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A Pilot Study of Differential Gene Expressions in Patients with Cough Variant Asthma and Classic Bronchial Asthma

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Abstract

Background Despite extensive exploration of asthma, the mechanism of asthma has not been fully elucidated. Cough variant asthma (CVA) is considered as precursor to classical asthma (CA). Comparative study between CA and CVA may be helpful in further understanding the pathogenesis of asthma.

Methods Peripheral blood mononuclear cells (PBMCs) were isolated from CVA, CA and healthy adults. Each group consisted of five cases. Total RNA was extracted from the PBMCs. Agilent 4 x 44 K human genome oligo microarray was used to detect whole genome expression. Allogeneic clustering, Gene Ontology and KEGG analysis were performed

to investigate differentially expressed genes (DEGs). Then, ten candidate genes were screened and verified by real-time PCR.

Results Gene expressions were significantly different among the three groups, with 202 DEGs between the CA and the CVA groups. The Gene Ontology analysis suggested that the DEGs were significantly enriched in ‘histone H4-K20 demethylation’ and ‘antigen processing and presentation of endogenous antigens’. *HDC*, *EGR1*, *DEFA4*, *LTF*, *GOS2*, *IL4*, *TFF3*, *CTSG*, *FCER1A* and *CAMP* were selected as candidate genes. However, the results of real-time PCR showed that the expression levels of *FCER1A*, *IL4* and *HDC* in the cough variant asthma group were significantly different from those in the other two groups ($P < 0.05$).

Conclusions The pathogenesis of CVA and CA may be related to genes such as *FCER1A*, *HDC* and *IL4*. Further studies incorporating a larger sample size should be conducted to find more candidate genes and mechanisms.

Keywords Cough variant asthma; classical asthma; whole-genome microarray; differentially expressed genes

Introduction

Asthma is a lifelong respiratory disease, usually characterized by chronic airway inflammation, hypersensitivity of the respiratory tract and reversible obstruction of airflow. Persistent inflammation in the airways leads to permanent airway remodeling. This disease is a major concern worldwide that creates a heavy burden on human health and society and reduces quality of life. According to a previous report, in 2016, 360 million people globally suffered from asthma, and approximately 420 000 people died from the disease(1). Both genetic factors (genetic polymorphisms and family history) and environmental factors (tobacco smoke, air pollution, allergens) contribute to the development of asthma(2). However, the exact pathogenesis of this disease has not been fully elucidated. In asthma genetics, a trend towards a few main topics developed over the last 2 years. Among them, some studies focused on overlapping and/or very specific phenotypes: within the allergy spectrum but also reaching beyond, looking for common genetic traits shared between different diseases or disease entities(3).

As a special type of asthma, cough variant asthma (CVA) is characterized by chronic cough as the main or only symptom and is also considered to be a peripheral airway inflammatory disease similar to asthma(4). CVA and asthma have many similarities. For example, these conditions have similar pathobiological bases, including eosinophilic inflammation, airway hyperresponsiveness and airway remodeling(5). However, the degree of airway inflammation, airway hyperresponsiveness and airway remodeling in CVA are minor. Some scholars regard CVA as the precursor to asthma, and 30%~40% of CVA cases will develop into typical asthma without proper treatment(6). Fortunately, early appropriate treatment in CVA is curative. Research has demonstrated that inhaled corticosteroids or a combination of inhaled corticosteroids and long-acting beta-agonists (ICS/LABA)

can alleviate the degree of inflammation and remodeling in asthmatics(7). Curing CVA and blocking CVA development to classical asthma are the first line of defense in the treatment of asthma. Unfortunately, the transformational mechanism of CVA to typical asthma has not been explored.

Asthma is caused by a complex interaction between genes and the environment, and gene expression is regulated by epigenetic modifications through gene and environment interactions. Are there differences in gene expression between CVA and classical asthma? Comparative studies between classical asthma and CVA may be helpful for further understanding the pathogenesis of asthma. Whole-genome microarray analysis is an important technique in the field of genetic research, and it possesses many advantages of which the greatest is the ability to improve traditional experiments by allowing the observation of differences in one or multiple genes in a single test(8). Here, we first performed a pilot study on gene expression differences among CVA patients, asthmatic patients and healthy adults by the whole-genome microarray technique. Then, we screened candidate genes by enrichment analysis and validated them by real-time PCR.

