

Identification of pathogenic genes and upstream regulators in allergic rhinitis

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ABSTRACT

Background: Allergic rhinitis (AR) is the main cause of irreversible blindness in older individuals. Our study aims to identify the key genes and upstream regulators in AR.

Methods: To screen pathogenic genes of AR, an integrated analysis was performed by using the microarray datasets in AR derived from the Gene Expression Omnibus (GEO) database. The functional annotation and potential pathways of differentially expressed genes (DEGs) were further discovered by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. We constructed the AR-specific transcriptional regulatory network to find the crucial transcriptional factors (TFs) which target the DEGs in AR. Electronic validation was performed to verify the DEGs obtained by integrated analysis.

Results: From two GEO datasets obtained, we identified 793 DEGs (460 up-regulated and 333 down-regulated genes) between AR and normal control (NC). After GO and KEGG analysis, chronic inflammatory response and MAPK signaling pathway were significantly enriched pathways for DEGs. The expression of 6 genes (CLC, CST1, CRTAM, ILK, STAT1, and POSTN) was detected. The 6 genes in GEO: GSE51392 dataset played the same pattern with that in our integrated analysis.

Conclusions: The dysregulation of 3 genes (CST1, CLC and STAT1) may be involved in the pathogenesis of AR. AP-1 was associated with AR by regulating CST1 and CLC. Our finding can contribute to developing new potential biomarkers, revealing the underlying pathogenesis, and further raising new therapeutic targets for AR.

1. Introduction

Traditionally, rhinitis can be categorized into allergic rhinitis (AR) and non-allergic rhinitis (NAR) based on clinical features and allergic sensitization to common allergens [1]. Allergic rhinitis is characterized as hypersensitive inflammation in the nasal mucosa and a large number of pro- and anti-inflammatory mediators are delivered into the nose during continuous inflammation, either through plasma extravasation or by secretion from serous and mucous glands [2,3]. It is believed that the pathogenesis of allergic rhinitis may be the result of environmental, immunological and genetic factors. Till now, many molecules have been shown to participate in the initiation and progression of allergic rhinitis [4–6]. Although various efforts have been exerted to verify the potential mechanisms of AR, the treatment of this disease is still quite difficult. Thus, it is very urgent to identify the key molecules that are involved in the AR pathologies, which may lead us to better understand the potential molecular mechanisms of pathogen and to further discover novel targets for the treatment of AR [7].

Transcription factors (TFs) are a family of proteins that enhance or inhibit gene transcription by binding to specific DNA sequences of target gene promoters [8]. TFs regulate the transcription of many genes, including cytokines, growth factors, intercellular adhesion molecules and apoptosis-inducing or apoptosis-inducing molecules [9]. Aberrantly expressed or activation/inactivation of TFs have been detected in the development and progression of many human and animal diseases, which as endogenous regulatory molecules may affect the mRNA expression levels of critical genes and modulate pathologic signaling pathways. However, the expression and function of TFs in the pathogenesis of AR remain unclear.

To find more candidate biomarkers for improving the diagnostic rate of AR, integrated analysis of AR microarray data was performed in our current study, and the TFs and their target genes that may be closely related to the development of AR were identified between AR and normal tissues. Moreover, a transcriptional regulatory network was also constructed to further understand the regulatory mechanism [10].

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2. Methods

2.1. Microarray expression profiling in GEO

The microarray data sets of AR and normal control (NC) were derived from the GEO database (<http://www.ncbi.nlm.nih.gov/geo>) [11]. The following key search terms were used: “rhinitis, allergic” OR “allergic rhinitis”, AND “*Homo sapiens*” AND “gse”. The selection criteria were as follows: (1) The selected data set must be genome-wide mRNA transcriptome data. (2) These data from allergic rhinitis nasal epithelial tissue samples and normal control nasal epithelial tissue samples. (3) Both normalized and raw datasets were considered, two sets of allergic rhinitis mRNA data sets were obtained after selected.

