Enzyme Mix, 0.5 µl of Oligo dT primer, 1 µl of miRNA specific stem-loop primers, and 6 µl of serum RNA extract or 500 ng RNA extract of rats was incubated at 37°C for 15 min and at 85°C for 5 sec on an Eppendorf Mastercycler EP Thermal Cycler PCR system (Eppendorf AG, Germany). Subsequently, the relative levels of miRNA transcripts were determined by RT-PCR using the 2x SYBR system and specific primers (RiboBio, China). The PCR amplifications were performed at 95°C for 30 sec and subjected to 40 cycles of 95°C for 5 sec, 60°C for 50 sec, and 70°C for 10 sec on an IQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The relative levels of miRNAs to the control U6 were analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data are present as median and range. The difference between groups was analyzed by non-parameter statistical analysis. The specificity and sensitivity of a diagnostic value were analyzed by receiver operating characteristic (ROC) curves and the area under the curve (AUC). The expression level of serum miRNA was analyzed after log transformation of some variants. All statistical analyses were performed using the SPSS 19.0 software (SPSS, Chicago, USA) and GraphPad Prism 5 software. A p value of <0.05 was considered statistically significant.

Results and discussion

MiRNAs regulate the pathogenesis of various lung diseases, such as lung cancer, COPD, and asthma [21]. However, little is known about the dynamic changes in the profiles of miRNAs

in the lungs of the different stages of COPD. In this study, we employed a rat model of COPD following forced smoking for varying time periods and we explored the global expression profiles of miRNA expression in the lungs of animals with different stages of COPD using microarray analysis.

Dynamic changes in the lung pathology in a rat model of emphysema

Animal models of emphysema were established to explore the lung pathogenic process of COPD. Elastase-induced and cigarette smoking exposure-related emphysema is a classical animal model [22], but neither of them can perfectly mimic the pathogenesis of COPD in humans. To understand the dynamic changes in the pathogenesis of COPD, we employed a rat model of emphysema induced by combining elastase with forced cigarette smoking, as described previously [16]. Wistar rats were exposed to cigarette smoking for different time periods. One group of Wistar rats was exposed to cigarette smoking for 15 weeks and intratracheally administrated with elastase, and another group of rats was exposed to normal air as the healthy controls. At the end of the experiment, the rats were sacrificed and their lung tissues were subjected to histological examination (Fig. 1). While normal morphology of the lung tissue section was observed in the healthy controls (Fig. 1A), the numbers of inflammatory infiltrates in the lung parenchyma and interstitial tissues and the thickness of airway wall increased in rats with cigarette smoking for 2-4 weeks (Fig. 1B-C). However, the numbers of inflammatory infiltrates in the lung tissues were obviously reduced and the thickness of airway wall was dramatically enlarged in rats with cigarette smoking for 6 weeks (Fig.1 D), consistent with the pathological characters of chronic bronchitis [23]. Furthermore,

the enlargement of alveolar space became more obvious and the numbers of inflammatory infiltrates were further reduced, accompanied by the pathogenic characters of emphysema in the lungs of rats with cigarette smoking for 8 weeks (Fig. 1E). Although there were only a few inflammatory infiltrates in the lung tissue sections, the alveolar airspace in the lungs was greatly enlarged in rats with cigarette smoking for 15 weeks and elastase injection, demonstrating the destruction of lung parenchyma and the development of emphysema in rats [24]. The degree of inflammation of each stage were evaluated by histopathologic inflammatory scoring system(Tab.2) .

Dynamic miRNA expression profiling in rat model of COPD

MiRNAs regulate the pathogenesis of COPD. However, little is known about the global profiles of miRNAs in the lung tissues during the development of COPD. We extracted total RNA from the lung tissues of the healthy control rats and the rats with cigarette smoking for 4 or 15 weeks given that they developed severe chronic bronchitis and obvious lung emphysema, respectively. The yielded RNA samples were subjected to microarray analysis of the global profiles of miRNAs in the lungs using the Rat miRNA OneArray (Fig. 2A). In comparison with that in the healthy controls, there were 30 and 37 differentially expressing miRNAs in the lungs of rats with 4-weeks and 15-weeks of cigarette smoking, respectively. There were 16 significantly up-regulated and 14 down-regulated miRNA expressions in the lungs of rats with 4-weeks of smoking, while 23 and 14 miRNAs were significantly up-regulated and down-regulated in the lungs of rats with 15-weeks of smoking. The unsupervised hierarchical clustering analysis revealed that many differentially expressing

miRNAs were clustered in the lungs of different groups of rats. For example, miR-21, miR-204, miR-147, and miR-667 were significantly up-regulated in the lungs of rats with 4-weeks of cigarette smoking and miR-30c-1*, miR-92a-2*, miR-1224, miR-667*, miR-92b*, and miR-296 in the lungs of rats with 15-weeks of cigarette smoking. In contrast, miR-345-5p, miR-181a, miR-181b, miR-145, miR-449a, and miR-351 were significantly down-regulated in the lungs of rats with 4-weeks and 15 weeks of cigarette smoking, respectively. Further qRT-PCR indicated that the relative levels of miR-146a, miR-147, miR-21, miR-92a-2*, and miR-206 were significantly up-regulated, while the levels of miR-181a expression were dramatically down-regulated in the lungs of rats with chronic bronchitis and emphysema (Fig. 2B). However, our findings were in disagreement with previous observations [14,25]. The difference may stem from varying animal models and samples tested. We analyzed miRNAs in the whole lungs of rats, which contain varying types of cells, and the mixed types of cells for miRNA analysis may explain the difference between our data and others.

