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Original Paper

Comprehensive Analysis of miRNA-mRNA-lncRNA Networks in Non-Smoking and Smoking Patients with Chronic Obstructive Pulmonary Disease

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Key Words
lncRNAs • miRNAs • genes • smoking • Chronic Obstructive Pulmonary Disease (COPD)

Abstract
Background/Aims: Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. This study aimed to identify overlapping or diverging dysregulated genes, lncRNAs, miRNAs and signaling pathways in smoking and non-smoking chronic obstructive pulmonary disease (COPD). ***Methods:*** Compared to normal controls, we identified the shared and divergent differentially expressed mRNAs (DEmRNAs), miRNAs (DEmiRNAs) and lncRNAs (DElncRNAs) in smoking and non-smoking COPD by RNA-sequencing and bioinformatics analysis. Functional annotation of DEmRNAs were performed. Both cis and trans-target DEmRNAs of DElncRNAs were identified. The target DEmRNAs of DEmiRNAs were identified as well. The DEmiRNA-DEmRNA-DElncRNA interaction network was constructed. QRT-PCR was performed to validat the selected DEmiRNAs, DEmRNA and DElncRNAs in COPD. ***Results:*** Compared to normal control, 1234 DEmRNAs, 96 DElncRNAs and 151 DEmiRNAs were identified in non-smoking patients with COPD; 670 DEmRNAs, 44 DElncRNAs and 63 DEmiRNAs were identified in smoking patients with COPD. Leukocyte transendothelial migration and pathways in cancer were significantly enriched pathways in non-smoking and smoking COPD, respectively. MiR-122-5p-A2M-LINC00987/A2M-AS1/linc0061 interactions might play key roles in COPD irrespective with the smoking status. Let-7-ADRB1-HLA-DQB1-AS1 might play a key role in the pathogenesis of smoking COPD while miR-218-5p/miR15a-RORA-LOC101928100/LINC00861 and miR-218-5p/miR15a-TGFβ3-RORA-AS1 interactions might involve with non-smoking COPD. ***Conclusion:*** We identified the shared and diverging genes, lncRNAs, miRNAs and their interactions and pathways in smoking and non-smoking COPD which provided clues for understanding the mechanism and developing novel diagnostic and therapeutic strategies for COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive disease that characterized by the presence of chronic bronchitis or emphysema which may result in the development of airway obstruction [1]. As a major global disease, COPD has been predicted to be the third leading cause of mortality worldwide by the year 2020[2]. COPD is a multifactorial complex disease that was associated with the interactions of genetic and environmental risk factors while its pathogenesis remains largely unknow [3].

Although cigarette smoking has long been widely known as the single most important risk factor for COPD[3], more than 25% patients with COPD were non-smokers [4-6]. However, previous studies mainly focused on COPD in smokers, studies of non-smoking COPD were limited [7]. The mechanism of COPD, especially in never-smokers, and the biomarkers and therapeutic targets of this disease at molecular levels have become targets of interest.

Besides key genes involved in the pathogenesis of COPD, non-coding RNAs including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) were reported to involve with COPD[8, 9]. MiRNAs are a class of small endogenous ncRNAs with 19–25 nucleotides that negatively regulate gene expression at the post-transcriptional level [8, 10]. LncRNAs are a class of ncRNAs that more than 200 nucleotides which could regulate the expression of protein-coding genes by cis- or trans-regulatory effects [11]. LncRNAs have been demonstrated to involve in various physiological and pathological processes [12].

In this study, we aimed to identify overlapping or diverging genes, lncRNAs, miRNAs and signaling pathways in smoking and non-smoking COPD. Compared to normal controls, the differentially expressed mRNAs (DEmRNAs), miRNAs (DEmiRNAs) and lncRNAs (DElncRNAs) in non-smoking COPD and smoking COPD were identified, respectively. Based functional annotation and bioinformatics analysis, the shared and divergent DEmiRNA-DEmRNA-DElncRNA interactions in non-smoking and smoking COPD were further identified which provided clues for understanding the mechanism and developing novel diagnostic and therapeutic strategies for COPD.

Materials and Methods

Patients and samples

Five smoking patients with COPD (S1-S5), five non-smoking patients with COPD (N1-N5) and five normal controls (C1-C5) were enrolled in this study from the Affiliated Changzhou No. 2 People's Hospital with Nanjing Medical University. The detailed information of all these 15 participants were displayed in Table 1. Since smoking patients with COPD in this hospital were all males, gender difference existed between smoking COPD group and normal controls in this study. However, different susceptibility of COPD between genders is believed to result from the different incidence of smoking between genders rather than to any gender driven susceptibility [13, 14]. Hence, we speculated that gender difference has little effect on our results. Due to restrictions in sample collection, significant differences existed between non-smoking patients with COPD and normal controls ($p<0.05$) as well as between smoking patients with COPD and normal controls ($p<0.05$). The blood samples were obtained from all these 15 participants. This study was approved by the Ethics Committee of The Affiliated Changzhou No.2 People's Hospital with Nanjing Medical University. The signed informed consent of all these participants was obtained. This research complied with the principles of the Declaration of Helsinki.

Library preparation and high-throughput sequencing

Total RNA was extracted from blood samples by using by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manual instruction. Nanodrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to check the concentration and purity of RNA. The integrity of RNA was checked by using 2% agarose gel. Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif.) was used to obtain the RIN value. RNA with amount > 5μg, concentration ≥ 200ng/ml, 1.8< OD260/280 <2.2 and RIN >7 was used to prepare cDNA library construction.

Firstly, we constructed the library for high throughput sequencing of lncRNA and mRNA as follow steps: 1). Remove the ribosomal RNA from total RNA by Ribo-Zero Magnetic kit (EpiCentre, Mandison, WI, USA); 2). Construct the library with Truseq RNA sample Prep Kit (Illumina, Inc., San Diego, CA, USA). RNA was purified and fragmented into 200~500 base pairs; the first cDNA strand was synthesized by RNA fragments primed with random hexamer primers; the second cDNA strand was synthesized with dUTP instead of dTTP; after purification by Qiaquick PCR purification kit, end repair, 3'end adenylation and adapter ligation were performed; the second cDNA strand was digested by using UNG enzyme (Illumina, Inc., San Diego, CA, USA) and polymerase chain reaction (PCR) was performed to construct library for high throughput sequencing of lncRNA and mRNA.

