Comprehensive analysis of miRNA–mRNA–lncRNA networks in severe asthma

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Aim: This study aimed to explore the molecular mechanism of severe asthma. **Materials & methods:** The shared and divergent differentially expressed mRNAs (DEmRNAs), miRNAs (DEmiRNAs) and lncRNAs (DElncRNAs) in asthma and severe asthma were identified by RNA-sequencing. Severe asthma-specific and shared DEmiRNA–DEmRNA–DElncRNA interaction networks were performed. **Results:** Compared with normal control, 1328 DEmRNAs, 608 DElncRNAs and 63 DEmiRNAs were identified in severe asthma. Compared with asthma, 95 DEmRNAs, 143 DElncRNAs and 96 DEmiRNAs were identified in severe asthma. MiR-133a-3p-*EFHD2/CNN2-AC144831.1* interactions and miR-3613-3p-*CD44/BCL11B-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2* interactions were speculated to involve with the development of severe asthma. The results of GSE69683 validation were generally consistent with our RNA-sequencing results. **Conclusion:** This study provides clues for understanding the mechanism of severe asthma.

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Keywords: asthma • comprehensive analysis • DEmiRNA–DEmRNA–DElncRNA interaction networks • lncRNA • miR-133A-3P • miR-361-3p • miRNA • mRNA • RNA-sequencing • severe asthma

Asthma is a common chronic disease of the airways that affects 300 million people worldwide, with that number expected to rise [1]. Asthma is characterized by airway inflammation, smooth muscle bronchoconstriction leading to airflow obstruction, and mucous hypersecretion [2]. Severe asthma is a subset of difficult-to-treat asthma, that requires treatment with high dose inhaled corticosteroids plus a second controller and/or systemic corticosteroids to prevent it from becoming 'uncontrolled' or that 'uncontrolled' despite this therapy [3]. Patients with severe asthma accounts for 3–10% [3,4] of the population of adults with asthma, but leads to increased morbidity and their care is estimated to make up more an 60% of the costs associated with asthma [5–9]. However, the pathology of severe asthma remains elusive. It is urgent to explore the pathology and develop biomarkers for severe asthma.

Non-coding RNAs (ncRNAs) are functional RNAs which have no ability of coding proteins. MiRNAs are a class of small ncRNAs (≈22 nts) which play multiple roles in various biological processes by regulating expression of their target genes [10]. Long noncoding cRNAs (lncRNAs) is a type of ncRNAs with a length of longer than 200 nts which are emerging as key regulators in various biological processes, including chromatin modification, transcription and post-transcriptional processing [11]. Accumulated evidences indicated that the interactions between lncRNAs and miRNAs, and their downstream target genes have been closely related to various diseases.

In this study, we obtained the mRNA, miRNA and lncRNA expression data from, asthma, severe asthma and normal controls by using RNA-sequencing. Moreover, miRNA–mRNA–lncRNA networks were constructed to elaborate the interactions and potential cross talk among the key lncRNAs, miRNAs and mRNAs in asthma and severe asthma.

Table 1. Patient characteristics.				
Indicators	Normal control	Asthma	Severe asthma	p-value
Mean age (years)	51.67 ± 12.01	52.33 ± 15.31	53.66 ± 11.68	0.9820
Mean BMI	21.31 ± 1.30	21.17 ± 1.72	20.73 ± 5.53	0.1118
Gender (male/female)	1/2	1/2	1/2	1
FEV1%	125.2 ± 1.51	73.83 ± 15.54	41.53 ± 16.84	0.0007
FEV1/FVC	87.63 ± 10.37	76.2 ± 17.94	52.10 ± 23.56	0.1245
FEV1%: Percent of predicted forced expiratory volume in 1 second; FEV1/FVC: FEV1 as percentage of forced vital capacity.				

Materials & methods

Patients & samples

Three adult patients with asthma, three with severe asthma and three control subjects enrolled in our study were from the Affiliated Changzhou No.2 People’s Hospital of Nanjing Medical University. The inclusion criteria were as follows: patients with asthma were diagnosed based on Bronchial Asthma Prevention Guidelines of Chinese Medicine Academy [12]; patients with severe asthma were diagnosed based on the expert consensus on the diagnosis and treatment of severe asthma [13]; participants more than 18; and participants did not take any drugs during the short period of sample collection time. Participants with abnormal liver function, coinfection, pulmonary embolism, chronic bronchitis, tuberculosis and blood system diseases were excluded. The detailed information of all participants were displayed in Table 1. All enrolled patients are stable at the time of drawing blood, and have no active asthma or exacerbation. Blood samples were collected by venipuncture from all subjects at 7:00–8:00 in the morning on an empty stomach 12 h after the last meal. The blood was stored in sterile PAXgene Blood RNA Tube after collection. Gently invert the PAXgene Blood RNA Tube eight- to ten-times. Store the PAXgene Blood RNA Tube upright for 12–24 h at room temperature (18–22°C) or 4°C. Leave at -20°C for 24 h and finally transfer to -80°C for long-term storage. PAXgene Blood RNA Tube is suitable for collecting whole blood and stabilizing RNA in cells, and can be stored for at least 50 months under -80°C conditions. The blood sample was stored in the central position of the thermal container with dry ice between withdrawal and extraction. This study has been approved by the Ethics Institute of Affiliated Changzhou No.2 People’s Hospital of Nanjing Medical University (2017KY024-01). The signed informed consents of all participants were obtained. This research complied with the principles of the Declaration of Helsinki.