Materials and Methods

Study subjects

Healthy adults from the physical examination center of the Sichuan Provincial People's Hospital were randomly selected as the healthy control group (g1, HC). Patients who were diagnosed with classical asthma (g2, CA) or CVA (g3, CVA) in the Outpatient Department of Respiratory Medicine of the Sichuan Provincial People's Hospital and West China Hospital of Sichuan University from March 1st, 2016, to December 31st, 2016, were screened for eligibility for this study. The diagnostic criteria of CA were from GINA 2015(9), CVA was diagnosed using the following criteria: chronic cough often accompanied by significant irritant cough at night, positive bronchial stimulation test or PEF mean variation rate > 10% or positive bronchial dilation test, effective to anti-asthma treatment(10). The inclusion criteria were as follows: 20–65 years of age, female, no family history of asthma, no smoking history, no oral or intravenous antiallergic medications and corticosteroids within 4 weeks, and no history of acute respiratory infection within 4 weeks. The exclusion criteria were as follows: severe asthma, other respiratory diseases (i.e. chronic obstructive pulmonary disease, bronchiectasis, pulmonary fibrosis, pneumonia, tuberculosis), serious cardiovascular diseases (cardiac function at or above grade II according to the NYHA standard), hepatorenal insufficiency, pregnancy and lactation, and malignant tumor. Each group consisted of five subjects. The study was approved by the Medical Ethics Committee of the Sichuan Provincial People's Hospital (approval number: 2016 No. 103), and all participants provided written informed consent in advance.

Methods

Peripheral blood mononuclear cell isolation and RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation using Lymphoprep (Axis Shield, Norway) following the manufacturer's instructions. Total RNA was extracted using TRIzol reagent following the manufacturer's instructions, purified and preserved at -80°C for further detection.

Genome-wide gene expression by GeneChip microarray and data analysis

The genome-wide gene expression analysis was completed with the assistance of Shanghai Biotechnology Corporation (Shanghai, China). An Agilent 4×44 K human genome oligo microarray was used for gene expression profiling of the RNA samples previously described. Finally, the differentially expressed genes (DEGs) were analyzed by cluster analysis and enrichment analysis (Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis). A total of 10 candidate genes were selected.

Candidate gene verification by real-time PCR

Real-time PCR was applied to fourteen samples as one sample in the CVA group did not have sufficient RNA. GAPDH was used as the internal reference gene. The twenty different primers used together with that for GAPDH are listed in Table 1. Real-time PCR was performed as follows: RNA samples were thawed at 4°C , and cDNA first chain reaction solution was prepared using a 20 μL total volume containing 4 μL of 5x RT Buffer, 1 μL of enzyme mix, 1 μL of primer mix, 14 μL of RNA template (0.5 μg) and H_2O in the PCR tube. SYBR Green qPCR was run in 384-well optical reaction plates, and the reactions were conducted using a 10 μL total volume containing 5 μL of 2xSYBR Green PCR buffer (ABI, USA), 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 5 ng of template, and X (up to 10) μL of dd H_2O . The reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Analysis was performed using a 7900 HT Sequence Detection System (ABI, USA).

Quality control

NanoDrop ND-2000 spectrophotometer (Thermo Scientific, US) and Agilent Bioanalyzer 2100 (Agilent Technologies, US) were used for RNA quality control. The RNA Integrity Number (RIN) software algorithm based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. Our study required $\text{RIN} \geq 6.0$ and $28\text{ s}/18\text{ s} \geq 0.7$; Coefficient of variation(CV) was used to judge whether the system was stable or not by comparing and analyzing the data between groups in GeneChip microarray. In the experiment, the CV value of 10 times repeated probe point signal was used to evaluate the stability of chip and technology; Melting-curve

analysis was used as the quality control standard of real-time PCR, and all samples were repeated for three times. All the melting-curve in our study were single-peak and had strong repeatability.