Secondly, we isolated the 18-30 nt RNA from the total RNA and performed adapter ligation and RT-PCR to construct the cDNA library for high throughput sequencing of miRNA by TruseqTM Small RNA sample prep Kit.

Thirdly, all libraries for high throughput sequencing of lncRNA and mRNA were amplified through PCR for 15 cycles. Then, purification was performed by Certified Low Range Ultra Agarose (Bio-Rad) and quantification was performed by Picogreen (Molecular probes) on TBS380 (Turner Biosystems). Bridge PCR was performed on cBot. Sequencing was performed by using a llumina HiSeq X Ten platform.

Lastly, all libraries for high throughput sequencing of miRNA were amplified through PCR for 12 cycles. Then, purification was performed by 6% Novex TBE PAGE gel (1.0 mm, 10 well) and quantification was performed by Picogreen (Molecular probes) on TBS380 (Turner Biosystems). Bridge PCR was performed on cBot. Sequencing was performed by a llumina HiSeq 4000 platform.

Quality control of raw sequence

By using Base Calling, all the raw data obtained from high-throughput RNA-sequencing was translated into raw FASTQ sequence data. To obtain the clean reads, sequence with low quality including adaptor sequences, sequences with quality score <20 and sequences with N base rate of raw reads >10% were removed by using cutadapt (http://cufflinks.cbcb.umd.edu/).

Clean reads mapping

By using TopHat (http://ccb.jhu.edu/software/tophat/index.shtml), clean reads from RNA-sequencing results of mRNA and lncRNA were aligned with the human reference genome, Ensembl V84. By using Cufflinks (http://cufflinks.cbcb.umd.edu/), the expression of lncRNA and mRNA was quantified and the normalized expression data were outputted. By using bowtie (http://bowtie-bio.sourceforge.net/index.shtml), clean reads from RNA-sequencing results of miRNA were aligned with the human reference genome, GRCH38. The expression of miRNA was quantified by using miRDeep2.

Identification of DEmRNAs, DEmiRNAs and DElncRNAs

Compared to normal controls, DEmRNAs, DEmiRNAs and DElncRNAs in smoking patients with COPD and non-smoking patients with COPD were identified by DEGseq (http://bioconductor.org/packages/DEGseq/), respectively. FDR<0.01 and |log2Fold Change|>1 were selected as the criteria of significance.

Functional analysis of DEmRNAs

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) molecular pathway enrichment analysis of DEmRNAs in smoking and non-smoking patients with COPD were performed by using online-based software GeneCoDis3 (http://genecodis.cnb.csic.es/analysis), respectively.

DEmRNA-DElncRNA interaction analysis

LncRNAs were reported to regulate genes that transcribed near them, consistent with activity in cis [15]. To identify the nearby DEmRNAs of DElncRNAs with cis-regulatory effects, DEmRNAs transcribed within a 100kb window up- or down-stream of DElncRNAs in smoking and non-smoking patients with COPD were searched, respectively.

In addition, DEmRNAs co-expressed with DElncRNAs were identified as well. We calculated the pairwise Pearson correlation coefficients between DElncRNAs and DEmRNAs in smoking and non-smoking patients with COPD, respectively. DElncRNA-DEmRNA pairs with PCC >0.9 were served as significant

Table 1. Patient characteristics. BMI: Body mass index. FEV_1%: percent of predicted forced expiratory volume in 1 second. FEV_1/FVC: FEV_1 as percentage of forced vital capacity

Parameter	Non-Smoking COPD							Smoking COPD							Normal control					
Index	N1	N2	N3	N4	N5	N6	N7	S1	S2	S3	S4	S5	S6	S7	C1	C2	C3	C4	C5	C6
Gender	Female	Female	Female	Male	Male	Female	Female	Male	Male	Male	Male	Male	Male	Male	Female	Female	Male	Female	Female	Female
Age	73	68	74	64	63	77	82	74	78	62	77	80	52	53	64	51	40	45	44	42
BMI	24.5	28.9	26.7	28.35	20.8	26.2	20.4		17.51	31.16	22.72	20.65	17.625	19.05	22.07	22.07	19.81	24.3	23.43	23.23
Allergy history	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Smoking	No	No	No	No	No	No	No	4/d*40y	20-40/d*50y	8/d*40y	20/d*60y	20/d*40y	20/d*20y	40/d*40y	No	No	No	No	No	No
Stage	III	III	II	II	III	III	II	III	III	II	II	II	III	II						
FEV1%	39.1	33	63.3	59.1	39.4	49.9	61.4	42.5	43.9	52.2	55.9	72.9	40.6	53	125	123.8	126.8	99.8	103.6	95.2
FEV1/FVC	50.11%	42.29%	76.30%	57.31%	35.45%	51.20%	48.51%	58.9	57.5	61.72	61.16	67.77	65.63	51.54	77.94	86.4	98.56	89.2	91.6	80.57
History of respiratory diseases	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Occupational exposure history (dust, soot, coal dust)	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No

DElncRNA-DEmRNA co-expression pairs. The DElncRNA-nearby target DEmRNAs pairs and DElncRNA-DEmRNA co-expression pairs were used to construct the DElncRNA-DEmRNA interaction network by using Cytoscape software (http://www.cytoscape.org/).

DEmiRNA-DEmRNA interaction analysis

Firstly, the pairwise Pearson correlation coefficients between DEmRNAs and DEmiRNAs were calculated. DEmiRNA-DEmRNA pairs with $p<0.05$ and $r<0$ were served as significant negative DEmiRNA-DEmRNA co-expression pairs. Then, we obtained the putative target DEmRNAs of DEmiRNAs by six bioinformatic algorithms (RNA22, miRanda, miRDB, miRWalk, PICTAR2 and Targetscan). Moreover, the confirmed target mRNAs of miRNAs were obtained by miRWalk. Among these obtained significant negative DEmiRNA-DEmRNA co-expression pairs, the confirmed DEmiRNA-DEmRNA pairs by miRWalk and the DEmiRNA-DEmRNA pairs recorded by≥4 algorithms were served as target DEmRNAs of DEmiRNAs in our study.