Library preparation & high-throughput sequencing

By using TRIzol reagent (Invitrogen, CA, USA), total RNA was extracted from blood samples according to the manual instruction. Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, DE, USA) was used to access the concentration and purity of RNA. The integrity of RNA was checked by using 2% agarose gel. The RNA integrity number (RIN) value was obtained by the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). The criteria for library construction were as follows: RNA with amount > 5 µg; concentration of RNA ≥200 ng/ml; 1.8 < OD260/280 < 2.2; and RIN >7.

First, library for high-throughput sequencing of lncRNA and mRNA was constructed as follow steps: the ribosomal RNA was removed from total RNA by Ribo-Zero Magnetic Kit (EpiCentre, WI, USA); library with Truseq RNA sample Prep Kit (Illumina, Inc., CA, USA) was constructed. The RNA was purified and fragmented into 200–500 base pairs with addition of fragmentation buffer; 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., CA, USA) and PCR was performed to construct library for high-throughput sequencing of lncRNA and mRNA.

Second, the 18–30 nts RNA was isolated from the total RNA and then, adapter ligation and RT-PCR was performed to construct the cDNA library for high-throughput sequencing of miRNA by Truseq™ Small RNA Sample Prep Kit.

Third, 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 a Illumina HiSeq X Ten platform.

At last, 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, ten well) and quantification was performed by Picogreen (Molecular Probes) on TBS380 (Turner Biosystems). Bridge PCR was performed on cBot. Sequencing was performed by a Illumina 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 from RNA-sequencing results for lncRNA and mRNA, 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/>). After sequence with low quality including adaptor sequences, sequences with quality score <20, sequences with N base and sequence less than 18bp were removed by using Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

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, DE miRNAs & DE lncRNAs

In RNA sequencing analysis, gene expression level is calculated by the amount of clean reads located in the genomic region. The criteria for measuring gene expression levels are RPKM (Reads Per Kilobase of exon model per Million mapped reads) and FPKM (Fragments Per Kilobase of exon model per Million mapped reads) values. Based on the alignment of all samples with the reference genome, the FPKM value of each gene in the sample is calculated, and this value is used as the expression level of the gene in the sample. Finally, the differential expression of all genes in each comparison was performed. Compared with normal control, differentially expressed mRNAs (DEmRNAs) and lncRNAs (DElncRNAs) in adult patients with asthma or severe asthma were identified by using limma package in R. Moreover, DEmRNAs and DElncRNAs between adult patients with asthma or severe asthma were identified by using limma package in R as well. The MRNAs and lncRNAs with p-value <0.01 was served as significant DEmRNAs and DElncRNAs, respectively.

Compared with normal control, differentially expressed miRNAs (DE miRNAs) in adult patients with asthma or severe asthma were identified by using DESeq (<http://bioconductor.org/packages/DESeq/>). Furthermore, DE miRNAs between adult patients with asthma and severe asthma were identified by using DESeq as well. When the selection criteria of DE miRNAs were set as $p < 0.01$, the same to the DEmRNAs, the amount of differentially expressed miRNA is too large to construct mRNA, lncRNA and miRNA network. Moreover, the miRNA acted as a link between mRNA and lncRNA, so it is more rigorous when screening differentially expressed miRNA, and the value of logFC (difference multiple) is also considered. Hence, differentially expressed miRNAs were screened according to the adjustment criteria. Hence, p-value <0.001 and $|\log_2 \text{Fold Change}| > 2$ were defined as the criteria of significance.

Venny 2.1.0 (<http://bioinfogp.cnb.csic.es/tools/venny/>) was used to obtain the shared DEmRNAs, DElncRNAs and DElncRNAs in comparisons of severe asthma versus normal control, asthma versus normal control and severe asthma versus asthma.

DE miRNA–DE mRNA interaction analysis

DE miRNA–DE mRNA interaction pairs in severe asthma versus normal control, asthma versus normal control and severe asthma versus asthma were obtained. As miRNAs tend to decrease the expression of their target mRNA, target genes were selected from DEmRNAs that expressed inversely with that of miRNA, to subject to further investigation. First, the putative targeted DEmRNAs of DE miRNAs were predicted by six bioinformatic algorithms (RNA22, miRanda, miRDB, miRWalk, PICTAR2 and Targetscan). Then, the confirmed targeted DEmRNAs of DE miRNAs were obtained from miRWalk. Third, the confirmed DE miRNA–DE mRNA pairs were derived from miRWalk and the DE miRNA–DE mRNA pairs recorded by ≥ 4 algorithms. Based on the obtained DE miRNA–

DEmRNA pairs, DEmiRNA–DEmRNA interaction networks in severe asthma versus normal control, asthma versus normal control and severe asthma versus asthma were constructed by using Cytoscape software. Shared DEmiRNA–DEmRNA pairs in both asthma and severe asthma compared with normal controls were obtained as well.