2.2. Statistical analysis

We processed missing data and normalized raw microarray data sets. Using the Linear Models for Microarray Data (Limma) package in R, DEGs between AR and NC were identified by two-tailed Student's t-test. Individual P-values were obtained. Multiple comparison correction was performed by Benjamini and Hochberg method to obtain false discovery rate (FDR). FDR of 0.05 was defined as the selected criterion of DEGs [11].

2.3. Functional annotation of DEGs

Gene ontology (GO) and the Kyoto encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed to detect the biological functions and potential pathway of DEGs [12]. The GO functions of the DEGs were determined according to different categories including biological process, molecular functions, and cellular components. In addition, pathway enrichment analysis was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [13].

2.4. Protein-protein interaction (PPI) network construction

In order to find candidate genes involved in the formation of AR, PPI networks of significantly DEGs were constructed according to the data from Biological General Repository for Interaction Datasets (BioGRID) (<http://thebiogrid.org/>). Based on the existing data of protein interaction in BioGRID database, Cytoscape was used to search the differential expression mRNAs up-regulated in the top 100 and the top 100 down-regulated mRNAs. After removing the non-differentially expressed genes, the protein network interaction map was drawn.

2.5. Screening TFs of the top20 DEGs and constructing TFs regulation network

For the top20 differentially expressed genes, the 2 kb upstream promoter region was first downloaded on the UCSC website. The TRANSFAC website's match tool was then used to analyze TFs capable of binding to the promoter region of the differentially expressed genes described above. TFs that displayed altered expression in AR were identified with the criterion of FDR < 0.001. Position Weight Matrix scan was used to scan the human genome sequence to obtain the protein-coding genes, which are regulated by differentially expressed TFs. After removing redundant information, the AR-specific transcriptional regulatory network was constructed by the Cytoscape software (<http://www.cytoscape.org/>).

2.6. Electronic validation of differentiation genes by GEO database

We employed the Gene Expression Omnibus GSE51392 (GEO: GSE51392) database to validate the expression of selected AR differentiation related genes. We compared the expression levels of these genes in case group with normal group. The expression of 6 genes (CLC,

Table 1

The top 20 differentially expressed mRNA (case vs normal).

ID	Symbol	Combined.ES	P.Value	FDR	Up/Down
1469	CST1	2.31E+00	8.78E-07	1.51E-02	Up
64943	NT5DC2	1.31E+00	3.27E-04	7.61E-01	Up
493869	GPX8	1.28E+00	3.28E-04	7.61E-01	Up
60625	DHX35	1.30E+00	3.65E-04	7.61E-01	Up
1178	CLC	1.35E+00	4.47E-04	7.61E-01	Up
85413	SLC22A16	1.22E+00	5.89E-04	7.61E-01	Up
10631	POSTN	1.25E+00	6.01E-04	7.61E-01	Up
3914	LAMB3	1.21E+00	6.14E-04	7.61E-01	Up
51301	GCNT4	1.22E+00	6.94E-04	7.61E-01	Up
90102	PHLDB2	1.19E+00	7.76E-04	7.61E-01	Up
9760	TOX	-1.63E+00	4.04E-05	2.84E-01	Down
390059	OR51M1	-1.49E+00	4.96E-05	2.84E-01	Down
56253	CRTAM	-1.31E+00	3.96E-04	7.61E-01	Down
10723	SLC12A7	-1.25E+00	4.32E-04	7.61E-01	Down
3695	ITGB7	-1.20E+00	7.79E-04	7.61E-01	Down
8938	BAIAP3	-1.19E+00	8.10E-04	7.61E-01	Down
84464	SLX4	-1.20E+00	1.26E-03	8.39E-01	Down
10058	ABCB6	-1.13E+00	1.58E-03	8.39E-01	Down
3109	HLA-DMB	-1.12E+00	1.69E-03	8.39E-01	Down
120224	TMEM45B	-1.10E+00	1.71E-03	8.39E-01	Down

CST1, CRTAM, ILK, STAT1, and POSTN) was detected. The difference of expression levels was displayed by box-plots.

3. Results

3.1. Differential expression analysis of genes in AR

The probe corresponding to multiple genes removed, and then there are multiple probes corresponding to the average amount of expression of the gene, after this treatment, take the intersection of 17,169 genes.

