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Identification of hub genes in airway epithelial cells of asthma patients by WGCNA and PPI network

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Bronchial asthma is a common chronic disease of airway inflammation, high mucus secretion and airway hyper responsiveness. The pathogenetic mechanisms of asthma remain unclear. In this study, we aimed at identifying genes playing an import role in disease-related pathways in airway epithelial cells of asthma patients. Microarray data GSE41861 of asthma airway epithelial cells was used to screen differentially expressed genes (DEGs) through GEO2R analysis. The weighted gene co-expression network analysis (WGCNA) was performed to identify gene co-expression network modules in bronchial asthma. The DAVID database was then used to perform functional and pathway enrichment analysis of these DEGs. In addition, we have conducted protein-protein interaction (PPI) network of DEGs by STRING, and eventually found key genes and significant modules. A total of 315 DEGs (111 up-regulated and 204 down-regulated) were identified between severe asthma and healthy individual, which were mainly involved in pathways of cilium assembly, cilium morphogenesis, axon guidance, positive regulation of fat cell differentiation, and positive regulation of cell substrate adhesion. A total of 60 genes in the black module and green module were considered to be correlated with the severity of asthma. Combining PPI network, several key genes were identified, such as BP2RY14, PTGS1, SLC18A2, SIGLEC6, RGS13, CPA3, and HPGDS. Our findings revealed several genes that may be involved in the process of development of bronchial asthma and potentially be candidate targets for diagnosis or therapy of bronchial asthma.

Keywords: Biomarkers, Differentially expressed genes, GEO, Protein-protein interaction, Weighted gene co-expression network analysis

Bronchial asthma (referred as asthma) is one of the most prevalent chronic diseases in the world, characterized by airway inflammation, high mucus secretion and airway hyper responsiveness. The onset of asthma is mainly caused by complex genetic and environmental factors[1]. At present, the main treatment of asthma includes anti-inflammatory, bronchial spasm and relaxation of smooth muscle. Although the above methods can effectively alleviate asthma symptoms, most of the asthma patients still suffer residual symptoms and get poor life quality after treatments due to side effects caused by contraindications and post-treatments[2]. Growing evidence revealed that some key genes associated with asthma may function in the development of asthma[3,4]. Studies have shown that CD4+ T cell subset imbalance may be the main reason for occurrences of asthma[5]. Furthermore, helper T cell (Th) 2 cell differentiation disorders and methylation of gene expression in nasal epithelial cells of patients with allergic asthma have a close relationship with the pathogenesis of asthma[6]. However, the associations between DEGs and the pathogenesis of asthma remains were still unclear. Therefore, it is necessary to explore DEGs in asthma patients, which may shed light on finding novel targets for asthma. With the advances of microarrays and high-throughput sequencing technologies, thousands of genes in the human genome were found, offering more molecular level information on asthma[7-9].

Airway epithelial cells are the first line barrier of the airway. Increasing studies have shown that airway epithelial cells in asthma patients became dysfunctional, which facilitates the formation of asthma[10,11]. The severity of dysfunction of the airway epithelial barrier is associated with the degree of an acute bronchial asthma attack. Airway epithelial cells are involved in bronchial airway inflammation and

Abbreviations: WGCNA, weighted gene co-expression network analysis; PPI, protein-protein interaction; GEO, Gene Expression Omnibus; DEGs, differential expression genes; FC, fold change; GO, Gene Ontology; MF, molecular function; BP, biological process; CC, cellular component; KEGG, Kyoto Encyclopedia of Genes and Genomes; ME, module eigengene; GS, Gene significance; MM, Module membership; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins

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airway remodeling by strengthening proinflammatory activity by releasing growth factors[12,13]. Thus, identifying DEGs in the airway epithelium of asthma patients could help in diagnosing asthma and finding new treatment strategies.

WGCNA is a commonly used method in co-expression module correlation analysis widely applied in various investigations, especially for the identification of candidate biomarkers or therapeutic targets for a variety of diseases[14]. Herein, we analyzed the microarray data of airway epithelial gene expression profile in severe asthma patients and constructed a co-expression network through WGCNA, which was applied to search the hub genes regulating the progression of asthmatic diseases in bronchial asthma airway epithelial cells.

Materials and Methods

Data collection and preprocessing

The gene expression profile dataset of asthma was obtained from the Gene Expression Omnibus (GEO) database (https: //www.ncbi.nlm.nih.gov/geo/) with accession number GSE41861. 138 bronchial epithelial brushing samples were included for further study, including 44 mild asthma samples, 37 moderate asthma samples, 10 severe asthma samples, and 47 matched healthy tissue samples Robust multi-array average (RMA) expression measurements were used to normalize the data in the Bioconductor package (http://www.bioconductor.org/). The probes were reannotated with the latest annotation provided in the limma package in the Bioconductor package.