DEmiRNA–DElncRNA interaction analysis

DEmiRNA–DElncRNA interaction pairs in severe asthma versus normal control, asthma versus normal control and severe asthma versus asthma were obtained. MiRNA may negatively regulate the expression of lncRNA through the mechanism similar to mRNA. So DEmiRNA–DElncRNA pairs in which DElncRNAs were expressed inversely with DEmiRNAs were retained for following research. The putative targeted DElncRNAs of DEmiRNAs were predicted by five bioinformatic algorithms including miRWalk, miRanda, PITA, RNAhybrid and Targetscan. Based on the obtained DEmiRNA–DElncRNA pairs, DEmiRNA–DElncRNA interaction networks in severe asthma versus normal control, asthma versus normal control and severe asthma versus asthma were constructed by using Cytoscape software. Shared DEmiRNA–DElncRNA pairs in both asthma and severe asthma compared with normal controls were obtained as well.

DEmRNA–DElncRNA interaction analysis

Previous study has indicated that lncRNAs could play a cis-regulatory role on the expression of genes that transcribed near them [14]. The DEmRNAs transcribed within a 10 kb window up- or 100 kb down-stream of DElncRNAs were searched and served as nearby DEmRNAs of DElncRNAs in this present study. Based on the DEmRNAs and DElncRNAs derived from shared DEmiRNA–DEmRNA pairs and DEmiRNA–DElncRNA pairs in both asthma and severe asthma compared with normal controls, DElncRNA–nearby DEmRNA pairs were obtained. In addition, DElncRNA–nearby DEmRNA pairs were searched based on the DEmRNAs and DElncRNAs derived from DEmiRNA–DEmRNA pairs and DEmiRNA–DElncRNA pairs between severe asthma and asthma.

Moreover, DEmRNAs coexpressed with DElncRNAs were identified as well. The pairwise Pearson correlation coefficients between DElncRNAs and DEmRNAs were calculated. DElncRNA–DEmRNA pairs with $p < 0.001$ and $|r| \geq 0.8$ were served as significant mRNA–lncRNA coexpression pairs. DElncRNA–DEmRNA coexpression pairs in severe asthma versus normal control, asthma versus normal control and severe asthma versus asthma were obtained. Moreover, shared DElncRNA–DEmiRNA coexpression pairs in both asthma and severe asthma compared with normal controls were obtained as well. DElncRNA–DEmRNA coexpression networks were constructed by using Cytoscape software (<http://www.cytoscape.org/>).

DEmiRNA–DEmRNA–DElncRNA interaction network

Based on the shared DEmiRNA–DEmRNA pairs, DEmiRNA–DElncRNA pairs and DEmRNA–DElncRNA interaction pairs in both asthma and severe asthma compared with normal controls, the shared DEmiRNA–DEmRNA–DElncRNA interaction network was constructed.

Based on the DEmiRNA–DEmRNA pairs, DEmiRNA–DElncRNA pairs and DEmRNA–DElncRNA interaction pairs between severe asthma and asthma, the severe asthma-specific DEmiRNA–DEmRNA–DElncRNA interaction network was constructed.

Functional analysis of DEmRNAs

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) molecular pathway enrichment analysis of DEmRNAs derived from the shared and severe asthma-specific DEmiRNA–DEmRNA–DElncRNA were performed by using online-based software GeneCoDis3 (<http://genecodis.cnb.csic.es/analysis>), respectively. Hierarchical clustering analysis of DEmRNAs were conducted by using ‘pheatmap’ in R version 3.3.3.

Validation in the Gene Expression Omnibus dataset

GSE69683 dataset was downloaded from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/), which consisted of 344 patients with severe asthma and 87 normal controls. The dataset GSE69683 examined the blood sample. The expression patterns of selected DEmRNAs were validated in GSE69683 dataset.

Table 2. Shared differentially expressed mRNAs, lncRNAs and miRNAs in asthma vs normal controls, severe asthma vs normal controls and severe asthma vs asthma.