DEmiRNA-DEmRNA-DElncRNA interaction network

Based on the DElncRNA-DEmRNA interaction analysis and DEmiRNA-DEmRNA interaction analysis, we identified the DEmRNAs which were interacted with both DElncRNAs and DEmiRNAs and constructed the DEmiRNA-DEmRNA-DElncRNA interaction network.

Confirmation by qRT-PCR

To verify the expression of DEmRNAs, DEmiRNAs and DElncRNAs of our RNA-sequencing results, we collected 20 blood samples from 20 individuals including 7 smoking patients with COPD (S1-S7) and 7 non-smoking patients with COPD (N1-N7) and 6 normal controls (C1-C6). Fifteen out of these 20 individuals (S1-S5, N1-N7 and C1-C5) were whom enrolled for RNA-sequencing as well. The detailed information of other five individuals (S6, S7, N6, N7 and C6) were displayed in Table 1.

Total RNA was isolated by using the Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. The mRNA template was reversely transcribed into cDNA using a FastQuant RT Kit according to the manufacturer's protocol. MiRNA reverse transcription was performed using miRcute miRNA First-strand cDNA Synthesis kits (TIAN- GEN) according to the manufacturer's instructions. The qRT-PCR reactions were performed using SuperReal PreMix Plus (Invitrogen, USA) in ABI 7500 Real-time PCR Detection System. Relative gene expression was analyzed using $2^{-\Delta\Delta Ct}$ method. The human 18srRNA were used as endogenous controls for mRNA and lncRNA and human U6 were used as endogenous controls for miRNA expression in analysis.

Results

Identification of DEmRNAs, DEmiRNAs and DElncRNAs

Compared to normal controls, 1234 DEmRNAs (440 up-regulated and 794 down-regulated DEmRNAs), 96 DElncRNAs (20 up-regulated and 76 down-regulated DElncRNAs) and 151 DEmiRNAs (103 up-regulated and 48 down-regulated DEmiRNAs) in non-smoking patients with COPD were identified. The top 10 DEmRNAs, DEmiRNAs and DElncRNAs between non-smoking patients with COPD and normal controls were displayed in Table 2.

Compared to normal control, 670 DEmRNAs (249 up-regulated and 421 down-regulated DEmRNAs), 44 DElncRNAs (15 up-regulated and 29 down-regulated DElncRNAs) and 63 DEmiRNAs (35 up-regulated and 28 down-regulated DEmiRNAs) in smoking patients with COPD were identified. The top 10 DEmRNAs, DEmiRNAs and DElncRNAs between smoking patients with COPD and normal controls were displayed in Table 2 as well.

Table 2. The top 10 DEmRNAs, DElncRNAs and DEmiRNAs in smoking and non-smoking COPD compared to normal controls. FC, fold change

Smoking COPD				Non-smoking COPD			
DEmRNA	Regulation	LogFC	FDR	DEmRNA	Regulation	LogFC	FDR
IFI27	up	5.76	FDR<0.01	PRSS33	down	-6.93	FDR<0.01
DSP	down	-4.28	FDR<0.01	XKR3	up	6.02	FDR<0.01
CTXN2	down	-3.95	FDR<0.01	IFI27	up	5.50	FDR<0.01
MTRNR2L6	down	-3.91	FDR<0.01	ALOX15	down	-5.03	FDR<0.01
CCDC3	up	3.69	FDR<0.01	FN1	up	4.77	FDR<0.01
HLA-DQB1	up	3.53	FDR<0.01	SIGLEC8	down	-4.34	FDR<0.01
MYOM2	down	-3.45	FDR<0.01	HDC	down	-4.29	FDR<0.01
MAOA	up	3.20	FDR<0.01	GBP6	up	3.98	FDR<0.01
TMTC1	up	3.06	FDR<0.01	ANKRD22	up	3.89	FDR<0.01
SLC12A1	down	-3.05	FDR<0.01	PTGDR2	down	-3.77	FDR<0.01
DElncRNA	Regulation	LogFC	FDR	DElncRNA	Regulation	LogFC	FDR
XIST	down	-10.42	FDR<0.01	FAM225A	up	2.39	FDR<0.01
TSIX	down	-3.87	FDR<0.01	A2M-AS1	down	-2.18	FDR<0.01
MIR600HG	down	-1.68	FDR<0.01	LOC105373098	up	2.13	FDR<0.01
LINC00278	up	1.53	FDR<0.01	THRA1/BTR	down	-1.94	FDR<0.01
PAX8-AS1	up	1.45	FDR<0.01	LOC101928100	down	-1.73	FDR<0.01
A2M-AS1	down	-1.40	FDR<0.01	CHRM3-AS2	down	-1.71	FDR<0.01
LINC00612	down	-1.22	FDR<0.01	LINC00987	down	-1.65	FDR<0.01
TRG-AS1	down	-1.02	FDR<0.01	LINC00612	down	-1.57	FDR<0.01
LOC102724404	down	-1.24	FDR<0.01	LINC00402	down	-1.48	FDR<0.01
SND1-IT1	down	-1.05	FDR<0.01	NFE4	up	1.42	FDR<0.01
DEmiRNA	Regulation	LogFC	FDR	DEmiRNA	Regulation	LogFC	FDR
hsa-miR-494-3p	up	2.06	FDR<0.01	hsa-miR-9-5p	up	9.84	FDR<0.01
hsa-miR-323a-3p	up	1.89	FDR<0.01	hsa-miR-708-3p	up	7.37	FDR<0.01
hsa-miR-127-3p	up	1.80	FDR<0.01	hsa-miR-1298-5p	up	7.01	FDR<0.01
hsa-miR-381-3p	up	1.72	FDR<0.01	hsa-miR-379-5p	up	6.36	FDR<0.01
hsa-miR-6868-3p	up	1.72	FDR<0.01	hsa-miR-136-3p	up	6.16	FDR<0.01
hsa-miR-409-3p	up	1.71	FDR<0.01	hsa-miR-218-5p	up	5.92	FDR<0.01
hsa-miR-654-3p	up	1.70	FDR<0.01	hsa-miR-9-3p	up	5.80	FDR<0.01
hsa-miR-320d	down	1.66	FDR<0.01	hsa-miR-129-5p	up	5.13	FDR<0.01
hsa-miR-320c	down	1.65	FDR<0.01	hsa-miR-200b-3p	up	5.10	FDR<0.01
hsa-miR-320b	down	1.64	FDR<0.01	hsa-let-7e-5p	up	4.06	FDR<0.01