Identification of differentially expressed genes

Differentially expressed genes were compared between cases of severe asthma samples and healthy samples using the Bioconductor package (http:// www.bioconductor.org/). If there were multiple probes corresponded to the same gene, their average expression level was considered as the gene expression value. One probe corresponded to multiple genes was omitted. Differentially expressed gene were defined by *P*-value <0.05 and log |fold change (FC)| >0.585.

GO and KEGG pathway enrichment analyses

To characterize the biological function of the genes identified in asthma, Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis were performed based on Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david.ncifcrf.gov/)[15]. *P*-value <0.05 was considered statistically significant. The results were visualized using ggplot2 package of R software[16].

Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) as a means to measure the correlation between traits and gene modules, which was performed by WGCNA package[17]. The power values were filtered, which could mainly affect the scale independence and the mean connectivity degree of gene co-expression modules (from 1 to 20). When the scale independent value was equal to 7, the appropriate power value was determined. After that, the cluster analysis was performed at the appropriate threshold value.

Module-trait relationship analysis

The relationships among different co-expression modules were analyzed. The strength of the relationship (strong or weak degree) was performed using a heatmap package (http://www.bioconductor.org /packages/release/bioc/html/heatmaps.html). To evaluate the significance of modules, module-trait relationships were determined via the correlation between the module eigengene (ME) and clinical traits. The clinical traits include severity, sex, age, and diseases.

PPI network construction

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; http://string-db.org/) was used to explore the interactions between proteins encoded by genes in asthma related modules[18]. PPI network was visualized by Cytoscape. After that, the CytoNCA software was used for the nodes topological analysis. The color of nodes represents log (Fold Change) of obtained proteins and the number of the connections is defined as the degree of target proteins in the PPI.

Results

Identification of differentially expressed genes in asthma

Raw expression profiles of asthma patients were downloaded from the GEO database. There were 10tissue samples from case of severe asthma compared to 47 matched healthy control samples. As a result, a total of 315 DEGs in severe asthma were identified, consisting of 111 up-regulated DEGs and 204 down-regulated DEGs (Fig. 1).

Functional enrichment analysis of DEGs in asthma

In explore the biological of aberrantly expressed genes in severe asthma, GO term and KEGG pathway enrichment analyses of 315 DEGs were performed.

The top 10 terms of BP category were “cilium assembly”, “cilium morphogenesis”, “axon guidance”, “positive regulation of fat cell differentiation”, “positive regulation of cell substrate adhesion”, “negative regulation of keratinocyte proliferation”, “negative regulation of Wnt signaling pathway”, “multicellular organism development”, “cellular response to calcium ion”, and “cell migration involved in sprouting angiogenesis”. “Extracellular space”, “proteinaceous extracellular matrix”, and “axoneme”, “cilium”, “ciliary transition zone”, “extracellular space”, “neuron projection” were the most significant enrichment in CC. The significant MF terms were “calcium ion binding”, “heparin binding”, “microtubule motor activity”, and “microtubule binding” (Fig. 2). Furthermore, the significant pathways identified by KEGG pathway analysis consist of other types of O glycan biosynthesis, mineral adsorption, and serotonergic synapse (Fig. 2).

Analysis of WGCNA and identification of key modules

To construct the gene co-expression network, 4144 genes with $P< 0.05$ were used for cluster analysis with the fashClust function of the WGCNA package. The cluster analysis of 138 samples was performed and the outlier samples were removed (Fig. 3A). The co-expression module was identified by hierarchical clustering and dynamic branch cutting (Fig. 3B). The results showed that each module is independent of each other, which proves the high independence among modules. Furthermore, the black module (r= 2e-08) and green module (r= 4e-08) were highly associated with the severity of asthma (Fig. 3C). Furthermore, both of the two modules were also positively related to the disease process, indicating that the genes in the two modules may be involved in the mechanisms of asthma. Therefore, we considered the black module and green module as the candidate modules of asthma for further analysis.

As shown in Figure 4A, 138 samples were divided into two clusters on the whole. When R^2 was greater than 0.9 for the first time, the soft-thresholding was selected to calculate the co-expression module (Fig. 4B). Finally, 11 co-expression modules were identified (Fig. 4C). Genes in the 11 co-expression modules are listed in (Table 1).