Symbol	Asthma vs control		Severe asthma vs control		Severe asthma vs asthma	
	Log ₂ FC	p-value	Log ₂ FC	p-value	Log ₂ FC	p-value
lncRNA						
RP5-857K21.7	-0.98	3.10E-03	-2.05	2.41E-04	-1.07	8.18E-03
RP11-58A11.2	0.67	1.13E-03	0.42	7.39E-03	-0.24	1.80E-04
RP11-461A8.4	-0.47	6.04E-03	-0.72	8.92E-04	-0.25	1.29E-03
mRNA						
PABPC1	-0.84	4.33E-04	-1.38	1.09E-04	-0.54	9.85E-03
PIP5K1B	0.53	3.58E-04	0.91	2.61E-04	0.38	9.56E-03
USP53	1.35	9.32E-04	0.50	8.45E-03	-0.86	8.06E-03
SNRPE	1.31	3.21E-04	0.69	5.35E-03	-0.62	6.47E-03
KRT5	0.49	2.54E-04	0.72	5.34E-06	0.22	8.51E-03
C12orf55	0.40	5.61E-03	0.73	4.19E-05	0.33	8.67E-03
MTRNR2L9	-3.59	2.16E-05	-4.65	2.35E-06	-1.06	6.56E-03
miRNA						
hsa-miR-98-5p	-2.77	<1.00E-16	2.06	<1.00E-16	4.83	<1.00E-16
hsa-miR-18a-5p	-2.01	1.54E-13	2.21	<1.00E-16	4.22	<1.00E-16

Results

The characteristics of all subjects for analysis

The clinical data of all subjects was displayed in Table 1. All subjects have no history of smoking. The mean age of three patients with asthma, three patients with severe asthma and three normal controls were 52, 53 and 51, respectively. Each group consists of two women and one man. All nine persons belong to Asians from China.

Analysis of transcriptome sequencing

We sequenced RNA libraries of nine blood samples from three patients with asthma, three patients with severe asthma and three normal controls. Counts of clean reads of sequencing results were displayed in Supplementary Table 1.

Identification of DEmRNAs, DEmiRNAs & DElncRNAs

A total of 505 DElncRNAs (20 upregulated and 76 downregulated DElncRNAs), 608 DElncRNAs (504 upregulated and 104 downregulated DElncRNAs) and 143 DElncRNAs (50 upregulated and 93 downregulated DElncRNAs) were identified in asthma versus normal controls, severe asthma versus normal controls and severe asthma versus asthma, respectively (Figure 1A). Moreover, a total of 217 shared DElncRNAs were identified in both asthma and severe asthma compared with normal controls (Figure 1B).

A total of 962 DEmRNAs (576 upregulated and 386 downregulated DEmRNAs), 1328 DEmRNAs (780 upregulated and 548 downregulated DEmRNAs) and 95 DEmRNAs (17 upregulated and 78 downregulated DEmRNAs) were identified in asthma versus normal controls, severe asthma versus normal controls and severe asthma versus asthma, respectively (Figure 1C). Moreover, a total of 487 shared DEmRNAs were identified in both asthma and severe asthma compared with normal controls (Figure 1D).

A total of 53 DEmiRNAs (18 upregulated and 35 downregulated DEmiRNAs), 63 DEmiRNAs (61 upregulated and two downregulated DEmiRNAs) and 96 DEmiRNAs (88 upregulated and eight downregulated DEmiRNAs) were identified in asthma versus normal controls, severe asthma versus normal controls and severe asthma versus asthma, respectively (Figure 1E). Moreover, a total of 13 shared DEmiRNAs were identified in both asthma and severe asthma compared with normal controls (Figure 1F).

Shared DEmRNAs, DElncRNAs and DEmiRNAs in asthma versus normal controls, severe asthma versus normal controls and severe asthma versus asthma were displayed in Table 2.

Figure 1. Differentially expressed lncRNAs, mRNAs and miRNAs in normal control, asthma and severe asthma (SA). (A, C & E) were hierarchical clustering analysis of DElncRNAs, DEmRNAs and DEmiRNAs in normal control, asthma and severe asthma. Row represented DElncRNAs, DEmRNAs and DEmiRNAs. Column represented samples. The color scale represented the expression levels. (B, D & F) shown the venny analysis of DEmRNAs, DElncRNAs and DEmiRNAs in severe asthma versus normal control (SA vs C), asthma versus normal control (A vs C) and severe asthma versus asthma (SA vs A). A: Asthma; C: Control; SA: Severe asthma.

DEmiRNA–DEmRNA interaction

A total of 653, 1056 and 410 DEmiRNA–DEmRNA interaction pairs were identified in asthma versus normal controls, severe asthma versus normal controls and severe asthma versus asthma. Based on these 410 DEmiRNA–DEmRNA interaction pairs in severe asthma versus asthma, the severe asthma-specific DEmiRNA–DEmRNA interaction network was constructed. The RAPH1 (degree = 28), BCL11B (degree = 27) and SNX1 (degree = 26) were three hub genes of the severe asthma-specific DEmiRNA–DEmRNA interaction network (Supplementary Figure 1A). MiR-93-5p (degree = 15), miR-3613-3p (degree = 15) and miR-15b-5p (degree = 13) were three hub