Moreover, 225 shared DEmRNAs (82 up-regulated and 143 down-regulated DEmRNAs), 15 shared DElncRNAs (3 up-regulated and 12 down-regulated DElncRNAs) and 31 shared DEmiRNAs (18 up-regulated and 13 down-regulated DEmiRNAs) were identified in both smoking and non-smoking patients with COPD compared to normal controls.

Functional annotation of DEmRNAs

Based on the functional annotation of DEmRNAs between non-smoking patients with COPD and normal controls, immune response (FDR=5.78E-17), response to external stimulus (FDR=1.10E-16), immune system process (FDR=2.75E-16), molecular transducer activity (FDR=2.19E-08) and cell periphery (FDR=8.43E-18) were significantly enriched GO terms (Table 3). Based on the KEGG enrichment analysis in non-smoking COPD (Fig. S1A - for all supplemental material see www.karger.com/10.1159/000494541/), Transcriptional misregulation in cancer (FDR=2.80E-03, Fig. S1B) and pathways in cancer (FDR=1.34E-02) were significantly enriched pathways.

According to the functional annotation of DEmRNAs between smoking patients with COPD and normal controls, cellular response to chemical stimulus (FDR=5.36E-07), regulation of cell proliferation (FDR=1.98E-05), cell surface receptor signaling pathway (FDR=2.21E-05), protein binding (FDR=1.17E-06) and plasma membrane (FDR=1.32E-08) were significantly enriched GO terms (Table 3&4). Based on the KEGG enrichment analysis in smoking COPD (Fig. S1C), leukocyte transendothelial migration (FDR=4.19E-02) was a significantly enriched pathway in smoking COPD (Fig. S1D).

Moreover, cytokine-cytokine receptor interaction, ECM-receptor interaction, PI3K-Akt signaling pathway and cell adhesion molecules were four shared pathways in both smoking and non-smoking COPD.

Table 3. The top 10 significantly enriched GO terms in smoking COPD

GO ID	GO name	observed gene count	false discovery rate
Biological progress			
GO.0070887	cellular response to chemical stimulus	84	5.36E-07
GO.0042127	regulation of cell proliferation	59	1.98E-05
GO.0007166	cell surface receptor signaling pathway	73	2.21E-05
GO.0007154	cell communication	135	2.48E-05
GO.0007155	cell adhesion	44	2.48E-05
GO.0030334	regulation of cell migration	33	2.48E-05
GO.0042221	response to chemical	109	2.48E-05
GO.0044700	single organism signaling	133	2.48E-05
GO.0071310	cellular response to organic substance	67	2.48E-05
GO.0007165	signal transduction	126	3.38E-05
Moulecular Function			false discovery rate
GO.0005515	protein binding	136	1.17E-06
GO.0005198	structural molecule activity	31	0.000236
GO.0005488	binding	233	0.000559
GO.0004872	receptor activity	50	0.00364
GO.0060089	molecular transducer activity	55	0.0184
GO.0003674	molecular_function	260	0.0342
GO.0016500	protein-hormone receptor activity	4	0.0342
GO.0050840	extracellular matrix binding	6	0.0342
GO.0008092	cytoskeletal protein binding	23	0.0354
GO.0005539	glycosaminoglycan binding	13	0.0366
Celluar compent			false discovery rate
GO.0005886	plasma membrane	139	1.32E-08
GO.0071944	cell periphery	138	4.24E-08
GO.0044459	plasma membrane part	83	2.17E-07
GO.0030054	cell junction	49	2.52E-06
GO.0031988	membrane-bounded vesicle	107	2.56E-06
GO.0098590	plasma membrane region	42	3.49E-06
GO.0016020	membrane	206	4.12E-06
GO.0031982	vesicle	108	4.12E-06
GO.0005737	cytoplasm	230	5.91E-06
GO.0044421	extracellular region part	110	5.91E-06

DElncRNA-DEmRNA interaction

A total of 42 DElncRNA-nearby target DEmRNA pairs were obtained which were consisted of 29 DElncRNAs and 40 DEmRNAs between non-smoking COPD and normal controls. A total of 1559 DElncRNA-DEmRNA co-expression pairs were obtained which were consisted of 575 DEmRNAs and 81 DElncRNAs between non-smoking COPD and normal controls. Taken together, a total of 1601 DElncRNA-DEmRNA interaction pairs including 593 DEmRNA and 83 DElncRNAs were used to construct the non-smoking COPD-specific DElncRNA-DEmRNA interaction network (Fig. S2A). LINC01136 (degree=144), LOC105373098 (degree=126), LOC100129940 (degree=124), BISPR (degree=119) and FAM225A (degree=116) were hub DElncRNAs of this network.

A total of 23 DElncRNA-nearby target DEmRNA pairs were obtained which were consisted of 15 DElncRNAs and 19 DEmRNAs between smoking COPD and normal controls. A total of 221 DElncRNA-DEmRNA co-expression pairs were obtained which were consisted of 157 DEmRNAs and 35 DElncRNAs between smoking COPD and normal controls. Taken together, a total of 244 DElncRNA-DEmRNA interaction pairs including 208 DEmRNA and 41 DElncRNAs were used to construct the smoking COPD-specific DElncRNA-DEmRNA interaction network (Fig. S2B). LOC102724404 (degree=28), HLA-F-AS1 (degree=27), TRG-AS1 (degree=21), LINC02141 (degree=15) and PAXIP1-AS1 (degree=15) were hub DElncRNAs of this network.