GO and pathway analysis of differentially expressing miRNAs in the lung of rats with COPD

We analyzed the predicted genes targeted by seven increased expressing miRNAs (rno-miR-146a, rno-miR-21, rno-miR-147, rno-miR-206, rno-miR-30c-1*, rno-miR-92a-2*, and rno-miR-1224) and three decreased expressing miRNAs (rno-miR-345-5p，rno-miR-181a，and rno-miR-125a-5p)using the miRanda, miRDB, miRNAWALK, RNA22 and Targetscan database.The reason we choosed these miRNAs that the differential multiple of these miRNAs were biggest and most of them were validated by qRT-PCR The function of

the predicted targeting genes was further analyzed by gene ontology (Goeast:http://omicslab.genetics.ac.cn/GOEAST/），and the potential pathways, in which these genes are involved, were analyzed using the KEGG PATHWAY database. Interestingly, some predicted genes targeted by these miRNAs were associated with important biological functions, including regulating cell communication, cell-cell singaling，cellular biosynthetic process, vasculature development, and anatomical structure development, as shown in Figure 3A. The predicted genes targeted by these miRNAs were involved in many signal pathways, including the MAPK signal pathway, Wnt signal pathway, TGF-beta signal pathway, calcium signal pathway, VEGF signal pathway, T cell receptor signal pathway, B cell receptor signal pathway，insulin signal pathway, and GnRH signal pathway in the rats with 4-weeks and 15-weeks of cigarette smoking in Figure 3B and Figure 3C, respectively. Intriguingly, these pathways regulate cell cycle, ubiquitin mediated proteolysis，cytokine-cytokine receptor interaction，leukocyte trans-endothelial migration，apoptosis, and cell adhesion molecules (CAMs). Therefore, these miRNAs may regulate inflammation, apoptosis, and proteolysis that are associated with the development and progression of COPD. Bioinformatics analysis indicated that the differentially expressing miRNAs might target many crucial signaling pathways, including the MAPK, Wnt, TGF-beta, Calcium, VEGF, T cell receptor signal pathways, and others. Indeed, these signal pathways have been associated with the pathogenesis of COPD. Renda T et al [26] identified aberrant activation of the p38 MAPK in the alveolar walls of COPD patients. Heijink IH et al [27] demonstrated the increased levels of Wnt-4 expression in primary bronchial epithelial cells from COPD patients, suggesting the activated WNT signaling is associated with lung tissue destruction and inflammation. Morris

DG et al [28] proposed that the decreased TGF-beta signaling may lead to increased MMP expression, which may contribute to emphysema development.

Differential expression of serum miRNAs in asymptomatic heavy smokers and COPD patients

Previous studies have suggested that the levels of some circulating miRNAs may be used as new biomarkers for the evaluation of various diseases. Mitchell et al. [7] suggest that the levels of serum miR-141 may be potential diagnostic biomarkers of prostate cancer. Furthermore, Bianchi F et al. [29] report that the detection of 34 serum miRNAs could identify the early stage of non-small cell lung carcinomas (NSCLCs) patients, even when they are asymptomatic.To determine the importance of differentially expressing miR-21, miR-181a, miR-147, miR-345-5p, miR-92a-2, and miR-146a in the development of COPD, a total of 40 asymptomatic heavy smokers and 49 COPD patients as well as age- and gender-matched healthy controls (HC) were recruited for testing the levels of serum miRNAs. Their demographic and clinical characteristics are shown in Table 1. As expected, there was no significant difference in the distribution of age and gender in this population, but the values of BMI and smoking index in the COPD patients were significantly greater than both the HC and asymptomatic heavy smokers ($P<0.05$). The COPD patients displayed variable levels of lung functions.

First, we tested the relative levels of these six miRNAs in 15 serum samples by qRT-PCR and we detected three miRNAs (miR-21, miR-181a, and miR-146a, data not shown). Further analysis indicated that there was no significant difference in the relative levels of serum miR-

146a among these three groups of subjects. Furthermore, we found that the relative levels of serum miR-21 in the asymptomatic heavy smokers and COPD patients were significantly higher than that in the HC corresponding to an increase fold-change of 2.53 and 3.16， respectively ($p<0.001$, Fig. 4A). In contrast, the relative levels of serum miR-181a in the asymptomatic heavy smokers and COPD patients were significantly lower than that in the HC corresponding to an decrease fold-change of 2.53 and 2.44， respectively ($p<0.001$, Fig. 4B.). As a result, the ratios of serum miR-21 to miR-181a in the asymptomatic heavy smokers and COPD patients were greater than that in the HC. In addition, there was no significant difference in the relative levels of serum miR-146a tested among the asymptomatic heavy smokers, COPD patients, and healthy controls ($P=0.0594$, $P=0.1701$, respectively, Fig. 4C). Moreover, the relative levels of serum miR-21 and miRNA-181a were associated negatively with GOLD stages of COPD patients. The relative levels of serum miR-21 in GOLD I and II were significantly higher than that in GOLD III and IV（$P=0.0345$, $P=0.0134$, respectively, Fig. 5A. The relative levels of serum miR-181a in GOLD IV were significantly lower than that in GOLD III ($P=0.0059$, Fig. 5B). In this study, we validated the differentially expressing miRNAs in an animal model of COPD and found that miR-21 and miR-181a were differentially expressed in the heavy smokers and COPD patients, related to that in the healthy controls, supporting the notion that the majority of miRNAs are conserved and orthologous in mammals (such as humans and mice)[30]. The significantly elevated levels of serum miR-21 and down-regulated serum miR-181a in the heavy smokers and COPD patients were accorded with that in the lungs of animals with COPD. Interestingly, Rabinowits G et al. [31] also reported that there was a close correlation of circulating miRNAs with that in lung