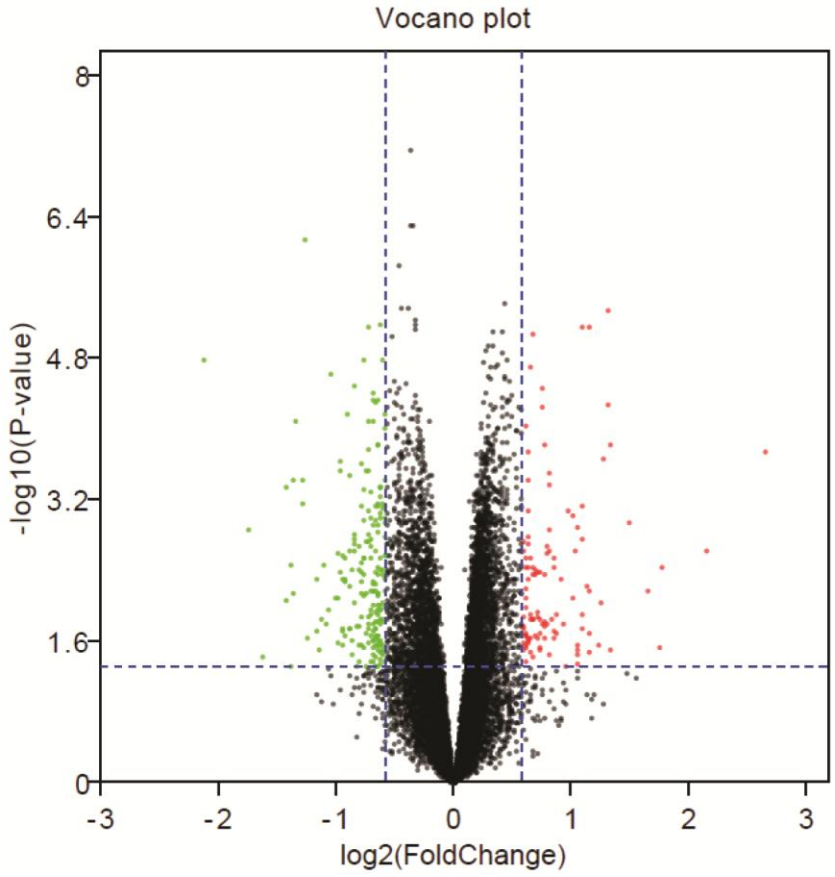

Fig. 1 — The volcano plot of the DEGs in severe asthma. Red: up-regulated genes and green: down-regulated genes

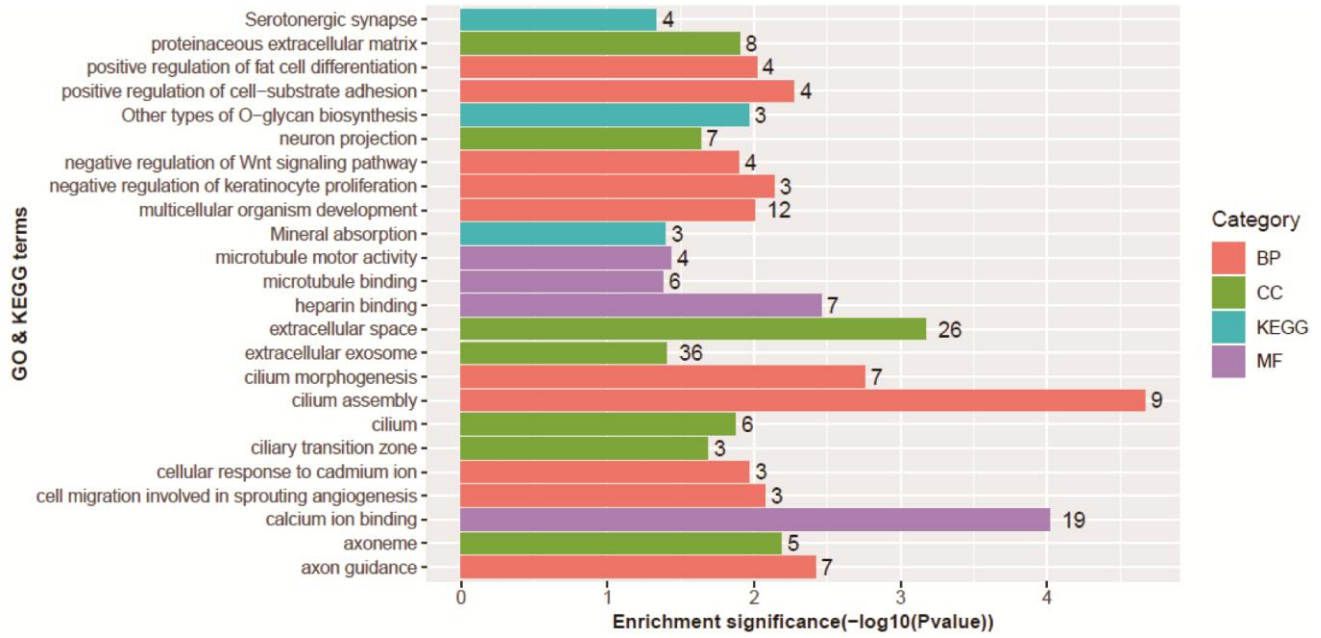

Fig. 2 — Functional enrichment analysis for DEGs in severe asthma, including GO and KEGG analysis. The y-axis suggests the name of functions. Fold line indicates the -log10 (*P*-value)