Two gene expression microarray datasets (GSE19187 and GSE44037) were enrolled. Compared with the normal controls, 793 DEGs in AR were obtained with P < 0.05, among which, 460 genes were up-regulated and 333 genes were down-regulated. The top 20 most significantly up or down-regulated genes were listed in Table 1. The DEGs were screened for clustering analysis. The heatmap after cluster analysis of two sets of cDNA microarrays is shown in Fig. 1.

3.2. Functional annotation

The DEGs were enriched with GO and enriched with KEGG using GeneCoDis3 (<http://genecodis.cnb.csic.es/analysis>). In Fig. 2, GO enrichment showed that the DEGs were significantly enriched in biological processes such as chronic inflammatory response, cell adhesion, signal transduction, membrane, endoplasmic reticulum, cytoplasm and other cellular components, nucleotide binding, hydrolase activity, and

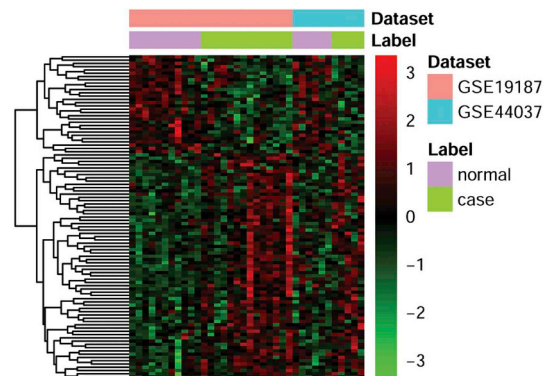


Fig. 1. Heatmap image displaying genes that were significantly up-regulated or down-regulated (P-value < 0.05) in AR compared to normal controls.

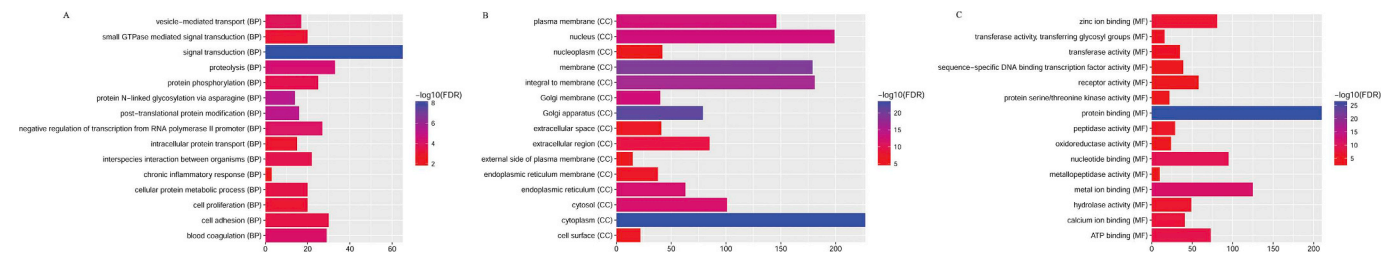


Fig. 2. Go functional enrichment of DEGs (FDR < 0.05). A: Biological process, B: Cellular components, C: Molecular functions.