Figure 1. Differentially expressed lncRNAs, mRNAs and miRNAs in normal control, asthma and severe asthma (SA) (cont.). (A, C & E) were hierarchical clustering analysis of DElncRNAs, DEmRNAs and DEmiRNAs in normal control, asthma and severe asthma. Row represented DElncRNAs, DEmRNAs and DEmiRNAs. Column represented samples. The color scale represented the expression levels. (B, D & F) shown the venny analysis of DEmRNAs, DElncRNAs and DElncRNAs in severe asthma versus normal control (SA vs C), asthma versus normal control (A vs C) and severe asthma versus asthma (SA vs A). A:Asthma; C: Control; SA: Severe asthma.

miRNAs of this network. Moreover, a total of 34 shared DEmiRNA–DEmRNA interaction pairs were identified in both asthma and severe asthma compared with normal controls. MiR-122-5p (degree = 10) and miR-133a-3p (degree = 7) were two hub miRNAs of this shared DEmiRNA–DEmRNA interaction network (Supplementary Figure 1B).

Table 3. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of differentially expressed mRNAs.

KEGG ID	KEGG pathways	FDR	Genes
Shared DEmRNAs in both asthma and severe asthma			
Kegg:04666	Fc gamma R-mediated phagocytosis	0.013354	ARPC5, RAC1
Kegg:04810	Regulation of actin cytoskeleton	0.014753	ARPC5, MSN, RAC1
Kegg:05200	Pathways in cancer	0.013003	SPI1, TGFβ1, RAC1
Kegg:04670	Leukocyte transendothelial migration	0.017984	MSN, RAC1
Kegg:05211	Renal cell carcinoma	0.010012	TGFβ1, RAC1
Kegg:05210	Colorectal cancer	0.013772	TGFβ1, RAC1
Kegg:05212	Pancreatic cancer	0.010012	TGFβ1, RAC1
Kegg:04380	Osteoclast differentiation	0.006689	SPI1, TGFβ1, RAC1
Kegg:05100	Bacterial invasion of epithelial cells	0.010012	ARPC5, RAC1
Kegg:05131	Shigellosis	0.01778	ARPC5, RAC1
Severe-asthma-specific DEmRNAs			
KEGG ID	KEGG pathways	Hyp.c	Genes
Kegg:03040	Spliceosome	0.028807	SF3A1, SNRPE, SF3B4
DEmRNA: Differentially expressed mRNA; KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: False discovery rate.			

DEmiRNA–DElncRNA interaction network

A total of 1147, 601 and 890 DEmiRNA–DElncRNA interaction pairs were identified in asthma versus normal controls, severe asthma versus normal controls and severe asthma versus asthma. Based on these 890 DEmiRNA–DElncRNA interaction pairs in severe asthma versus asthma, the severe asthma-specific DEmiRNA–DElncRNA interaction network was constructed (Supplementary Figure 2A). AP000569.8 (degree = 68), C14orf64 (degree = 47) and AC010976.2 (degree = 46) were three hub lncRNAs of the severe asthma-specific DEmiRNA–DElncRNA interaction network. MiR-1827 (degree = 22), miR-491-5p (degree = 20) and miR-378g (degree = 19) were three hub miRNAs of this network. Moreover, a total of 25 shared DEmiRNA–DElncRNA interaction pairs were identified in both asthma and severe asthma compared with normal controls (Supplementary Figure 2B). MiR-3130-5p (degree = 6) and miR-122-5p (degree = 6) were two hub miRNAs of this shared DEmiRNA–DElncRNA interaction network. RP11-361M10.5 (degree = 5) was the hub lncRNA of this network.

DElncRNA–DEmRNA interaction network

A total of 64 DElncRNAs and 54 DEmRNAs were obtained from the severe asthma-specific DEmiRNA–DElncRNA and DEmiRNA–DEmRNA interaction network, respectively. Based on these 64 DElncRNAs and 54 DEmRNAs, two DElncRNA-nearby DEmRNA pairs (AC005237.4-*PASK* and CTD-2376I20.1-*EVL*) and 184 DElncRNA–DEmRNA coexpression pairs were identified (Supplementary Figure 3A).

A total of 17 DElncRNAs and 27 DEmRNAs were obtained from the shared DEmiRNA–DElncRNA and DEmiRNA–DEmRNA interaction network in both asthma and severe asthma, respectively. Based on these 17 DElncRNAs and 27 DEmRNAs, no DElncRNA-nearby DEmRNA pairs were identified while 13 DElncRNA–DEmRNA coexpression pairs were identified (Supplementary Figure 3B).

Shared DEmiRNA–DEmRNA–DElncRNA interaction network in both asthma & severe asthma

The shared DEmiRNA–DEmRNA–DElncRNA interaction network in both asthma and severe asthma was consisted of ten DEmiRNAs, 17 DElncRNAs and 27 DEmRNAs (Figure 2A). MiR-133a-3p (degree = 8), miR-517a-3p (degree = 4) and miR-517b-3p (degree = 4) were three hub miRNAs. Hierarchical clustering analysis of these ten DEmiRNAs, 17 DElncRNAs and 27 DEmRNAs were displayed in Figure 2B. Subnetwork of miR-133a-3p and expression of miR-133a-3p-*EFHD2/CNN2*-AC144831.1 interactions in normal control, asthma and severe asthma were displayed in Figure 4A & B.