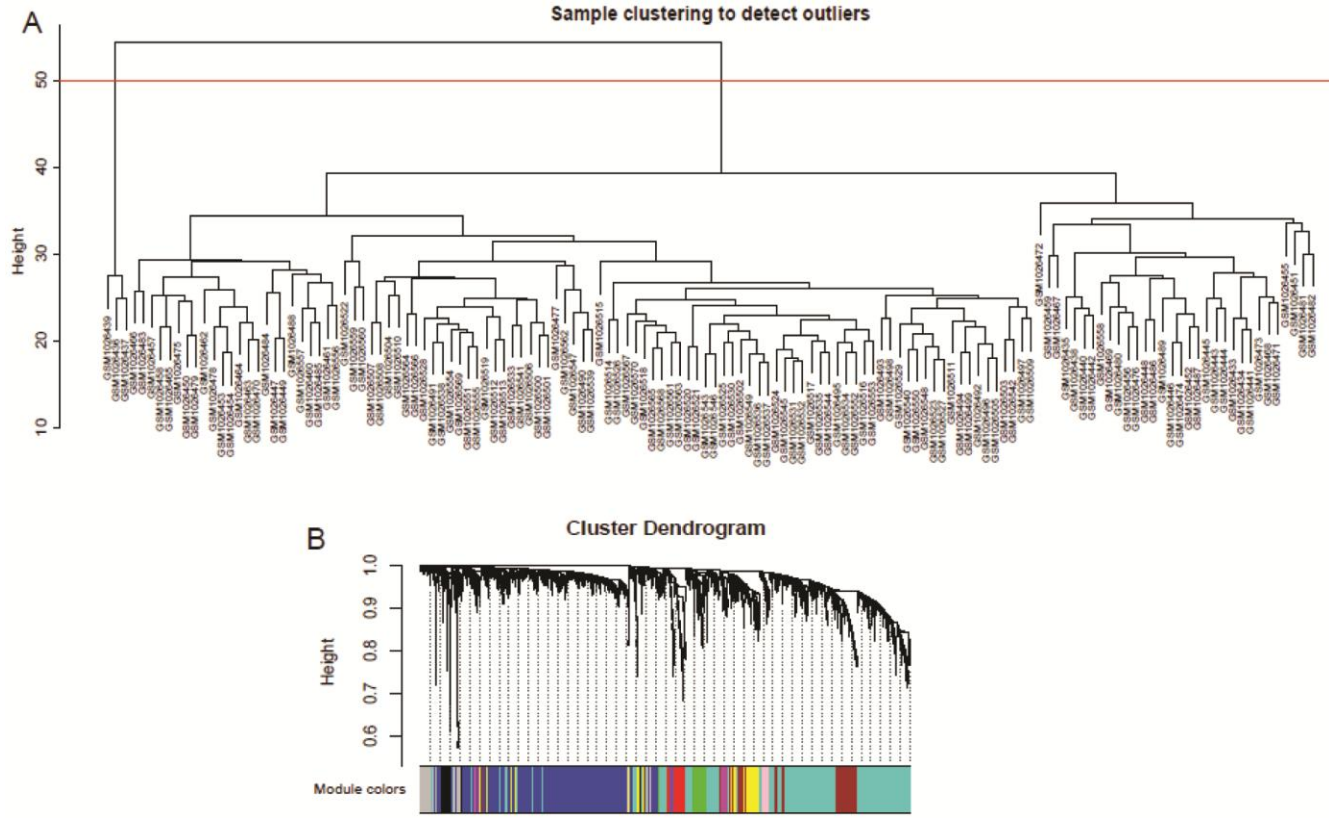

Fig. 3 — The most significant module screened by WGCNA (A) The sample dendrogram and trait heatmap based on the expression data of GSE41861; (B) Calculation chart of adjacency matrix weighting parameters (power). The most fit power value is seven; and (C) The correlation between modules and clinical traits. The clinical traits include disease, sex, age and severity. The corresponding correlations and *P*-values were presented. Both black and green module were positively correlated with severity and disease