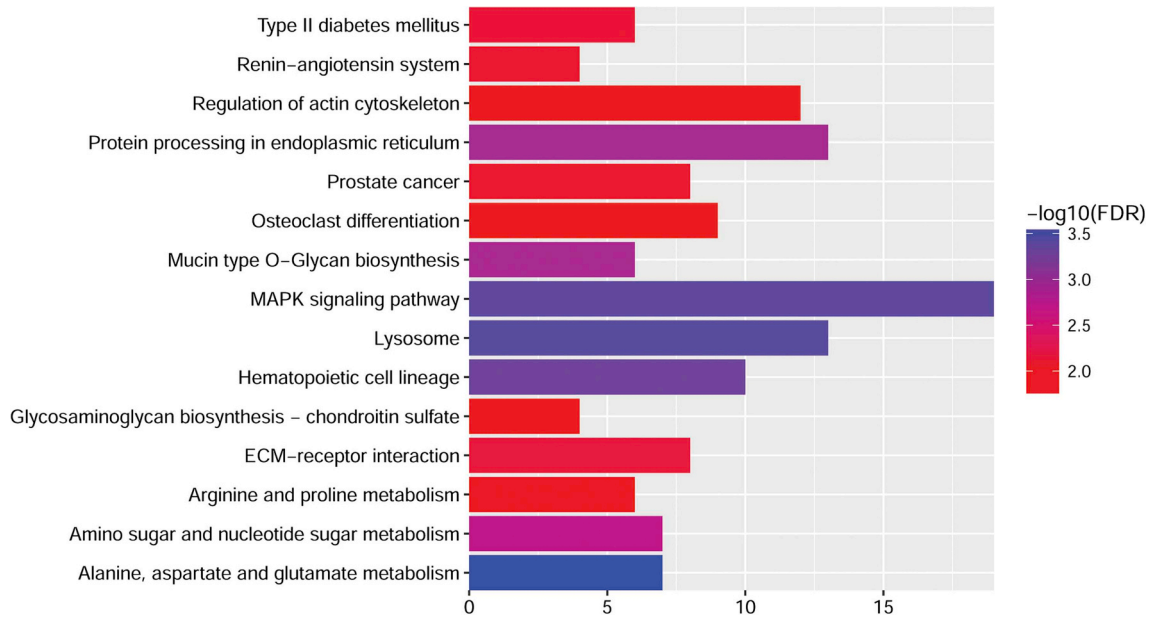


Fig. 3. KEGG analysis of DEGs in AR.

protein binding.

Furthermore, in Fig. 3, the results of KEGG pathway enrichment showed that DEGs were enriched in MAPK signaling pathway, Mucin type O-Glycan biosynthesis, ECM-receptor interaction (ECM-receptor interaction) and other signaling pathways.

3.3. PPI network construction

After removing the non-differentially expressed genes, the protein-protein interaction (PPI) network was established. The result is shown in Fig. 4. The network consists of nodes and edges. The nodes in the network represent the proteins and the lines represent the interaction between them. There are 179 nodes and 192 edges in the figure. Among them, the genes with higher degree were SHMT2 (degree = 22), ILK (degree = 15), CREB3 (degree = 15), TRAF1 (degree = 11), HUWE1 (degree = 10), FBXO6 (degree = 9), MTOR (degree = 8), RHOA (degree = 7), SORT1 (degree = 7), SNRNP200 (degree = 6), AMOT (degree = 6).

3.4. TFs of the top20 DEGs and TFs regulation network

A total of 372 TFs binding relationships were obtained (including multiple binding sites on the same transcription factor in a gene). Among which, 44 TFs were involved. In Table 2, the top6 TFs with the most downstream genes (the top 20 differentially expressed genes) include: Pax-4, 1-Oct, Nkx2-5, Pax-6, AP- S3.

Cytoscape software (version 3.5.0) was used to generate the transcription factor and its differentially expressed regulatory network of target genes in Fig. 5. The figure shows there were 64 nodes (including

44 transcription factors and 20 differentially expressed target genes) and 134 edges. The highest degree of 7 target genes were PHLDB2 (degree = 12), CRTAM (degree = 12), OR51M1 (degree = 11), NT5DC2 (degree = 9), LAMB3 (degree = 8), BAIAP3 (degree = 8), CST1 (degree = 8).

3.5. Validation of DEmRNAs in GEO GSE51392 dataset

To define the key genes that play important role in AR, we searched the online GEO GSE51392 repository of high throughput gene expression data and hybridization arrays, chips, microarrays. The results indicated that three DEmRNAs (STAT1, CLC and CST1) were closely related to AR. POSTN is related to chronic sinusitis and CRTAM, ILK are related to asthma. In Fig. 6, the genes of CLC, CST1, ILK and POSTN were up-regulated, the genes of CRTAM and STAT1 were down-regulated. These results were consistent with the integrated analysis in the GSE19187 and GSE44037 datasets.