adenocarcinoma tumors. It is possible that these differentially expressing miRNAs may have originated from the lungs. However, there were some differentially expressing miRNAs in the lungs of animals with COPD, but were not detected in the peripheral blood of human patients with COPD. In our study, we did not detect significant difference in the level of serum miRNA-146a among the healthy controls, heavy smokers, and COPD patients. We are interested in further investigating the mechanisms underlying the differential expression of miRNAs between different species during the pathogenic process of COPD.

The ROC analysis of the levels of serum miR-21 and miR-181a.

Heavy smoking is a high risk for development of COPD. Evidentially, the incidence of COPD in individual heavy smokers is nearly five-fold higher than that in the general population (Global incidence of COPD is 9–10%) [32-33]. Furthermore, many patients with early stage of COPD are usually asymptomatic and they would not be tested by spirometry. Indeed, only 45% of patients with mild symptoms are diagnosed [34]. Hence, there is an urgent need to discover new biomarkers for the diagnosis of COPD at the subclinical stage, because early intervention can delay the pathogenic process of COPD. We found that the lung pathogenic characteristics of animals in this study were similar to that in human patients with early stage of COPD [35]. Notably, significantly altered levels of serum miRNA expression were detected in animals with early stage of COPD, and the same pattern of change in the levels of serum miR-21 and miR-181a were detected in both heavy smokers and COPD patients. The ratios of serum miR-21 to miR-181a tended to be associated negatively with the GOLD stages in COPD patients. Similarly, a previous study has shown that plasma

metabolite profiles in a group of asymptomatic heavy smokers were similar to the emphysema group of patients [36]. These findings suggest that the pathogenic process of emphysema may already exist in those heavy smokers, although they had no clinical symptoms. Finally, we tested whether the levels and ratios of serum miR-21 and miR-181a could be risk factors for heavy smokers to develop COPD by the ROC analysis. We found that the areas under the ROC curves (AUC) were 0.815 (95% CI: 0.723–0.906) with a sensitivity of 73.2% and specificity of 75.0% for miR-21 and 0.767 (95% CI: 0.666–0.869) with a sensitivity of 62.5% and specificity of 75.6% for miR-181a, respectively (Fig. 5A and B). The AUC was 0.910 (95% CI: 0.846–0.974) with a sensitivity of 85.4% and specificity of 85.0% for the ratio of serum miR-21 to miR-181a in distinguishing heavy smokers from normal subjects. (Fig. 5C). Previous studies have shown that miR-21 and miR-181a can regulate matrix metalloproteinases (MMP), cell growth, apoptosis, and NK cell cytotoxicity. For example, miR-21 can down-regulate MMP inhibitor expression, enhance MMP activity, and inhibit RECK expression, thereby promoting MMP-9 expression [37-38]. Consequently, the imbalance of MMPs/anti-metalloproteases (TIMPs) contributes to the pathogenesis of COPD [39]. In contrast, miR-181a is a positive regulator of cell apoptosis and promotes stem cell differentiation to mature epithelial cells, but inhibits inflammatory cytokine production, which may inhibit inflammation and enhance tissue repairs [40-42]. Therefore, the higher ratios of serum miR-21 to miR-181a may be associated with the prognosis of COPD onset in heavy smokers.

Conclusions

In this study, we analyzed the dynamic miRNA expression profiling in an animal model

of COPD and we found that miR-206，miR-146a，miR-147，miR-21，miR-181a, and miR-92a-2* were significantly dysregulated in the mice at different stages of emphysema. These miRNAs were associated with various pathways in regulating broad biological functions. Further validation analysis revealed that significantly increased levels of serum miR-21 and decreased miR-181a expression were detected in heavy smokers and COPD patients and that the ratios of serum miR-21 to miR-181a tended to be associated negatively with the GOLD stages in COPD patients. Therefore, a high ratio of serum miR-21 to miR-181a expression may be a risk factor of the development of COPD and can be associated with the prognosis of COPD onset in heavy smokers. We recognized that our study had limitations of a small sample size, retrospective association, and the lack of functional studies of these miRNAs in the pathogenesis of COPD. Further perspective investigations on the value of these miRNAs in the prediction and diagnosis of COPD at early stage in a bigger population are warranted.

Competing interests

Authors declare that they have no competing interests.

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Table 1 The demographic and clinical characteristics of participants

	Healthy control	Heavy smokers	COPD patients
Case number	41	40	49
Age, yr,	50 (40-71)	58 (40-73)	64 (48-75)
Gender M/F	35/6	38/2	45/4
BMI,	23.55 (19.65-31.62)	24.33 (18.94-30.84)	20.8 (16.6-32.79)*
Smoking index, （pack-years）	0	27 (20-90)	42 (20-105.75)#
Lung function （ FEV1, % reference）			
I（≥ 80%）			4, 86.5 (82-93)%
II（50%-80%）			10, 60.4 (53-69)%
III（30%-50%）			16, 40.5 (30-47)%
IV（＜30%）			19, 24.0 (15-29)%

Data are expressed as median and range. The lung function is expressed as the case number, median (range). *p<0.05 vs. the HC and asymptomatic heavy smokers. #p<0.05 vs. the asymptomatic heavy smoker.