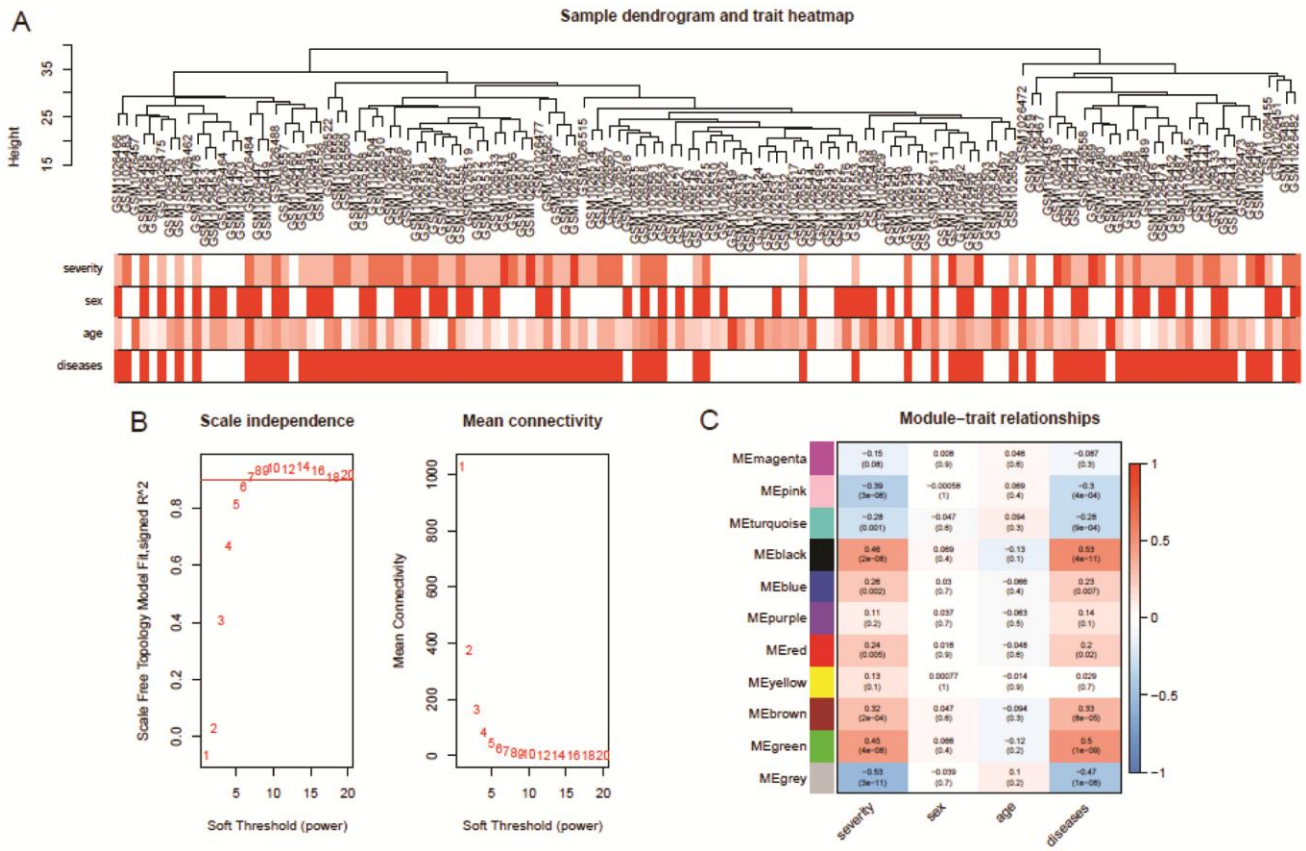

Fig. 4 — Clustering dendrograms of asthma (A) The sample clustering tree; and (B) The cluster dendrogram of genes in GSE41861. Each branch indicates one single gene, and each color indicates one single co-expression module

Table 1 — Gene details in the co-expression modules.

Module	Number of genes
black	223
blue	1314
brown	371
green	142
grey	165
magenta	91
pink	96
purple	48
red	124
turquoise	1354
yellow	333

Identification of hub genes in the black module and green module

Using WCGNA package, the gene co-expression network was established. After that, the black and green modules were visualized by Cytoscape. Furthermore, we screened out the top 30 genes for intra-module connectivity by sequencing candidate genes between nodes. Figure 5A and B showed the top 30 hub genes in the black module and green module. We chose the bigger nodes as hub genes, including P2RY14, PTGS1, SLC18A2, SIGLEC6, RGS13, CPA3 and HPGDS in black module, and MRPL42, OCIAD1, VDAC3, RTN4IP1, PDE6D, C11orf71 in green module.

Pathway enrichment analysis of genes in two key modules

To explore the biological features of gens in the two modules, the KEGG pathway enrichment analysis was performed (Table 2 and Fig. 6A & B). The genes in the black module were enriched in pathways of metabolism of biological compounds such as hematopoietic cell lineage, arachidonic acid metabolism, and salivary secretion. In the green module, the genes were mainly enriched in several pathways like signaling pathways in cancer, bacterial invasion of epithelial cells, transcriptional misregulation in cancer, regulation of actin cytoskeleton, and Rap1 pathway.