4. Discussion

AR, an airway allergic disease, is caused by hypersensitivity to one or multiple antigens resulting in chronic allergic inflammation of the nasal mucosa. The pathogenesis of AR is rather complex. Although many studies have shown that a large number of genes are involved in the pathogenesis of AR, it is unclear how these genes are regulated [14]. Since the effective treatment is deficient, finding novel strategies of treatment for AR has long been an essential goal. In our study, 793 DEGs (460 genes were up-regulated and 333 genes were down-regulated) and 372 TFs binding relationships in AR were obtained.

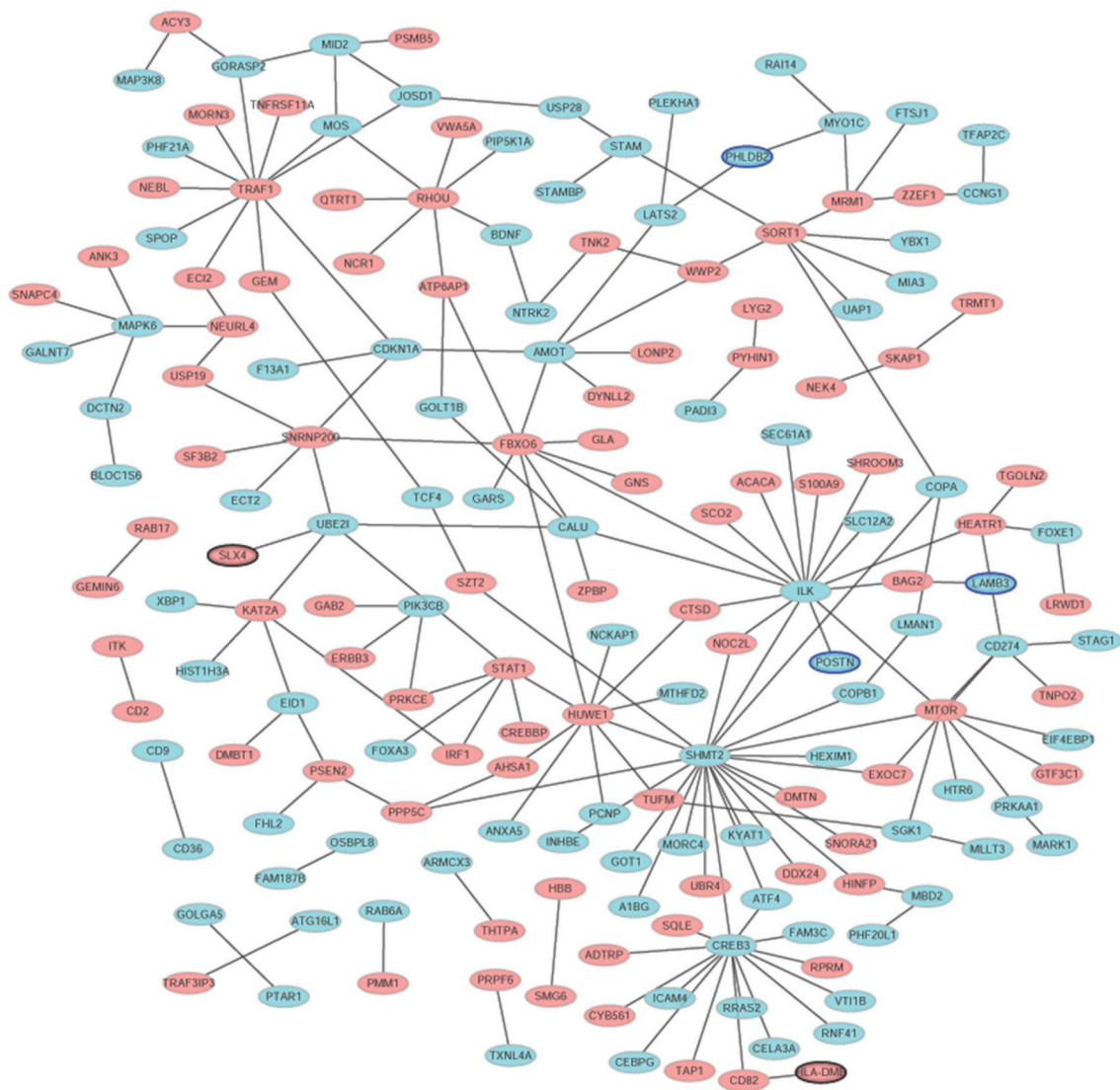


Fig. 4. The PPI network of top 100 significantly DEGs in AR. The blue ellipses were represented the proteins encoded by down-regulated DEGs and the red ellipses were represented the proteins encoded by up-regulated DEGs. Among which, ellipses with black border were derived from the top 10 down-regulated DEGs in AR. The ellipses with blue border were represented the top 10 up-regulated DEGs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Cystatin SN (cystatin 1, CST1) is a member of the cystatin superfamily that inhibits the proteolytic activity of cysteine proteases, plays an important role in the processes of inflammation and tumorigenesis [15]. Previous study found CST1 is associated with pulmonary fibrosis in systemic sclerosis patients [16,17]. Lisa Giovannini-Chami et al. using nasal epithelial cells