Based on the functional annotation of these 27 DEmRNAs derived from shared DEmiRNA–DEmRNA–DElncRNA interaction network, cytoskeleton organization (false discovery rate [FDR] = 1.59E-02), inflammatory response (FDR = 1.93E-02), protein binding (FDR = 1.15E-05) and nucleus (FDR = 9.57E-04) were significantly enriched GO terms (Supplementary Table 2). Based on the KEGG enrichment analysis, Fc gamma R-mediated phagocytosis (FDR = 0.0133538) was a significantly enriched pathways (Table 3).

Figure 2. Shared differentially expressed miRNA–mRNA–lncRNA interaction network in both asthma and severe asthma. **(A)** Shared DEMiRNA–DEmRNA–DElncRNA interaction network in both asthma and severe asthma. The rectangles, ellipses and rhombuses represented DEMiRNAs, DEmRNAs and DElncRNAs, respectively. Red and green color represented up and downregulation, respectively. **(B)** Hierarchical clustering analysis of DElncRNAs, DEmRNAs and DEMiRNAs derived from this network in normal control, asthma and severe asthma. Row represented DElncRNAs, DEmRNAs and DEMiRNAs. Column represented samples.

Severe asthma-specific DEMiRNA–DEmRNA–DElncRNA interaction network

The severe asthma-specific DEMiRNA–DEmRNA–DElncRNA interaction network was consisted of 196 DEMiRNAs, 139 DElncRNAs and 95 DEmRNAs (Figure 3A). MiR-3613a-3p (degree = 31), miR-1827 (degree = 30) and miR-93-5p (degree = 28) and miR-15b-5p (degree = 28) were four hub miRNAs. Hierarchical clustering analysis of these 196 DEMiRNAs, 139 DElncRNAs and 95 DEmRNAs were displayed in Figure 3B. Subnetwork of miR-3613-3p and miR-93-5p and expression of miR-3613-3p-*CD44*/*BCL11B*-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions and miR-93-5p-*BCL11B*-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions in normal control, asthma and severe asthma were displayed in Figure 4C–F.

Based on the functional annotation, regulation of transcription, DNA-dependent (FDR = 0.0020768), metal ion binding (FDR = 0.0322671), nucleus (FDR = 0.000048193) were significantly enriched GO terms (Supplementary Table 3). Based on the KEGG enrichment analysis, Spliceosome (FDR = 0.028807) was the only one significantly enriched pathway (Table 3).

Validation in GEO dataset

The expression patterns of selected DEmRNAs (*SNRPE*, *SF3B4*, *RAC1*, *PABPC1*, *C12orf55*, *BCL11B* and *CD44*) were verified using GSE69683 dataset. The dataset GSE69683 consisted of 334 patients with severe asthma and 87 normal controls, which were all from the USA. The dataset GSE69683 examined the blood sample. As shown in Figure 5, *BCL11B* was downregulated and *CD44* was upregulated, which was inconsistent with

Figure 2. Shared differentially expressed miRNA–mRNA–lncRNA interaction network in both asthma and severe asthma (cont.). (A) Shared DEMiRNA–DEmRNA–DElncRNA interaction network in both asthma and severe asthma. The rectangles, ellipses and rhombuses represented DEMiRNAs, DEMRNAs and DElncRNAs, respectively. Red and green color represented up and downregulation, respectively. **(B)** Hierarchical clustering analysis of DElncRNAs, DEMRNAs and DEMiRNAs derived from this network in normal control, asthma and severe asthma. Row represented DElncRNAs, DEMRNAs and DEMiRNAs. Column represented samples.

our RNA-sequencing results. But for most of the selected DEMRNAs, the expression was consistent with our RNA-sequencing results, such as *SF3B4*, *RAC1*, *PABPC1*, *SNRPE* and *C12orf55*. The *SF3B4*, *RAC1* and *PABPC1* were downregulated, while *SNRPE* and *C12orf55* were upregulated in severe asthma suggesting that the results of RNA-sequencing were convincing.

Discussion

Severe asthma is a complex disease affecting millions of individuals worldwide, and is increasingly recognized as a major unmet need [7]. Although severe asthma accounts for only a minority of patients with asthma, it contributes

Figure 3. Severe asthma-specific differentially expressed miRNA–mRNA–lncRNA interaction network in both asthma and severe asthma. (A) Severe asthma-specific DEmiRNA–DEmRNA–DElncRNA interaction network in both asthma and severe asthma. The rectangles, ellipses and rhombuses represented DEmiRNAs, DEmRNAs and DElncRNAs, respectively. Red and green color represented up and downregulation, respectively. (B) Hierarchical clustering analysis of DElncRNAs, DEmRNAs and DEmiRNAs derived from this network in normal control, asthma and severe asthma. Row represented DElncRNAs, DEmRNAs and DEmiRNAs. Column represented samples. DElncRNA: Differentially expressed lncRNA; DEmRNA: Differentially expressed mRNA; DEmiRNA: Differentially expressed miRNA.