Table 4. The top 10 significantly enriched GO terms in non-smoking COPD

GO ID	GO name	observed gene count	false discovery rate
Biological progress			
GO.0006955	immune response	117	5.78E-17
GO.0009605	response to external stimulus	145	1.10E-16
GO.0002376	immune system process	149	2.75E-16
GO.0006952	defense response	117	2.93E-15
GO.0007166	cell surface receptor signaling pathway	148	1.13E-14
GO.0051239	regulation of multicellular organismal process	156	1.27E-12
GO.0002682	regulation of immune system process	107	2.42E-12
GO.0070887	cellular response to chemical stimulus	150	1.12E-11
GO.0042221	response to chemical	212	1.23E-11
GO.0050793	regulation of developmental process	140	1.62E-11
Moulecular Function			
GO.0060089	molecular transducer activity	117	2.19E-08
GO.0004871	signal transducer activity	105	1.61E-07
GO.0004872	receptor activity	97	3.44E-07
GO.0038023	signaling receptor activity	85	3.22E-06
GO.0008201	heparin binding	23	4.96E-06
GO.0005515	protein binding	227	1.86E-05
GO.0003674	molecular_function	510	5.82E-05
GO.1901681	sulfur compound binding	25	9.31E-05
GO.0005539	glycosaminoglycan binding	24	0.000131
GO.0004888	transmembrane signaling receptor activity	73	0.000398
Celluar compent			
GO.0071944	cell periphery	274	8.43E-18
GO.0005886	plasma membrane	269	2.06E-17
GO.0044459	plasma membrane part	153	3.87E-12
GO.0044425	membrane part	306	3.54E-08
GO.0016020	membrane	381	3.35E-07
GO.0031224	intrinsic component of membrane	271	3.35E-07
GO.0005887	integral component of plasma membrane	99	8.29E-07
GO.0005615	extracellular space	87	8.52E-07
GO.0044421	extracellular region part	195	8.52E-07
GO.0031226	intrinsic component of plasma membrane	101	9.12E-07

DEmiRNA-DEmRNA interaction network

A total of 936 DEmiRNA-DEmRNA interaction pairs including 666 DEmRNAs (151 up-regulated and 515 down-regulated DEmRNAs) and 109 DEmiRNAs (81 up-regulated and 29 down-regulated DEmiRNAs) were identified in non-smoking COPD. hsa-miR-26b-5p (degree=128), hsa-miR-181d-5p (degree=110), hsa-miR-27b-3p (degree=108), hsa-miR-15a-5p (degree=106) and hsa-miR-15b-5p (degree=105) were the top 5 DEmiRNAs that owned most of target DEmRNAs in non-smoking COPD.

A total of 378 DEmiRNA-DEmRNA interaction pairs including 222 DEmRNAs (63 up-regulated and 159 down-regulated DEmRNAs) and 28 DEmiRNAs (16 up-regulated and 12 down-regulated DEmiRNAs) were identified in smoking COPD. hsa-miR-30a-5p (degree=42), hsa-miR-381-3p (degree=41), hsa-miR-329-3p (degree=38), hsa-miR-452-5p (degree=32) and hsa-miR-409-3p (degree=31) were the top 5 DEmiRNAs that owned most of target DEmRNAs in smoking COPD.

DEmiRNA-DEmRNA-DElncRNA interaction network

Non-smoking COPD-specific DElncRNA-DEmiRNA-DEmRNA interaction network was consisted of 579 nodes and 3224 edges. STRBP (degree=11), BACH2 (degree=11), SMC1A (degree=10), DDX3X (degree=9), IKZF1 (degree=9), CLCN4 (degree=9), ARSD (degree=9),