from children with dust mite allergy showed that CST1 was the most highly upregulated gene and that CST1 was also strongly induced by stimulation with IL-4 and IL-13 [18,19]. Yoshimasa Imoto et al. observed that the expression of CST1 was upregulated specifically in seasonal allergic rhinitis (SAR) patients during natural

allergen exposure [20]. In our study, CST1 was significantly up-regulated in AR patients, furthermore, CST1 was the top1 differentially expressed mRNA. In the TFs regulation network, CST1 was regulated by AP-1, 1-Oct and Pax-4.

Galectin-10 (CLC), known as Charcot-Leyden protein, is a member of the b-galactoside binding lectins family [21], produced by a restricted panel of inflammatory cell types, involved in allergic response [22,23], leading to consider CLC as a potential novel actor of the aspirin induced inflammatory process. CLC mRNA has been found to be over-expressed in asthma [24]. In nasal lavage fluid, CLC/galectin-10 is

Table 2
The top6 TFs with the most downstream regulatory genes and their target genes.

Factor.name	Number of regulated genes	Regulated genes
Pax-4	12	LAMB3,PHLDB2,GPX8,SLC12A7,HLA-DMB,SLC22A16,CRTAM,TMEM45B,ITGB7,BAIAP3,SLX4,CST1
1-Oct	10	ABCB6,PHLDB2,NT5DC2,GCNT4,HLA-DMB,OR51M1,CRTAM,TMEM45B,POSTN,CST1
Nkx2-5	9	PHLDB2,GPX8,GCNT4,HLA-DMB,SLC22A16,OR51M1,BAIAP3,CLC,DHX35
Pax-6	7	LAMB3,NT5DC2,SLC12A7,HLA-DMB,TOX,TMEM45B,POSTN
AP-1	7	LAMB3,SLC12A7,OR51M1,TMEM45B,ITGB7,CLC,CST1
COMP1	7	LAMB3,PHLDB2,GPX8,HLA-DMB,TOX,ITGB7,CLC