Figure 4. Severe asthma-specific and shared DEmiRNA-DEmRNA-DElncRNA interactions. (A, C & E) were subnetworks of miR-133a-3p, miR-3613-3p and miR-93-5p. The rectangles, ellipses and rhombuses represented DEmiRNAs, DEmRNAs and DElncRNAs, respectively. Red and green color represented up and downregulation, respectively. (B, D & F) were expression of miR-133a-3p-EFHD2/CNN2-AC144831.1, miR-3613-3p-CD44/ BCL11B-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 and miR-93-5p- BCL11B-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions in normal control, asthma and severe asthma. DElncRNA: Differentially expressed lncRNA; DEmRNA: Differentially expressed mRNA; DEmiRNA: Differentially expressed miRNA.

Figure 5. Validation of selected differentially expressed mRNAs in GSE69683. The x-axis shows healthy normal control (blue colour) and severe asthma (red colour) groups and y-axis shows a log2 transformation to the intensities.

to the majority of the morbidity, mortality and economic costs of the disease. However, the molecular mechanism of severe asthma remains unclear.

To better understanding the pathogenesis and developing potential biomarkers of asthma and severe asthma, this present study utilized next-generation sequencing to provide a quantitative and comprehensive analysis of the

coding and noncoding transcriptome in asthma and severe asthma. These analyses revealed significant differences in the patterns of miRNA, lncRNA and mRNA expression in asthma and severe asthma, as well as the dynamic changes of DEMiRNAs, DELncRNAs and DEMRNAs. Generally, our data suggest that distinct populations of miRNAs, lncRNAs and mRNAs are involved in the pathogenesis of asthma and severe asthma. Recent years, ncRNAs have been found to be associated with wide range of biological regulatory functions [15]. However, most of the DELncRNAs obtained in this study were with unknown function, which is mainly due to the few researches for them. Hence, shared and severe asthma-specific DEMiRNA–DEmRNA–DELncRNA interaction networks were constructed. In these networks, DEMRNAs were coexpressed with DELncRNAs, and both DEMRNAs and DELncRNAs were targets of DEMiRNAs.

A total of 13 shared DEMiRNAs, 217 shared DELncRNAs and 487 shared DEMRNAs were identified in both asthma and severe asthma compared with normal controls. The shared and severe asthma-specific DEMiRNA–DEmRNA–DELncRNA interaction network in both asthma and severe asthma were consisted of ten DEMiRNAs, 17 DELncRNAs and 27 DEMRNAs. MiR-133a-3p-EFHD2/CNN2-AC144831.1 interactions involve with both asthma and severe asthma. Upregulated miR-133a-3p and its downregulated targets including EFHD2, CNN2 and lncRNA, AC144831.1 were first found in both asthma and severe asthma compared with normal controls.

It is reported that miR-133a-3p might be regulated by IL-17 [16]. The IL-17-producing T cells has key role in asthma, which was upregulated in both patients with asthma [17,18] and animal models of asthma [19]. The IL-17A response may be variable during the development of allergic airway inflammation in asthma animal models and be affected by experimental conditions, such as sensitization procedure, dosing pathways, antigenic doses, sampling time, etc [20–23]. In fact, a clinical trial demonstrated the high-dose and low-responder subpopulations in a therapeutic effect of brodalumab, a human anti-IL17RA monoclonal antibody, which inhibits the biologic activity of IL-17A, in patients with inadequately controlled moderate to severe asthma [24]. Chiba *et al.* reported that IL-17A upregulates the RhoA protein through downregulation of miR-133a-3p, which is one of the causes of asthmatics [16]. Therefore, we presumed that IL-17 might involve in the development of asthma by regulating miR-133a-3p. Although, no previous study reported that the association between EF-hand domain family member D2 (*EFHD2*) and asthma, *EFHD2* and *CNN2* shared with the same binding miRNA, miR-133a-3p which suggested that *EFHD2* might play a role in the process of both asthma and severe asthma by interacting with miR-133a-3p-*CNN2*. AC144831.1 was a target of miR-133a-3p that coexpressed with both *EFHD2* and *CNN2* which suggested that miR-133a-3p-*EFHD2/CNN2*-AC144831.1 interactions play an important role in the occurrence and development of asthma and severe asthma.