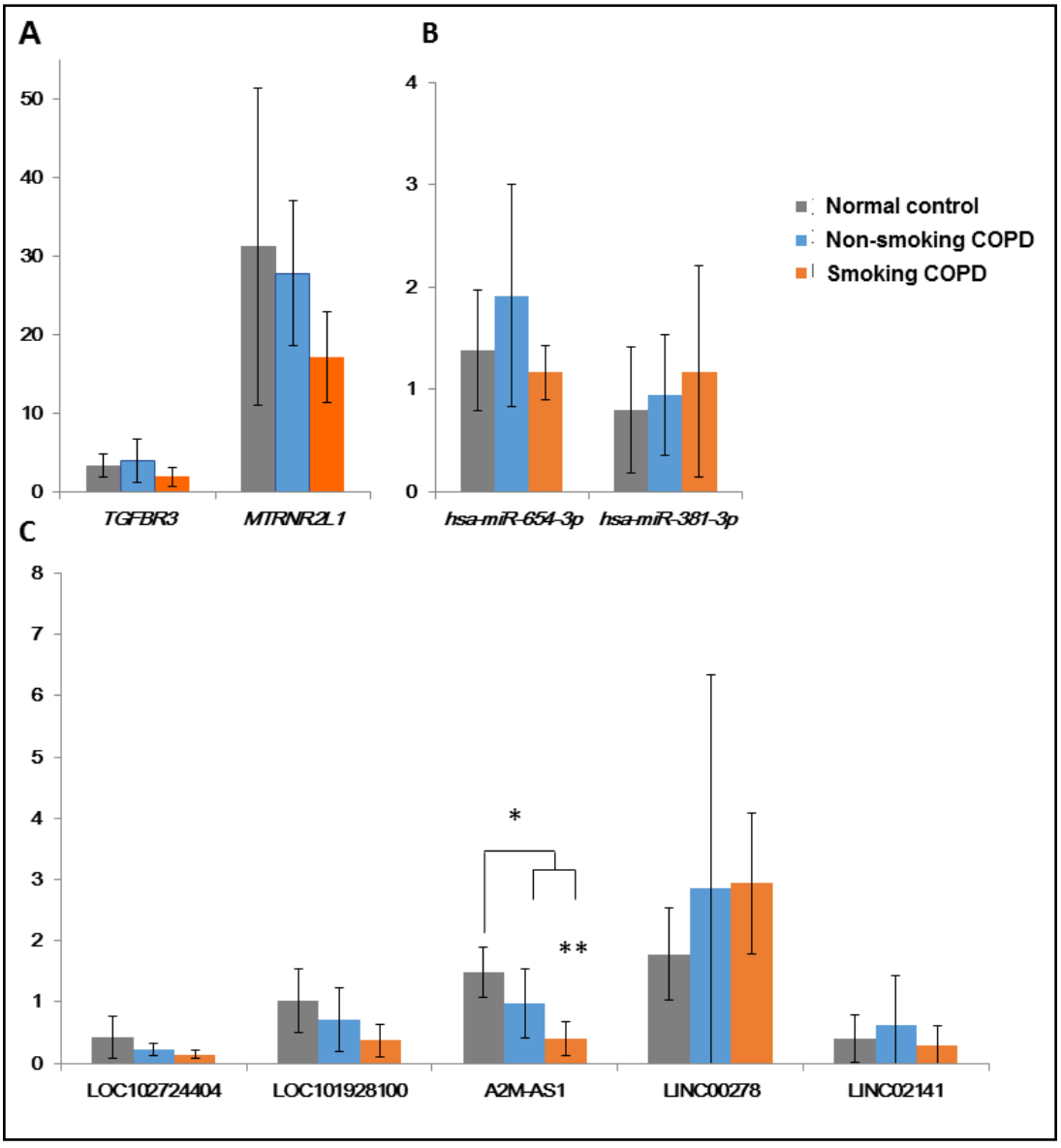

Fig. 1. Expression levels of candidate DEmRNAs, DEmiRNAs and DElncRNAs in COPD based on RNA-sequencing results. The X-axis represented normal control, non-smoking COPD (Non_smo COPD) and smoking COPD (smo_COPD) group, the Y-axis represented expression levels. The names of DEmRNAs, DEmiRNAs and DElncRNAs were above the box plots.

DOK6 (degree=9) and TMTC1 (degree=9) were hub DEmRNAs of this network. MiR-218-5p/miR15a-RORA-LOC101928100/LINC00861 interactions and miR-218-5p/miR15a-TGFβ3-RORA-AS1 interactions were found in non-smoking COPD-specific DElncRNA-DEmiRNA-DEmRNA interaction network (Fig. S3A and Fig. 1).

Smoking COPD-specific DElncRNA-DEmiRNA-DEmRNA interaction network was consisted of 187 nodes and 420 edges. RORA (degree=49), SGCD (degree=43), KLF12 (degree=43), C1orf21 (degree=39), DOK6 (degree=36), TGFBR3 (degree=33), PLXNA4 (degree=32), ATK3 (degree=31) and BCL11B (degree=30) were hub DEmRNAs of this network. Let-7-ADRB1-HLA-DQB1-AS1 interactions were found in smoking COPD-specific DElncRNA-DEmiRNA-DEmRNA interaction network (Fig. S3B and Fig. 1).

MiR-122-5p-A2M-LINC00987/A2M-AS1/linc00612 were shared interactions in both non-smoking and smoking COPD (Fig. S3C and Fig. 1).