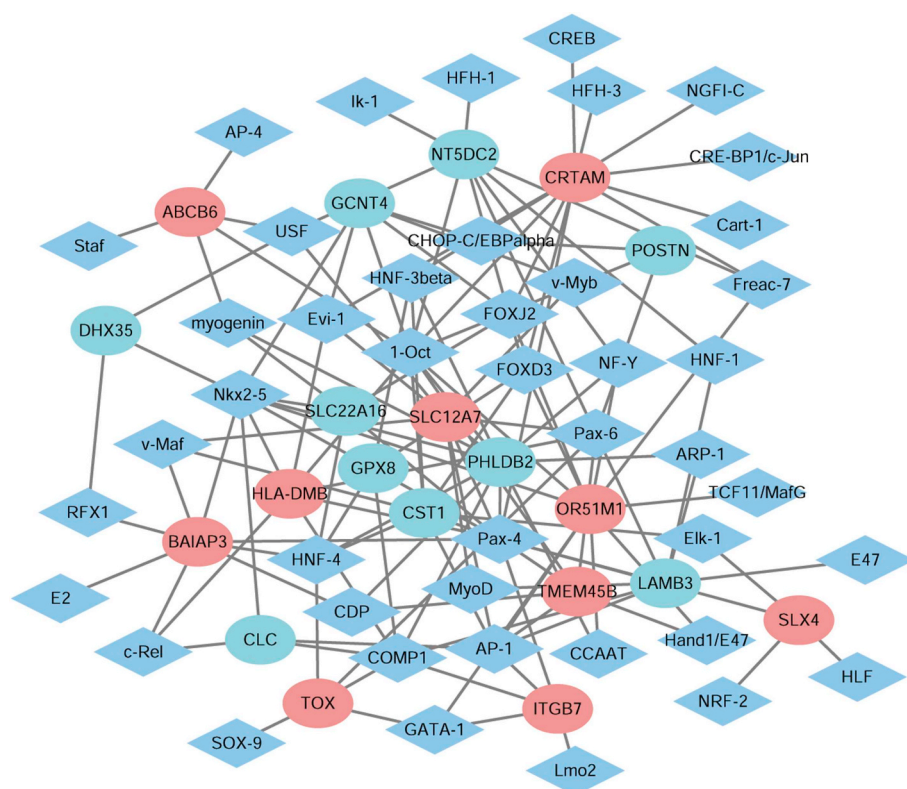


Fig. 5. The AR-specific transcription factors regulation network diagram. Blue diamond were represented transcription factors, ovals were represented top20 genes, red ovals were represented up-regulated genes, green ovals were represented down-regulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