To better research the mechanism of severe asthma, we constructed the severe asthma-specific DEMiRNA–DEmRNA–DELncRNA interaction network. The expression of miR-3613-3p-*CD44/BCL11B* interaction was significantly altered in severe asthma compared with asthma. The *CD44* is a major hyaluronan-binding protein and decreased on alveolar macrophages was reported to involve with deranged hyaluronan homeostasis in asthma [25]. In addition, accumulated evidence showed that toll-like receptor (TLR) signaling plays an important role in innate immunity of asthma and *CD44* serves as a negative regulator of TLR signaling [17,18,26]. Decreased expression of *CD44* could induce impaired hyaluronan clearance and upregulate TLR2 and TLR4 on macrophages which resulted in persistent inflammation [25]. In this present study, *CD44* was significantly downregulated in severe asthma compared with asthma which provided evidence for previous studies and highlighted the importance of *CD44* in severe asthma.

BCL11B is expressed in thymocytes which are critical for Type-2 T-helper (Th2) lineage licensing and the development of T-regulatory cells [27]. Th2 cells are formed following the activation of naive CD4⁺ T cells in the presence of IL-4, and are critical in allergic diseases such as asthma [19]. Downregulated *BCL11B* was found in severe asthma compared with asthma.

Correspondingly, miR-3613-3p was significantly upregulated in severe asthma compared with asthma which suggested that miR-3613-3p plays a role in severe asthma by regulating both *CD44* and *BCL11B*. Four targets (LINC00158, CTA-217C2.1, AC010976.2 and RP11-641A6.2) of miR-3613-3p that coexpressed with both *CD44* and *BCL11B* were first found to be aberrant expressed between severe asthma and asthma. Hence, we speculated that miR-3613-3p-*CD44/BCL11B*-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions contributed to the development of asthma to severe asthma.

In addition, *BCL11B* and the above four lncRNAs (LINC00158, CTA-217C2.1, AC010976.2 and RP11-641A6.2) were shared targets of another severe asthma-specific miRNA, miR-93-5p. Downregulated miR-93 in the CD3(+) IL-22(+) compared with CD3(+)IL-22(-) T cells which was speculated to impact the T-cell

responses in asthma [28]. These finding emphasized the importance of miR-93-5p-*BCL11B*-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions in the development of severe asthma.

Function annotation of DEmRNAs derived from the shared DEmiRNA–DEmRNA–DElncRNA interaction network in both asthma and severe asthma indicated that Fc γ R-mediated phagocytosis involve with asthma and development of severe asthma. Spliceosome was the only one enriched pathway according to function annotation of DEmRNAs derived from severe asthma-specific DEmiRNA–DEmRNA–DElncRNA interaction network, which suggested that spliceosome and three spliceosome-related genes (*SF3A1*, *SNRPE* and *SF3B4*) involve with the development of severe asthma.

In conclusion, we identified the shared and diverging genes, lncRNAs, miRNAs and signaling pathways in asthma and severe asthma. 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 severe asthma.

Limitation

There is a limitation of our study. The small sample size (three sample per group) for RNA-sequencing might influence the quality of our results. Although the validation based on GSE69683 suggested that our RNA-sequencing results were generally convincing, studies with larger sample size need to be conducted to confirm this conclusion. Moreover, further experiment was needed to solve the biological significances of important mRNAs, lncRNAs and miRNAs in severe asthma.

Future perspective

Our study determined the DEmRNA, DEmiRNA and DElncRNA expression data from normal controls, asthma and severe asthma by using RNA sequencing. MiR-133a-3p-*EFHD2/CNN2-AC144831.1* interactions might play an important role in both asthma and severe asthma. MiR-3613-3p-*CD44/BCL11B*-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions and miR-93-5p-*BCL11B*-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2 interactions were speculated to involve with the development of asthma to severe asthma. This study provides clues for understanding the mechanisms of severe asthma and developing novel therapeutic strategies for severe asthma. In the future, we plan to first validate the expression of the identified key mRNAs, lncRNAs and miRNAs in a larger-scale study, and further investigate the biological significances of important mRNAs, lncRNAs and miRNAs in model systems or cell lines. These experiments may contribute to understanding the pathological mechanism of severe asthma and finding potential drug targets for severe asthma therapy.

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Ethical disclosure

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Summary points

- Compared with normal control, 962 differentially expressed mRNAs (DEmRNAs), 545 differentially expressed long noncoding RNAs (DElncRNAs) and 53 differentially expressed miRNAs (DEmiRNAs) were identified in asthma; 1328 DEmRNAs, 608 DElncRNAs and 63 DEmiRNAs were identified in severe asthma.
- A total of 95 DEmRNAs, 143 DElncRNAs and 96 DEmiRNAs were identified between asthma and severe asthma.
- Fc γ R-mediated phagocytosis and spliceosome were significantly enriched pathways for DEmRNAs derived from shared and severe asthma-specific DEmiRNA–DEmRNA–DElncRNA interaction network, respectively.
- MiR-133a-3p-*EFHD2/CNN2-AC144831.1* interactions might play an important role in both asthma and severe asthma.
- MiR-3613-3p-*CD44/BCL11B-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2* interactions and miR-93-5p-*BCL11B-LINC00158/CTA-217C2.1/AC010976.2/RP11-641A6.2* interactions were speculated to involve with the development of asthma to severe asthma.

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