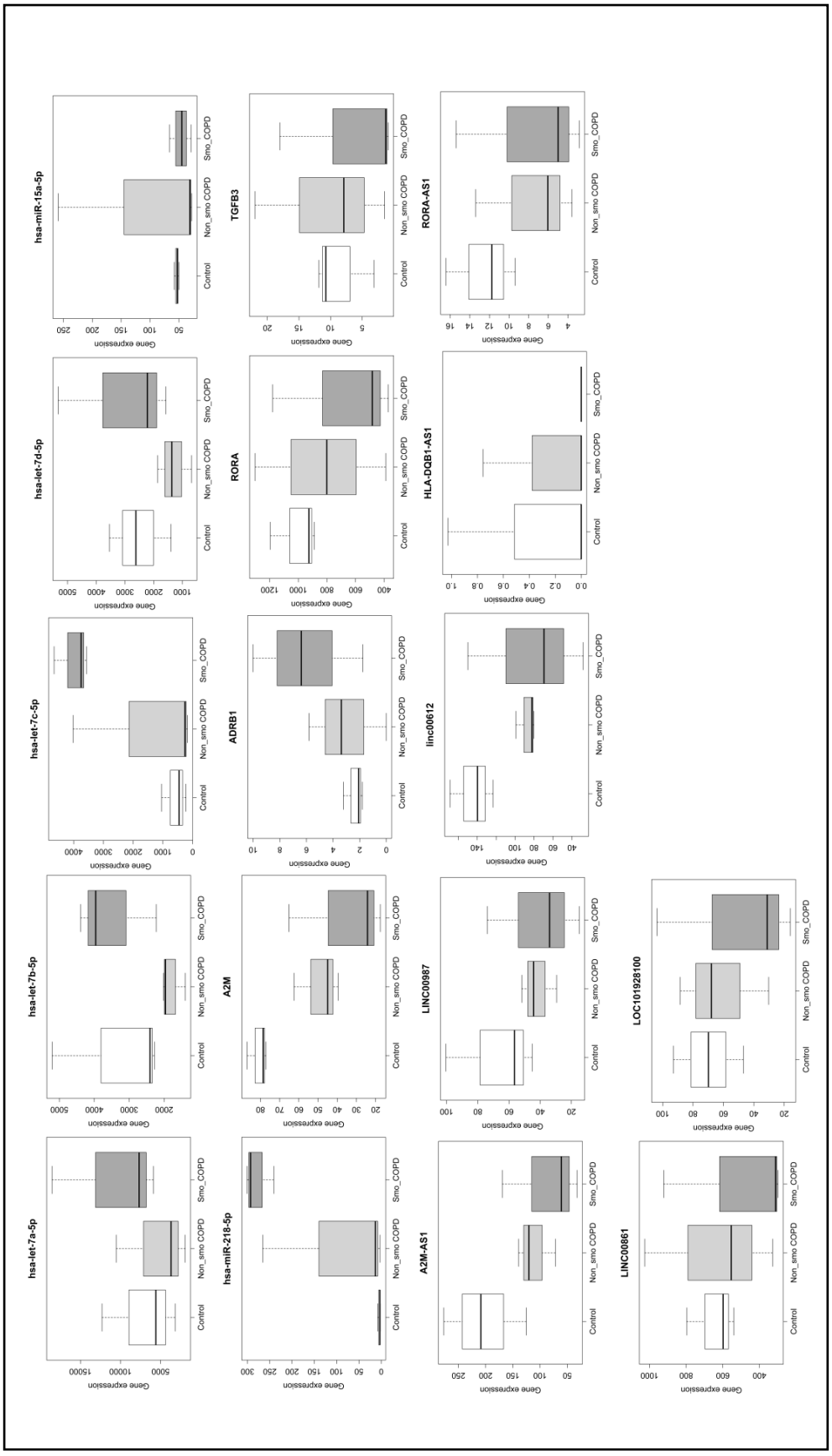

Fig. 2. QRT-PCR results of selected DEmRNAs, DEmiRNAs and DElncRNAs in COPD. A: qRT-PCR results of DEmRNAs in COPD. B: qRT-PCR results of DEmiRNAs in COPD. C: qRT-PCR results of DElncRNAs in COPD. The X-axis represented the DEmRNAs/DEmiRNAs/DElncRNAs and the Y-axis represented the relative expression levels.

Confirmation by qRT-PCR

We performed the confirmation of two DEmRNAs (TGFB3 and MTRNR2L1), two DEmiRNAs (has-miR-654-3p and has-miR-381-3p) and five DElncRNAs (LOC102724404, LOC101928100, A2M-AS1, LINC00278 and LINC02141) by qRT-PCR. Based on our RNA-sequencing results, TGFB3 and MTRNR2L1 was down-regulated in non-smoking and smoking COPD, respectively; has-miR-654-3p and has-miR-381-3p were two shared up-regulated miRNAs in both non-smoking COPD and smoking-COPD; LOC102724404 and A2M-AS1 were two shared down-regulated miRNAs in both non-smoking COPD and smoking-COPD; LOC101928100 was down-regulated while LINC00278 and LINC02141 were up-regulated in non-smoking COPD. Except for TGFB3 and has-miR-654-3p, the qRT-PCR results were consistent with our RNA-sequencing results, generally (Fig. 2).

Discussion

To better research the pathogenesis and developing potential biomarkers of non-smoking COPD, DEmiRNAs, DEmRNAs and DElncRNAs between non-smoking patients with COPD and non-smoking controls were identified. Previous study reported that smoking COPD may has an autoimmune component which contributes to the airway inflammation in COPD even after smoking cessation [16]. Smoking-induced inflammatory and oxidative lung injury could result in production of auto-antibodies that against antigens present in tobacco or endogenous auto-antigens [17] which suggested that smoking was a potential biological foundation for the occurrence of smoking COPD. Moreover, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [18] indicated that approximately 50% of smokers eventually develop COPD. Smokers have at least a one in two chances of developing COPD if they continue smoking lifelong [19]. Hence, smoking-related genes might have a potential effect on the occurrence of COPD no matter with their expression if altered in the process of smoking COPD. In this study, we identified DEmiRNAs, DEmRNAs and DElncRNAs between smoking patients with COPD and non-smoking controls which might involve with mechanism of smoking-induced alteration including the mechanism of smoking COPD process and the potential biological foundation for occurrence of smoking COPD. Furthermore, we identified the overlapping or diverging genes, lncRNAs, miRNAs and signaling pathways in smoking and non-smoking COPD.

Based the KEGG enrichment analysis, four shared pathways including cytokine-cytokine receptor interaction, ECM-receptor interaction, PI3K-Akt signaling pathway and cell adhesion molecules were identified in both smoking and non-smoking COPD. ECM-receptor interaction was reported to involve with the vascular remodeling of COPD-induced pulmonary hypertension [20]. PI3K-Akt signaling pathway was a known pathway involved with COPD as well. PI3K activation and up-regulated downstream kinase phosphorylated AKT were found in the lungs and cells of COPD patients [21]. Moreover, polymorphisms of PTEN, an inhibitor of PI3K-Akt signaling pathway were reported to be genetic risk factors for COPD[22]. Significantly increased soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble platelet endothelial cell adhesion molecular-1 (sPECAM-1) have been observed during acute exacerbation of COPD and after treatment, respectively which suggested the potential roles of cell adhesion molecules in COPD [23]. The KEGG enrichment analysis in this present study provided evidence for previous studies and suggested that these four pathways involve with COPD irrespective of smoking.

Leukocyte transendothelial migration was a significantly enriched pathway in smoking COPD. Neutrophils, a type of leukocytes was reported to play a fundamental role in the development and progression of COPD. Moreover, enhanced neutrophil–endothelial interaction was reported to increase the susceptibility of smokers who develop COPD which was regulated by a1-antitrypsin [9]. Taken together, we speculated that leukocyte transendothelial migration involve with smoking COPD. Pathways in cancer and

transcriptional misregulation in cancer were two significantly enriched pathways in non-smoking COPD which suggested that the association between cancer and non-smoking COPD. Whether the risk for lung cancer of patients with non-smoking COPD was higher than that in patients with smoking COPD needs further research.

Both miRNAs and lncRNAs could regulate gene expression. To deeper research the biological functions of DElncRNA, DEmRNAs and DEmiRNAs in COPD, we constructed DElncRNA-DEmRNA-DEmiRNA interaction network and identified several DElncRNA-mRNA-DEmiRNA interactions involved with COPD.