Table 2 Comparison of histopathologic score of lung tissue at different stage of COPD animal model

	Control	2w	4w	6w	8w	12w
Score	0	7.6±0.9*	19.0±1.6*#	11.2±1.3*#	6.6±1.5*#	6.2±1.3*#
p		<0.01	<0.01	<0.01	<0.01	<0.01
#P			<0.01	<0.01	0.24	0.08

Data are expressed as mean±standard deviation. *p vs. the control and different stage of COPD animal model. #p vs. the 2w COPD animal model.

Figure legends:

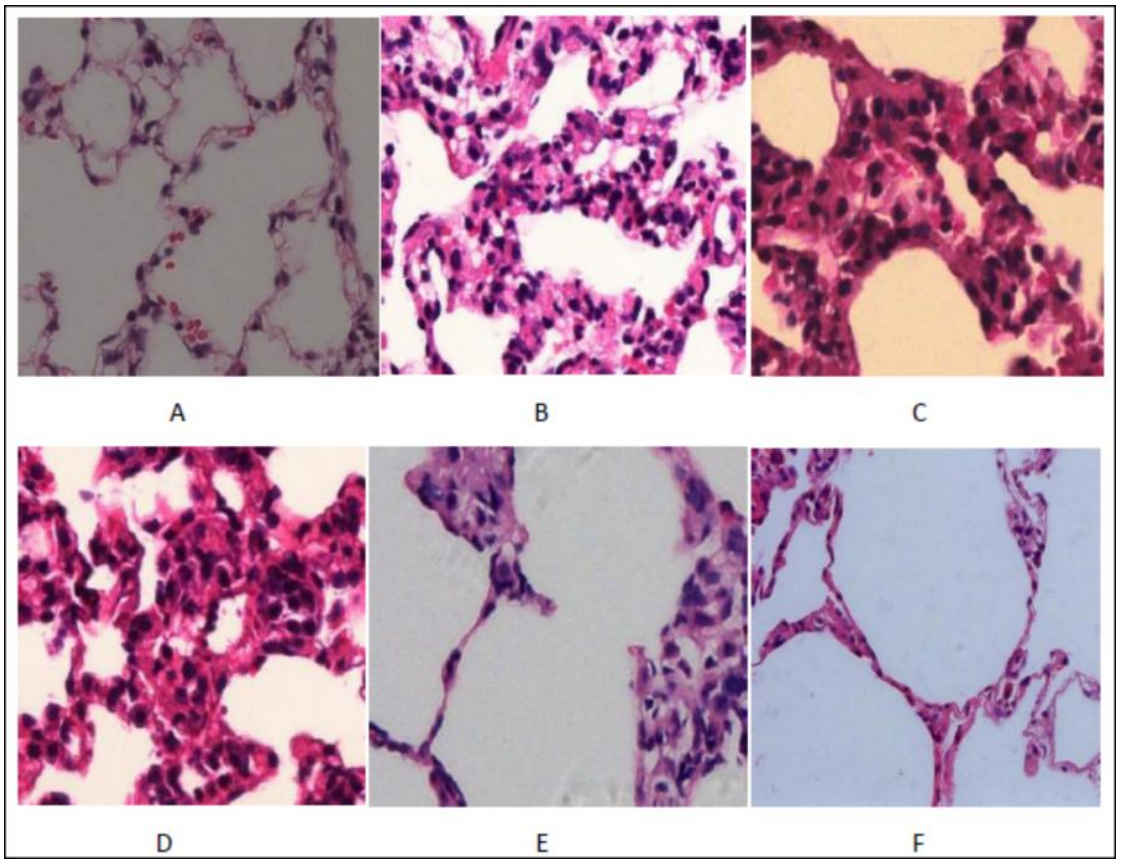
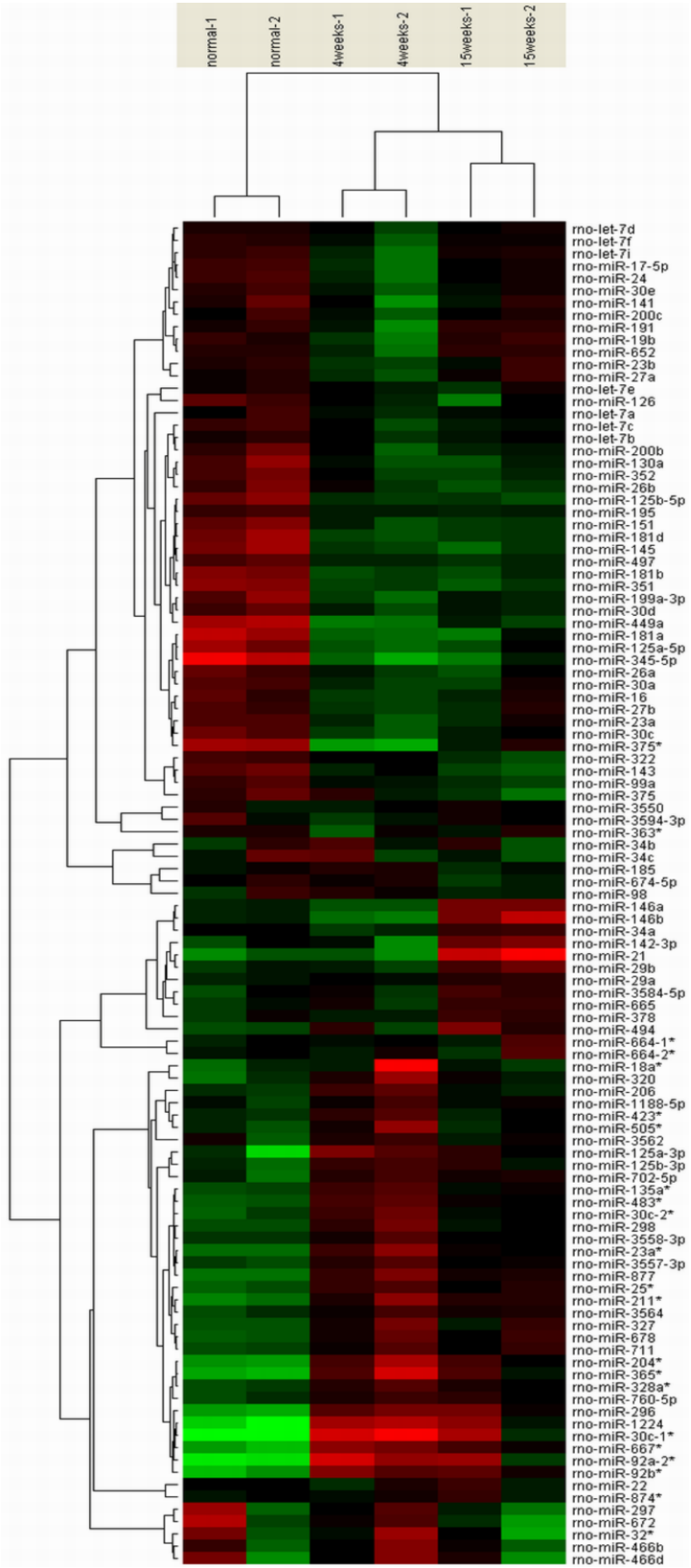
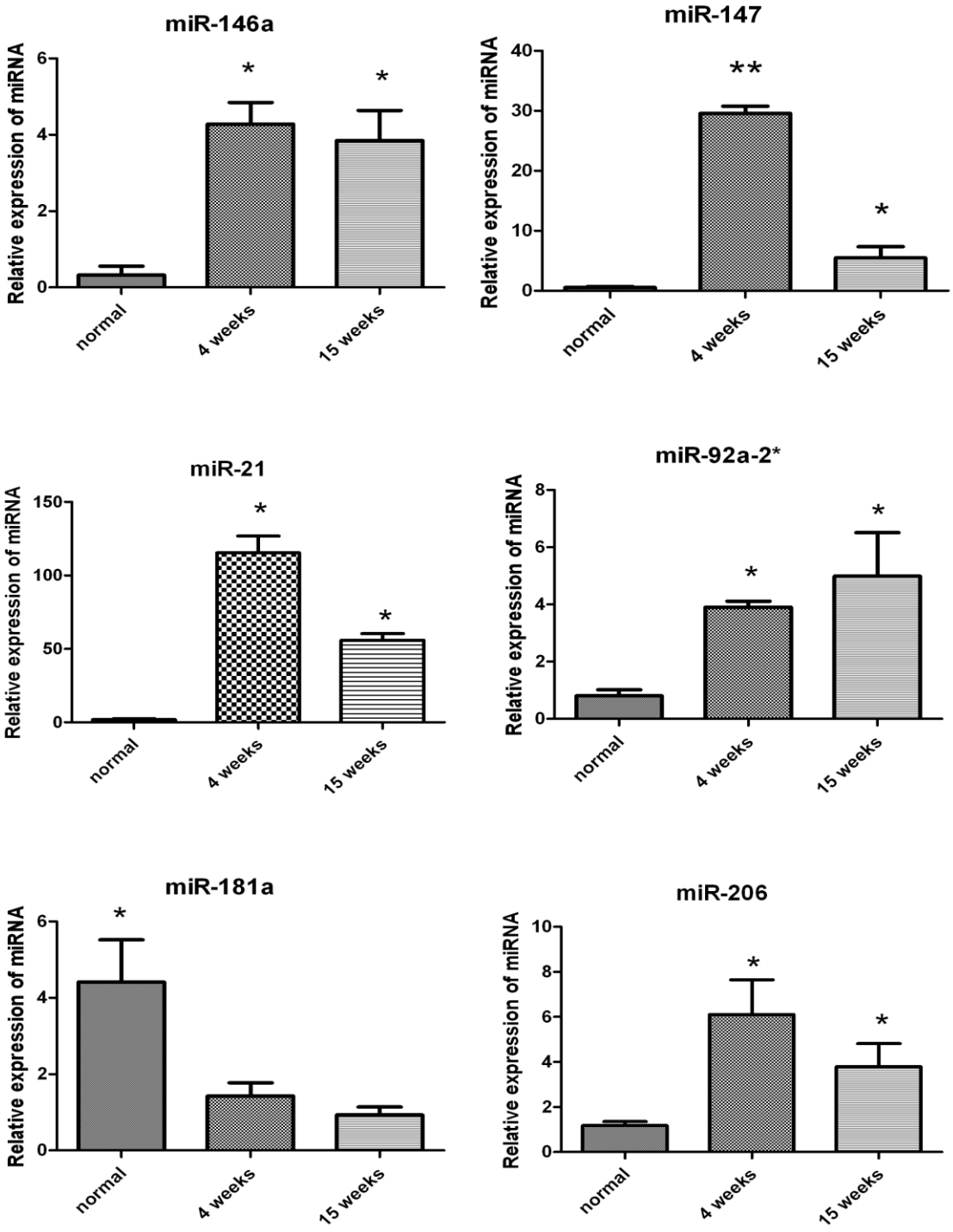


Figure 1. Histological examination of the lung tissue sections.