Statistical analysis

Normally distributed data are expressed as the mean $\pm$ standard deviation ($\bar{x} \pm SD$), Nonnormally distributed data are expressed as the median (Interquartile range, IQR). The expression level of each mRNA is expressed as a log2 transformed value and was normalized to a mean of zero. ANOVA was used for comparisons between groups. DEGs were analyzed by cluster analysis. A p values of less than 0.05 was considered statistically significant. Statistical analysis was performed with SPSS (Professional Version 22).

Results

Characteristics of individuals in each group

The characteristics of the individuals in each group are summarized in Table 2. Age, body mass index (BMI), FEV1/FVC (FEV1: Forced expiratory volume in one second; FVC: forced vital capacity) and FEV1%/Pred were not significantly different among the HC, CA and CVA groups. Fraction of exhaled nitric oxide (FeNO) between CA and CVA groups was not significantly different (20(16.5), 24(26.5), $p > 0.05$); All individuals from the CA and CVA groups had a history of allergic rhinitis. The course of disease between the CA and CVA groups was significantly different (8 ± 11.38 years vs 1.8 ± 1.6 years, $P < 0.01$). All individuals had normal white blood cell counts; one patient with asthma had an elevated percentage of eosinophils in blood ($\geq 5\%$); One patient with classical asthma had an elevated IgE level (≥ 100 IU/ml); four and three individuals from the CA and CVA groups, respectively, were using inhaled corticosteroids; and no individual was using oral corticosteroids.

Gene expression and cluster analysis

The DEGs showed a two-fold increase or decrease in cluster analysis. The cluster analysis divided the fifteen samples into three categories, which were completely consistent with the experimental grouping (Figure 1). From the gene expression level, we found 779 DEGs between the HC group and the CA group; 264 genes were upregulated, and 515 genes were downregulated. There were 119 DEGs between the HC group and the CVA group; 44 genes were upregulated, and 75 genes were downregulated. When the CVA group was compared with the CA group, there were 202 DEGs, of which 141 genes were upregulated and 61 genes were downregulated.

Enrichment analysis and candidate gene selection

The enrichment analysis included FF pathway analysis. GO analysis suggested that the DEGs between the HC and CA groups were significantly enriched in biological processes including ‘histone H4-K20 demethylation’, cellular components such as ‘CCR4-NOT core complex’, and molecular functions including ‘histone demethylase activity (H4-K20 specific)’. Between the HC and CVA groups, the DEGs were significantly enriched in biological processes including ‘histone H4-K20 demethylation’ and ‘DNA methylation on cytosine’ and molecular functions including ‘histone demethylase activity (H4-K20 specific)’. Between the CA and CVA groups, the DEGs were significantly enriched in biological processes including ‘antigen processing and presentation of endogenous antigens’, cellular components such as ‘MHC class I protein complex’, and molecular functions including ‘TAP binding’ (see additional information in Figure 2).

KEGG pathway enrichment analysis showed that the DEGs between the HC and CA groups were significantly enriched in biological pathways including ‘ECM-receptor interaction’ and ‘systemic lupus erythematosus’ (Table 3). Between the HC and CVA groups, the DEGs were significantly enriched in biological pathways such as ‘asthma’, ‘systemic lupus erythematosus’, and ‘Fc epsilon RI signaling pathway’ (Table 4). Between the CA and CVA groups, the DEGs were significantly enriched in biological pathways such as ‘pantothenate and CoA biosynthesis’, ‘asthma’ and ‘Fc epsilon RI signaling pathway’ (Table 5). Between the CA group and the CVA group, we identified the KEGG pathway of hsa05310, which is described as ‘asthma’, as shown in Figure 3.

Combining the DEGs with GO and KEGG analysis, we screened ten DEGs (*HDC*, *EGR1*, *DEFA4*, *LTF*, *G0S2*, *IL4*, *TFF3*, *CTSG*, *FCER1A* and *CAMP*) as candidate genes and completed the verification test by real-time PCR.