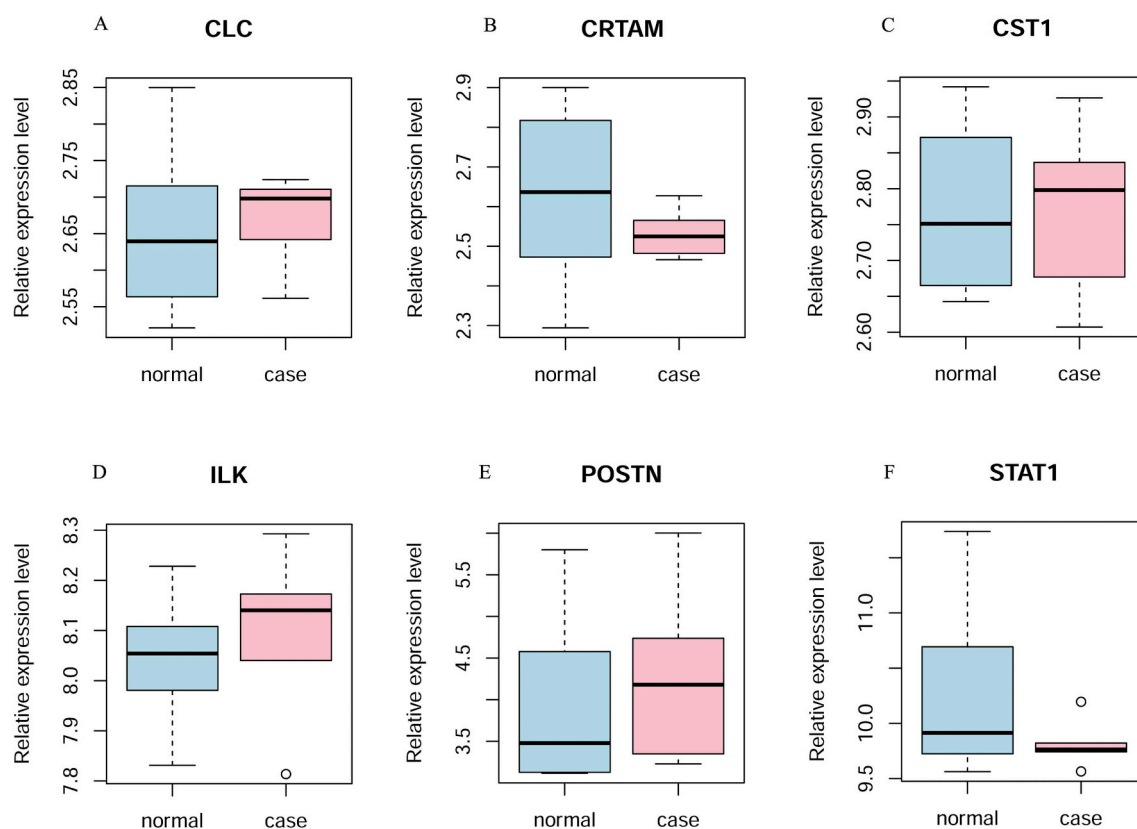


Fig. 6. The validation of the expression levels of CLC, CST1, CRTAM, ILK, STAT1, and POSTN in AR based on GEO: GSE51392 database. The x-axis shows the case and normal groups and y-axis shows expression reads counts.

detected in allergic individuals during pollen season, at considerable higher levels compared to healthy controls, but not outside season [25]. We observed CLC was dramatically up-regulated in AR group compared with healthy control and CLC was regulated by the TFs of COMP1, AP-1 and Nkx2-5. CLC was the top5 differentially expressed mRNA.

Signal transducer and activator of transcription (STAT) 1 signaling pathway mediates biological functions of interferon (IFN) gamma, which is a key cytokine-regulating T helper 1 (Th1) differentiation. In the study of Hattori H, STAT1-1- mice showed impaired nasal eosinophilia and markedly reduced histamine-induced nasal hyperresponsiveness after SEA sensitization. Moreover, levels of Th2-associated SEA-specific IgG1 and IgE antibodies were lower in STAT1-/- mice. Anti-CD3 stimulated nasal lymphocytes from STAT1-/- mice also produced less amounts of Th2-associated cytokines IL-4, IL-5, IL-10, and IL-13 compared with WT mice, but both produced comparable levels of IFN-gamma [26]. In our results, STAT1 was down-regulated in the AR patients compared with normal control.

After function annotation analysis, we found that chronic inflammatory response was a significantly enriched pathway for DEGs. And previous studies have reported that nasal Th2-mediated inflammation was confirmed by cytometric evaluation of nasal lavage fluid from NAR patients after allergen challenge: increases in mast cells, basophils, eosinophils, CD3⁺ T cells and CD4⁺ T cells when exposed to grass pollen [27,28].

Since TFs were indicated to regulate the production of mRNAs, we therefore predicted the TFs that could regulate the production of differentially expressed mRNAs. We selected 20 most differentially regulated mRNAs (10 up-regulated and 10 down-regulated) for the prediction. AP-1 was one of the first mammalian transcription factors to be identified unravelled. AP-1 activity is induced by a plethora of physio-1, but its physiological functions are still being logical stimuli and environmental insults. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. AP-1 activity is induced by growth factors, cytokines, neurotransmitters, polypeptide hormones, cell-matrix interactions, bacterial and viral infections, and a variety of physical and chemical stresses. These stimuli activate mitogen activated protein kinase (MAPK) cascades13 that enhance AP-1 activity through the phosphorylation of distinct substrates [29–31]. In our KEGG analysis, MAPK signaling pathway was one of the significantly enriched pathways for DEGs. From these results we indicated that, in AR patients, mitogen activated protein kinase induced the transcription factor of AP-1, then AP-1 regulated the targeted gene CLC or CST1, thus these two genes were up-regulated.

5. Conclusion

In our study, three DEGs (CST1, CLC and STAT1) were found to involve with the process of AR. From two GEO datasets obtained, we identified 793 DEGs (460 up-regulated and 333 down-regulated genes) between AR and normal control (NC). After GO and KEGG analysis, chronic inflammatory response and MAPK signaling pathway were significantly enriched pathways for DEGs. AP-1 was associated with AR by regulating CST1 and CLC. Our finding can contribute to developing new potential biomarkers, revealing the underlying pathogenesis, and further raising new therapeutic targets for AR.

Declaration of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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