We identified that miR-122-5p-A2M-LINC00987/A2M-AS1/linc0061 interactions might play key roles in COPD irrespective with the smoking status. A2M, alpha 2-Macroglobulin is a major human plasma protease inhibitor that was speculated to play a role in the regulation of protease activity in the lung [24]. Dysregulation of A2M was found in smoking and non-smoking patients with COPD compared with normal controls in both previous study [25] and this present study which suggested that A2M was a potential biomarker of COPD.

Three shared DElncRNAs in both non-smoking and smoking COPD including LINC00987, A2M-AS1 and linc00612 were all co-expressed with A2M. MiR-122-5p was a shared DEmiRNA in both non-smoking and smoking COPD that could regulate expression of A2M according to the DEmiRNA-DEmRNA interaction network in this study. Compared to normal controls, miR-122 was reported to be down-regulated in lungs of human and rats exposed to cigarette smoke [26] while was not differentially expressed in patients with COPD in previous study [27]. In this study, miR-122-5p was up-regulated in bloods of non-smoking and smoking patients with COPD. All these findings suggested that miR-122-5p might be a potential regulator of COPD. Hence, we speculated that miR-122-5p, LINC00987, A2M-AS1 and linc00612 might involve with COPD by regulating A2M.

Let-7-ADRB1-HLA-DQB1-AS1 interactions were smoking COPD-specific which might play a key role in the pathogenesis in smoking COPD. Down-regulation of let-7c has been observed in smokers with COPD compared with never smokers and the expression of let-7c was found to be associated with forced expiratory volume in 1 second (FEV_1) in previous study [28]. Moreover, let-7c was reported to be a tumor suppressor of lung cancer and serve as an important pathogenic link between COPD and lung cancer [2, 29]. In our study, let-7a-5p, let-7b-5p, let-7c-5p and let-7d-5p were the top 4 down-regulated DEmiRNAs that owned most of target DEmRNAs between smoking COPD and normal controls which suggested the importance of let-7 in smoking COPD. Among shared DEmRNAs of let-7a-5p, let-7b-5p, let-7c-5p and let-7d-5p, ADRB1 (adrenoceptor beta 1) is a subtype of adrenergic receptors. Adrenergic receptors have been reported to play crucial roles in bronchomotor tone [29]. ADRB1 accounts for 30% of adrenergic receptors in the respiratory system [29]. Moreover, the antagonists of adrenergic receptors were reported to involve with the management of exacerbated COPD [28]. In this present study, ADRB1 was up-regulated in smoking COPD compared to normal controls which emphasized its importance in smoking COPD. HLA-DQB1-AS1 was a co-expressed lncRNA of ADRB1. We firstly found the up-regulation of HLA-DQB1-AS1 in patients with smoking COPD compared to normal controls. We speculated that let-7c-ADRB1-HLA-DQB1-AS1 interactions play a key role in smoking COPD.

Among non-smoking COPD-specific DEmiRNAs in this study, miR-15a was one of the top 5 miRNAs that owned most of target DEmRNAs that up-regulated in the blood of patients with COPD compared to normal controls. Previous study also detected the overexpression of miR-15a in vastus lateralis muscle of patients with acute exacerbations of COPD [30]. Moreover, miR-15a was speculated to prevent the progression of acute exacerbations of COPD by inhibition of Wnt signaling [31].

MiR-218-5p was reported to be down-regulated in lung tissues of patients with COPD and cigarette smoke–exposed mice and indicated that miR-218-5p play a protect role in cigarette smoke–induced inflammation and COPD [32]. In this present study, miR-218-5p was up-regulated in bloods of non-smoking patients with COPD which suggested that miR-218-5p might be a potential regulator of non-smoking COPD as well.

TGFβ3 (transforming growth factor beta receptor 3) and RORA, (Retinoic acid receptor-related orphan receptor-α) were two shared targets of both miR-15a and miR-218-5p that were down-regulated in non-smoking patients with COPD compared to normal controls. TGFβ3 serve as a "switch" that blunted Tgfbr1/Smad2/3 and potentiated Acvrl1/Smad1 signaling in lung fibroblasts [33]. Previous studies have demonstrated the importance of TGF-β pathway in COPD [34, 35]. Moreover, polymorphisms of TGFβ3 have been demonstrated to be associated with the susceptibility of COPD[36]. RORA plays a key role in the regulation of lipid and cholesterol metabolism and inflammation [37, 38]. Decreased expression of several macrophage markers (F4/80, Mac-2, Mpeg1 and Msr1) and proinflammatory cytokines (interleukin-1a, -1b and -6, and tumor necrosis factor-α) were found in RORA deficient staggerer mice compared to the wild-type counterparts [39]. The inhibition of the pro-inflammatory transcription factor nuclear factor-kB RORA could be regulated by RORA as well [40]. In addition, RORA involve with the response to DNA damage, and loss of RORA protect against airspace enlargement in animal models of emphysema [41]. Moreover, variants of RORA were reported to be associated with the risk of COPD. All these findings suggested that RORA was a potential regulator of non-smoking COPD.

Two non-smoking specific DElncRNAs (LOC101928100 and LINC00861) and RORA-AS1 were co-expressed with RORA and TGFβ3, respectively. Although no previous study reported the association between COPD and these three lncRNAs, we speculated that miR-218-5p/miR15a-RORA-LOC101928100/LINC00861 and miR-218-5p/miR15a-TGFβ3-RORA-AS1 interactions play important roles in non-smoking COPD. Additionally, RORA was a nearby target gene of RORA-AS1 which suggested that RORA-AS1 might involve in COPD by its cis-regulatory role on the expression of RORA.

Conclusion

In conclusion, we identified the shared and diverging genes, lncRNAs, miRNAs and signaling pathways in smoking and non-smoking COPD. Moreover, the network of lncRNA-miRNA-mRNA interactions will facilitate further experimental studies and may be used to refine biomarker predictions for developing novel therapeutic approaches in COPD. However, there are two limitations in this study. First, age and gender differences existed between the groups resulted from restrictions in sample collection. Lack of the comparison of mRNA, lncRNA and miRNA expression profiles between smoking patients with COPD and smoking controls was another limitation in this study. This comparison and further research with age-matched and sex-matched samples were needed to confirm our finding.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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