Wistar rats were exposed to cigarette smoking for 2, 4, 6, or 8 (B, C, D, E) weeks. One group of rats was exposed to cigarette smoking for 15 weeks and treated with elastase (F), and another group of rats was exposed to normal air as the healthy controls (A). At the end of the experiment, the rats were sacrificed and their lung tissues were subjected to H&E staining, followed by histological examination in a blinded manner. Data shown are representative images (magnification × 200) from the individual groups.



2A

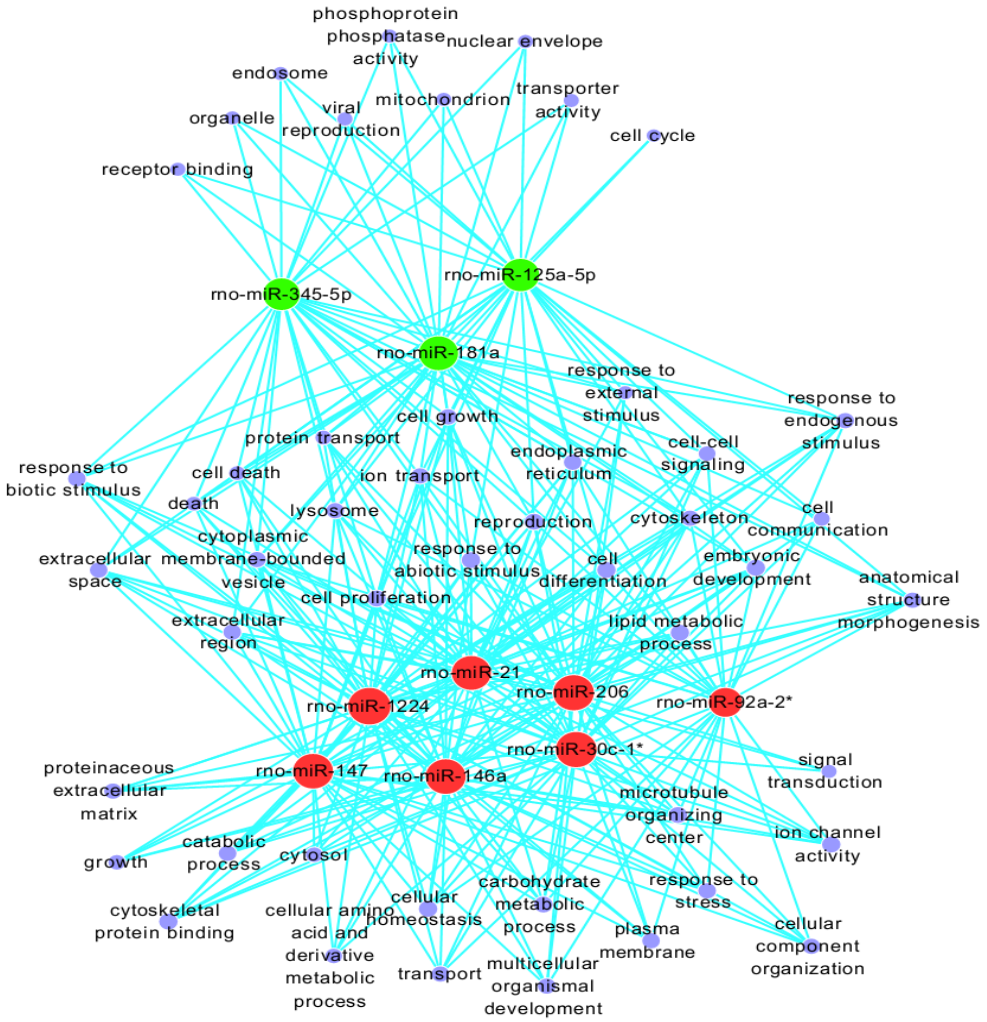


2B

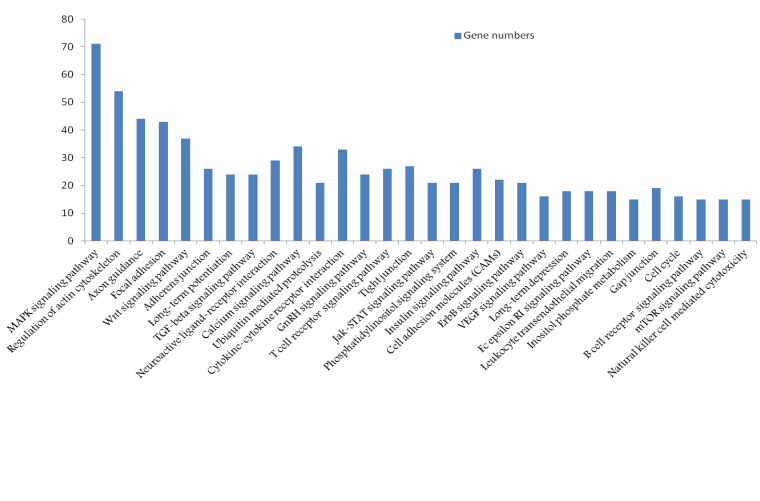
Figure 2. Microarray analysis of miRNA expression.