PPI network construction

To order to gain insights into the potential interactions among proteins encoded by genes in asthma related modules, the PPI network was constructed using STRING. The top 30 genes in the black and green module were screened for analysis of protein interactions. In the black module, 8 main

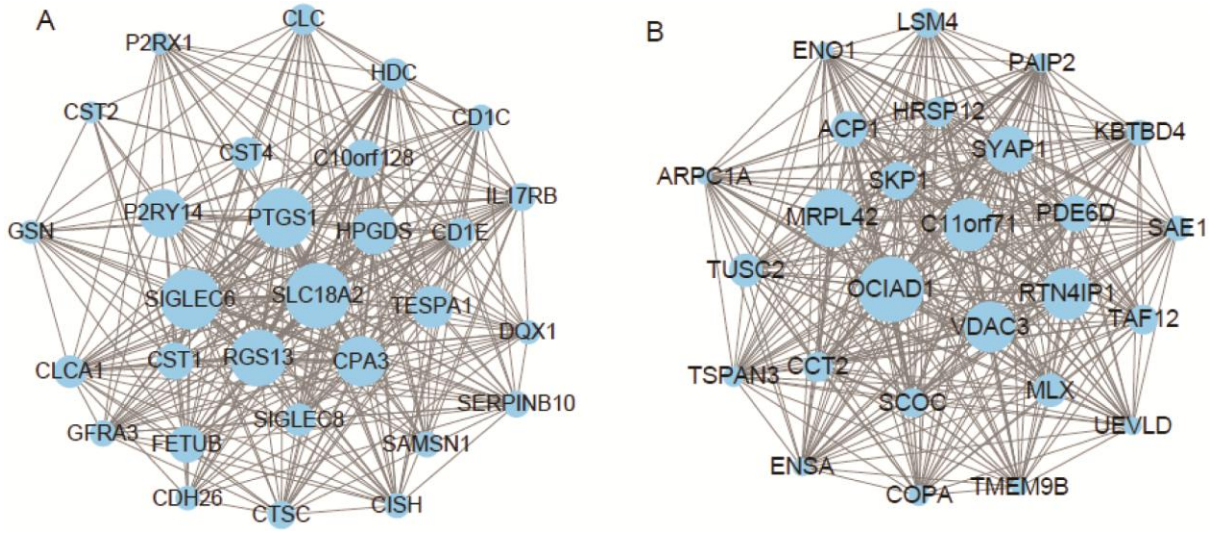

Fig. 5 — .Co-expression network of top 30 hub genes in the black module and green module (A) Co-expression network of the top 30 hub genes in black module; and (B) Co-expression network of the top 30 hub genes in green module. Nodes represent hub genes and lines represent weight. The bigger the node, the stronger the intra-modular connectivity

Table 2 — KEGG pathway enrichment analysis of black module and green module

Category	Term	Count	%	P Value
Black module				
KEGG	hsa04640:Hematopoietic cell lineage	5	5.050505051	0.001146584
KEGG	hsa00590:Arachidonic acid metabolism	3	3.03030303	0.042238213
KEGG	hsa04970:Salivary secretion	3	3.03030303	0.07764556
Green module				
KEGG	hsa05100:Bacterial invasion of epithelial cells	7	4.964539007	4.01E-05
KEGG	hsa05130:Pathogenic Escherichia coli infection	4	2.836879433	0.00847848
KEGG	hsa05202:Transcriptional misregulation in cancer	6	4.255319149	0.012023357
KEGG	hsa05131:Shigellosis	4	2.836879433	0.015728246
KEGG	hsa04810:Regulation of actin cytoskeleton	6	4.255319149	0.029294385
KEGG	hsa04015:Rap1 signaling pathway	6	4.255319149	0.029294385
KEGG	hsa04666:Fc gamma R-mediated phagocytosis	4	2.836879433	0.032042962
KEGG	hsa04350:TGF-beta signaling pathway	4	2.836879433	0.032042962
KEGG	hsa05200:Pathways in cancer	8	5.673758865	0.042341311
KEGG	hsa05016:Huntington's disease	5	3.546099291	0.074018393
KEGG	hsa04110:Cell cycle	4	2.836879433	0.082964999
KEGG	hsa01130:Biosynthesis of antibiotics	5	3.546099291	0.097959728

up-regulated nodes including CST1, CPA3, POSTN, CLCA1, ITLN1, CCL26, P2RY14, and SERPINB 10 and 4 down-regulated nodes (C3, PCDH17, STEAP4, and TMEM150C) were identified in the PPI network (Fig. 6C). The nodes with the highest score were considered as hub genes, such as ATCB, ENO1, RHOA, C1orf43, and 4 down-regulated genes including ITPKB, KANSL1, SCAF4, TRAPPC10 in green modules (Fig. 6D).

Discussion

Asthma originates from the interaction between genetic and environmental factors. Although most asthma patients could achieve relieved symptoms through prolonged inhalation of corticosteroids, some patients with severe asthma may develop resistance to long-term hormone maintenance therapy[19]. Microarrays and high-throughput sequencing have been widely applied to predict potential therapeutic targets for asthma[20].