Real-time PCR for candidate gene verification

The real-time PCR results showed that the gene expression levels of *FCER1A*, *HDC* and *IL4* in the CVA group were upregulated compared with those in the CA group ($P < 0.05$). The expression levels of these three genes were also upregulated in the CVA group compared with those in the HC group ($P < 0.05$). Among the three groups, the relative expression levels of the other seven genes, *EGR1*, *DEFA4*, *LTF*, *G0S2*, *TFF3*, *CAMP* and *CTSG*, were not significantly different ($P > 0.05$). The details are shown in Table 6.

Discussion

As an atypical type of asthma, CVA is the most common cause of chronic cough in China and has a similar pathogenesis to asthma. Some scholars regard CVA as the precursor to asthma. Genetic factors together with environmental exposures promote the development of asthma; many studies on genetic mechanisms have been completed and have revealed more than 400 genes related to asthma(11). Over the past 3 years, well powered genetic studies of asthma have increased the

number of independent asthma-associated genetic loci to 128 (12). Despite the substantial efforts made to study asthma, only a few susceptibility genes, such as *IL1*, *IL33*, thymic stromal lymphopoietin (*TSLP*), *HLA*, IL-1 receptor–like 1 (*IL1RL1*), the 17q12-21 locus encoding ORM1-like 3 (*ORMDL3*), and *ADAM33*, have been identified by genome-wide association studies (GWAS)(11). The contribution of genetic factors to the risk of asthma has been confirmed in previous studies, where heritability estimates ranged from 35% to 80%(13). The wide range of heritability estimates explained the role of environmental exposures in the risk of asthma. Thus, it can be concluded that asthma is a complex multifactorial disease. Study also found that different respiratory symptom with asthma associated variants gene locus(14), and in view of the relationship between CVA and asthma, our study used CVA to provide novel insights that may help open new avenues for future research on asthma.

On the basis of gene expression and cluster analysis, we found that gene expression profiles among the HC, CA and CVA groups were significantly different. All the profiles were consistent with their groups, indicating that our groupings and sample collections were correct.

After candidate gene screening and validation, we found that the expression of three genes, *IL4*, *FCER1A* and *HDC*, was upregulated in the CVA group compared with the HC and CA groups. This finding indicated that some genes, such as *IL4*, *FCER1A* and *HDC*, may be related to the process of transformation from CVA to asthma. These three genes were closely related to asthma. The *IL4* gene is located at 5q31.1, which is in a susceptibility region for asthma. Interleukin-4, the genetic product of *IL4*, causes naive CD4⁺ T cells to highly express *STAT6* and *GATA3* and differentiate into Th2 cells, which play a critical role in the pathogenesis of asthma(15). *FCER1A* encodes the alpha subunit of IgE receptors, which also plays a central role in asthma. Candidate gene studies and GWAS showed that single nucleotide polymorphisms (SNPs) in *FCER1A* were major genetic determinants of IgE levels(16–19). *HDC* encodes L-histidine decarboxylase, which can catalyze histidine to histamine. Histamine is a major component participating in the pathophysiology of asthma. Ohtsu et al.(20) found that tissues from *HDC*-deficient mice lacked histamine-synthesizing activity and that the numbers of mast cells were decreased. Many researchers agree that *HDC* is closely related to asthma(21). To the best of our knowledge, we first found that *IL4*, *FCER1A* and *HDC* were highly related to CVA. However, these genes were not highly expressed in the CA group in our study, which was not consistent with previous studies. The endotypes of asthma can be divided into the ‘Th2-high’ and ‘Th2-low’ subtypes; they are relatively stable subgroups and can be defined by underlying distinct genetic or molecular characteristics(22). The Th2-high subtype occurs in many but not all patients(23). In the early stage of asthma, its gene expression may be up-regulated. Furthermore, a small sample size may result in errors. Therefore, we speculated that the differences between subtypes (CVA and CA) and the sample size could explain the results.