The levels of miRNA in the lung tissues from normal controls, 4-week CS, and 15 week

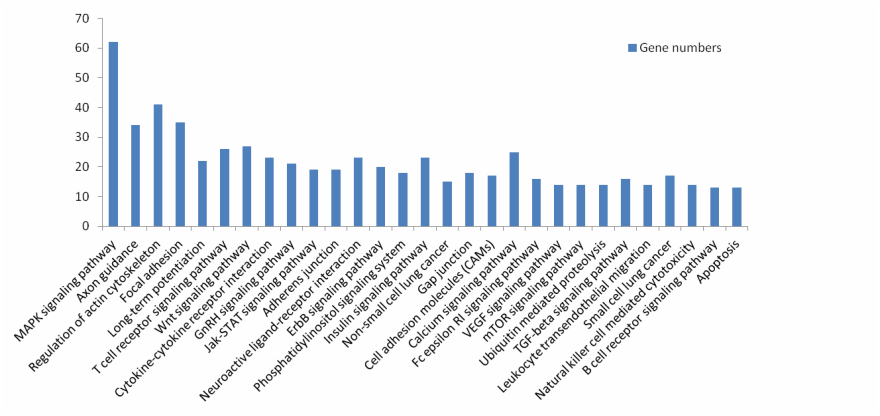
CS groups of rats were determined by microarray, and the relative levels of interesting miRNAs were further determined by quantitative real-time PCR. Data are a representative image of unsupervised hierarchical clustering of miRNA. The red blocks indicate the relatively higher levels of expressing miRNAs, while the green blocks represent the relatively lower levels of expressing miRNAs. (B) Quantitative RT-PCR analysis of the relative levels of miR-146a, miR-147, miR-21, miR-92a-2*, miR-181a, and miR-206 expression in the lungs of different groups of rats.*p<0.01 vs. the controls.



3A



3B



3C

Figure 3. The biological processes and regulatory networks of dysregulated miRNAs.

The biological processes and regulatory networks of dysregulated miRNAs were analyzed by five(miRanda,miRDB,miRNAWALK,RNA22,Targetscan) database and Go Ontology. (A) The associated biological processes and regulatory networks of differentially expressing miRNAs**. (**B) and (C) The numbers of genes targeted by these differentially expressing miRNA in the lung of 4-week CS and 15-week CS groups of rats, respectively. Data are expressed as the mean number of the genes targeted by these differentially

expressing miRNAs.

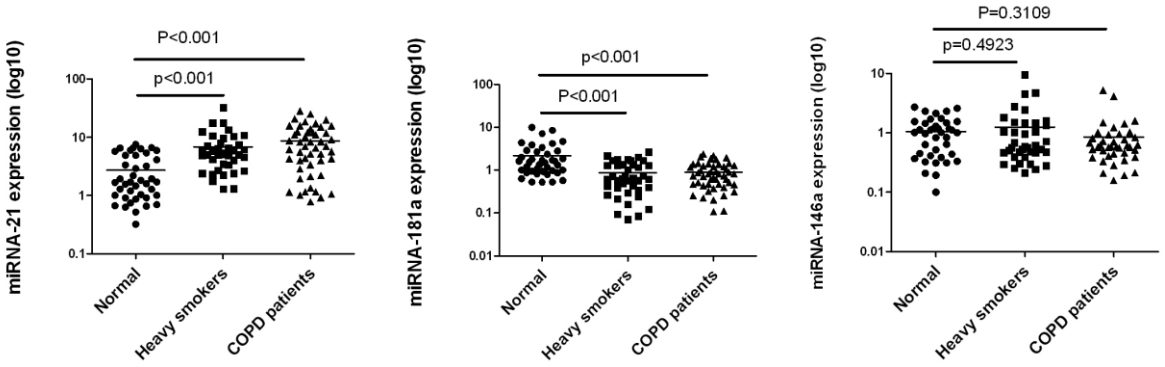


Figure 4. The relative levels of serum miRNAs in healthy controls, heavy smokers, and patients with COPD by quantitative RT-PCR.

Data shown are individual values of the mean levels of serum miRNAs from two separate experiments. A total of 41, 41, 39 healthy controls, 40, 40, 38 heavy smokers, and 49, 49, 40 COPD patients for measuring miR-21, miR-181a, and miR-146a, respectively.