In this study, the data from GSE41861 were used to analyze DEGs in asthmatic individuals. The results showed that there were plenty of DEGs between asthmatic patients and healthy individuals, including 111 up-regulated and 204 down-regulated genes. To better understand the function of DEGs, GO and

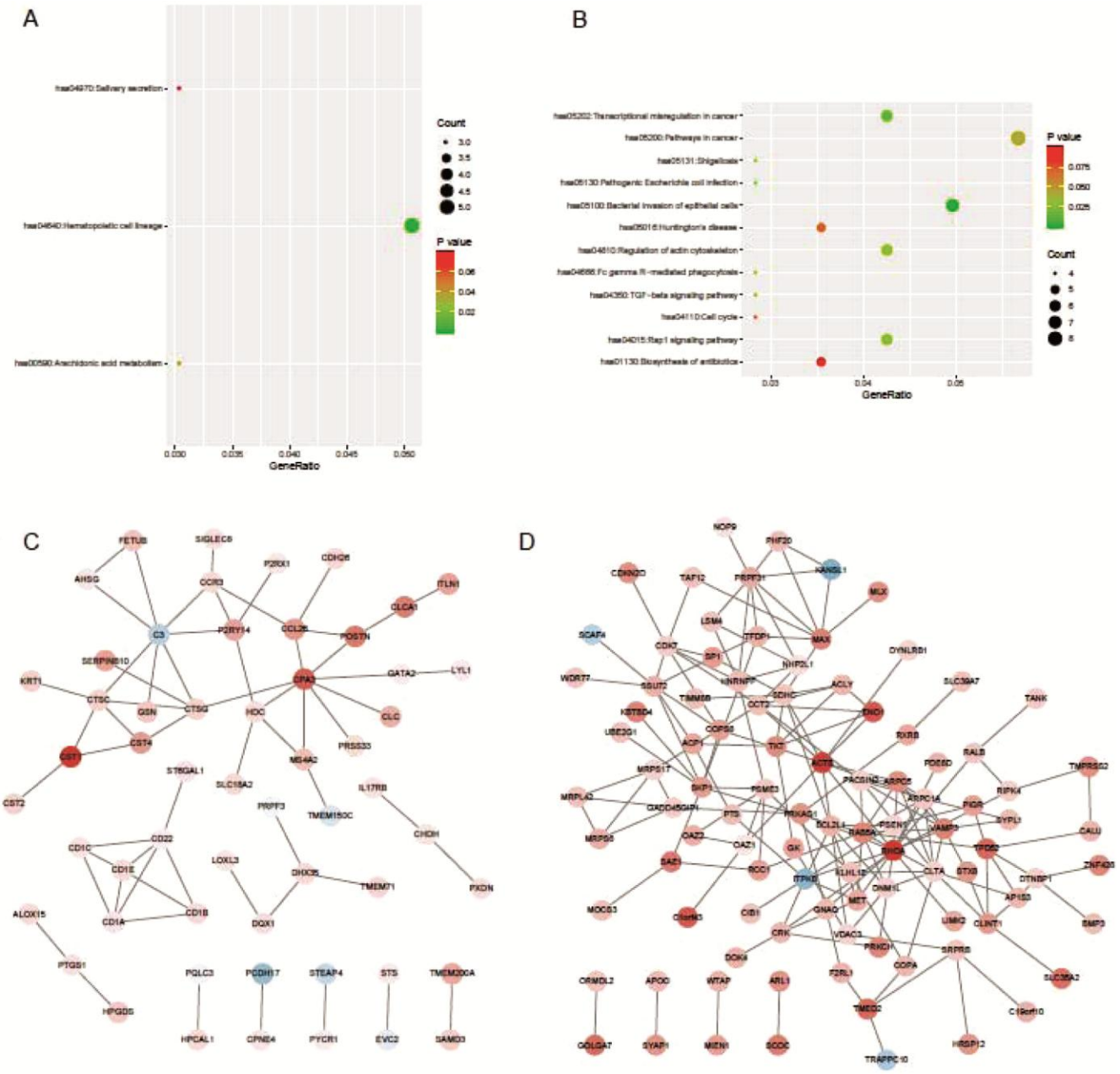

Fig. 6 — KEGG analysis and PPI network of genes in black and green modules (A & B) KEGG pathway enrichment analysis of genes in black module and blue module. The bigger the node size, the bigger the count; Red color means bigger *P* value and blue color means smaller *P* value; and (C & D) PPI network of top 30 hub genes in the black module and green module. Red represents up-regulated genes and green represents down-regulated genes. The darker the color, the more significant the difference