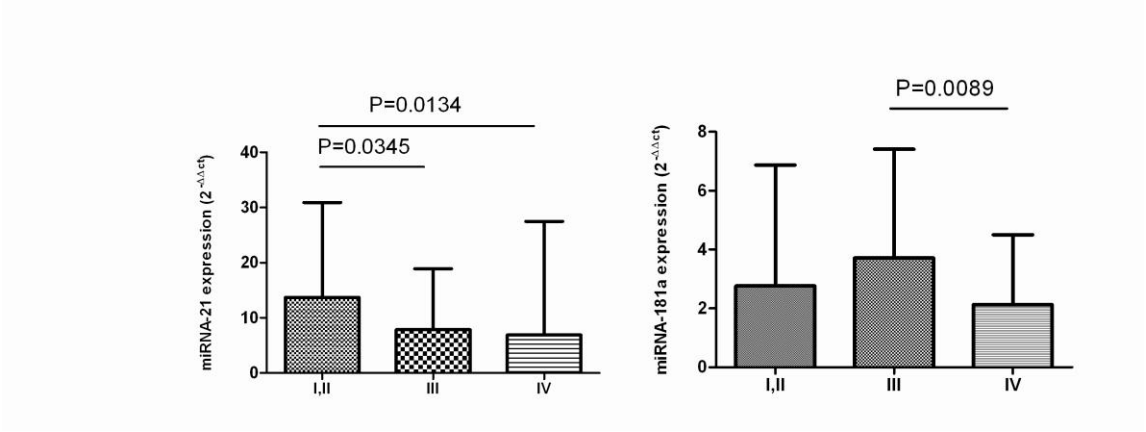
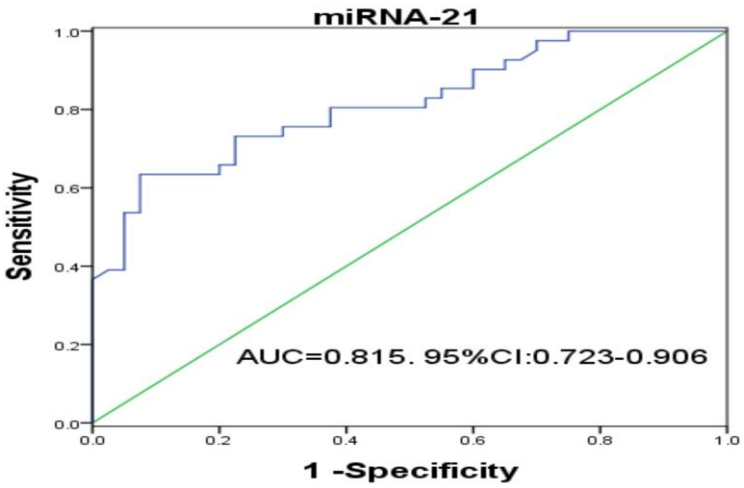
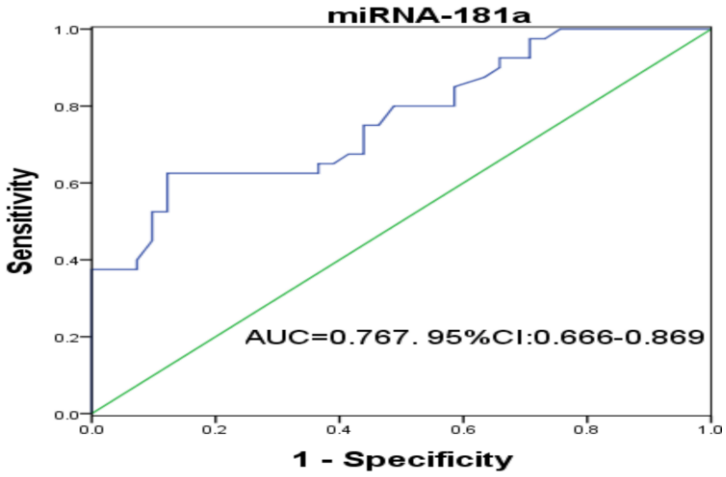


Figure 5. Stratification analysis.

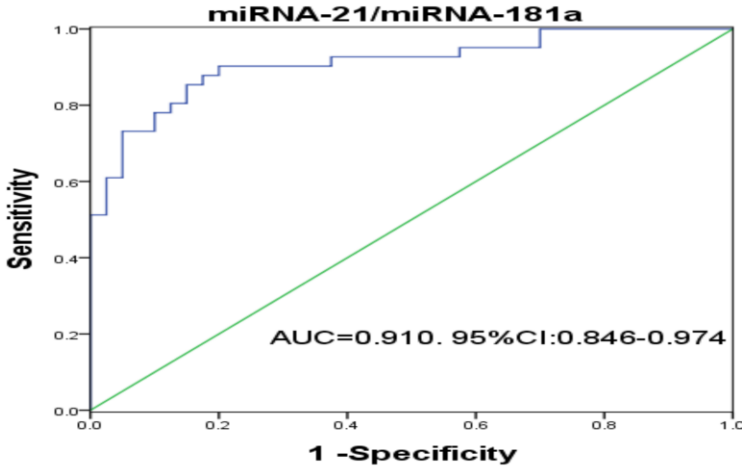
The levels of serum miR-21 and miR-181a in the patients with different GOLD stages of COPD were analyzed. Data are expressed as the mean ± SD of individual groups of patients. There were 14 patients at GOLD I/II group, 16 patients at the GOLD III ,and 19 patients at GOLD IV.



6A



6B



6C

Figure 6. The ROC analysis.

The sensitivity and specificity of the relative levels of miR-21 and miR-181a for predicting the development of COPD in heavy smokers (n=40) were analyzed by ROC analyses. Data shown are the ROC curves. (A) The AUC curve of miR-21. (B) The AUC curve of miR-181a. (C) The AUC curve of the ratios of serum miR-21 to miR-181a levels.