KEGG analyses were further performed. The results showed that these DEGs were mainly enriched in several signal pathways such as calcium ion binding, heparin binding, microtubule motor activity, and microtubule binding. Voltage-gated Ca^{2+} channels (VGCCs) play a classical role in asthma, which are activated after depolarization of the cell membrane, triggering calcium influx, and airway smooth muscle contraction[21]. Therefore, a large number of early asthma mechanism studies focused on this pathway[22,23]. The traditional asthma relief drug, the crude extract of Magnolia Sinensis, promotes bronchial smooth muscle relaxation by blocking VGCCs and phosphodiesterase[24]. It was found that potassium channels can change membrane potential and eventually activate VGCCs; therefore, potassium

channel has gradually become a new research hotspot[25]. Damage and repair of airway epithelial cells is a typical pathological feature of airway remodeling. Epithelial tissue changes in asthma patients mainly include epithelial cell shedding, injury, cilia reduction, cell-to-cell adhesion, and goblet cell proliferation[26]. Related literature indicates that airway epithelial cells in patients with severe asthma have significantly abnormal proliferation compared with normal and mild asthma patients, leading to airway remodeling and a sharp decline in lung function[27]. Due to various internal and external injury factors, the barrier function of the airway epithelium, adhesion, and repair of epithelial cells are impaired. In addition, various inflammatory factors and cytokines are released and participate in the inflammatory responses[28].

Asthma is a complex inflammatory disease, characterized by airway inflammation and remodeling. The airway of asthma patients is in a chronic inflammatory state, and chronic inflammatory stimulates airway remodeling, forming a vicious circle[29]. Consistent with the results of this study, the signaling pathways involved in DEGs were concentrated in microtubule motion, followed by calcium ion binding. Detection of these signaling pathways will help to understand the development of asthma.

WGCNA has been used to screen for novel biomarkers[30,31]. In the co-expression networks, genes with similar expression patterns are clustered together in the same module, and these genes may have similar regulatory functions[14]. Therefore, in our study, we constructed a gene co-expression network for asthma. To explore the genetic mechanisms behind clinical features, the relationship between modules and clinical features was analyzed. The association between clinical features and gene module is helpful to understand the pathogenesis of asthma and screen potential biomarkers. The results showed that the black module and green module were highly associated with the severity of asthma. The two modules were positively correlated with the disease process, suggesting that the genes in the two modules may play an important part in asthma. Therefore, the two modules were considered as the most relevant modules to asthma. After that, the genes in the black module and green module were used to construct the PPI network. The hub genes were identified through the PPI network, among them, human OCIAD1 was considered as a hub gene with the highest degree of connectivity. It has been reported that the OCIAD1 (ovarian cancer immune response antigen domain protein 1) gene encodes a type 1 transmembrane protein that is highly conserved during evolution[32]. Studies have reported that OCIAD1 expression in ovarian cancer and thyroid cancer affects integrin function[33]. Overexpression of OCIAD1 increases the interaction between cell integrin and extracellular matrix, thereby regulating the migration and metastasis of cancer cells[34]. Furthermore, OCIAD1 is involved in many physiological and pathological processes such as extracellular recognition, intercellular adhesion, cytoskeleton formation, and intracellular signaling[33]. This indicates that OCIAD1 has a certain role in cell adhesion, which may be related to the genetic regulation pathway of asthma. Therefore, the hub gene is mainly related to calcium binding and cilia change in asthma. By regulating or monitoring the core gene, it may be a potential target for treating asthma or may become a new biomarker for asthma[34].

Actin β (β-actin, ACTB), widely distributed in eukaryotic cells, is an essential protein for cytoskeleton synthesis and the basis of cell migration[28]. As a rich and highly conserved cytoskeletal structural protein, ACTB is regulated by the Rho/ROCK signaling pathway upstream proteins ROCK, profilin1, and other actin-binding proteins. ACTB participates in maintaining cell and tissue morphology, promoting cell migration, division, growth and signaling, and cytoskeletal formation[30]. Studies have shown that selective inhibitors of the Rho /Rho kinase signaling pathway can significantly inhibit 5-HT-induced proliferation of rat airway smooth muscle cells, and arrest cells in the G0/G1 phase. Inhibition of Rho kinase reduces airway remodeling and airway hyper responsiveness, as well as airway inflammation and oxidative stress. The Rho/Rock kinase signaling pathway mediates airway smooth muscle contraction, myofibroblast differentiation and ASMCs maturation, airway wall mesenchymal cell proliferation and migration, and migration of inflammatory cells during the development of chronic airway inflammatory diseases including asthma[35].

Conclusion

In our study, we identified DEGs of asthma airway epithelial cells in severe asthma. Furthermore, gene co-expression modules were constructed by WGCNA from 4144 genes in asthma airway epithelial cells.

Among them, the black module and green module were identified, which were both correlated with the severity of asthma. The genes in the two modules were used to construct a PPI network, several hub genes were identified, which could become potential diagnostic or therapeutic targets for bronchial asthma.

Conflict of interest

All authors declare no conflict of interest.

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