In terms of signaling pathway analysis, we found that the signaling pathway of ‘asthma’ (hsa05310, Figure 3) had a high enrichment factor in the CVA group compared with the HC or CA group. This pathway contains seven signaling pathways related to the pathogenesis of asthma, and in our study, we found that two of these pathways, the ‘Jak-STAT signaling pathway’ (between the HC and CVA groups) and the ‘Fc epsilon RI signaling pathway’ (between the HC and CVA groups and the CA and CVA groups) had high enrichment factors. The Jak–STAT signaling pathway is a paradigm of receptor-mediated signal transduction and a shining example of the clinical translation of basic immunology(24). Asthma is associated with Th2 cell orientations, and the expression of IgE is an important part of the pathogenesis of asthma. The Jak-STAT signaling pathway was hypothesized to play a critical role in the development of asthma by regulating the level of IgE and the balance between Th1 and Th2 cells(25). Mast cells are another important member that participates in the pathogenesis of asthma. During allergic reactions, mast cell activation is mediated through FcεRI, which is defined as a receptor binding to monomeric IgE on the mast cell surface. Following its crosslinking, the IgE-bound FcεRI with the cognate antigen results in the release of arachidonic acid metabolites, vasoactive amines (early phase) and cytokines and chemokines (late phase)(26). Multiple signaling pathways contribute to the development of FcεRI-mediated mast cell responses, among which the Fc epsilon RI signaling pathway is the most important. Based on the above findings, we can conclude that CVA has a similar pathogenesis to asthma. The mechanistic differences between CVA and classical asthma may be explained by the fact that CVA has a higher Th2 cell level, IgE level and mast cell activation to some degree. This hypothesis may also be consistent with the theory that CVA is the precursor to classical asthma. Unfortunately, we did not detect subsets of T cells, and the IgE levels in the two groups did not support these conclusions, but we could not eliminate this possibility. Another explanation is that different phenotypes of asthma may have different gene expression levels(27). We verified that CVA is one of the phenotypes of asthma and has specific gene transcript differences from classical asthma.

The DEGs related to the signaling pathway described above included IL4, MS4A2, FCER1A, IL5RA and CLC. All of them were upregulated in the CVA group, but we only identified IL4 and FCER1A. In the future, we will continue to identify the other genes. The DEGs EGR1, G0S2, DEFA4, LTF, TFF3, CTSG and CAMP were not validated by real-time PCR, but we could not confirm that the difference among the seven genes was not statistically significant between the groups.

There are some limitations in our study. First, we did not perform the induced sputum test, and the subtypes of asthma were unknown; the gene expression profiles of different phenotypes of asthma might be different. Second, our study only had five cases in each group, and all the individuals were female. The reason why we chose females is that all the individuals in the CVA group were female by chance. To allow comparisons between the groups and avoid significant differences, we chose females in all the other groups, which was also consistent with the

epidemiological history of asthma. The incidence rate of asthma is higher in females than in males. Admittedly, our study was only a pilot study and had a very small sample size. Thus, the results and conclusions may be limited.

In summary, in this pilot study, we found that gene expression in PBMCs among patients with CVA, patients with classical asthma and healthy adults was significantly different. Some DEGs functionally related to asthma, antigen presentations and expression were identified between the CVA and classical asthma groups or between the CVA group and healthy adults. Here, we first consider that the pathogenesis of CVA and classical asthma may be related to certain genes, such as FCER1A, HDC and IL4, and related to some mechanistic processes, such as Th2 cell orientations, IgE levels and mast cell activation. Once again, we showed that CVA is a phenotype of asthma and a precursor to asthma. Further studies may be designed to increase the sample size and find more candidate genes and mechanisms.

Conclusions

In this pilot study, we first found that gene expression in PBMCs among patients with CVA, patients with and healthy adults was significantly different. Some DEGs between the CVA and classical asthma groups were functionally related to asthma, antigen presentations and expression. Here, we first consider that the pathogenesis of CVA and classical asthma may be related to genes such as FCER1A, HDC and IL4. Further studies may be performed to increase the sample size and find more candidate genes and mechanisms.

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Competing interests

The authors declare that they have no competing interests.

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Table 1 List of primers used in real-time PCR for the detection of the 10 candidate genes.

Primer Name	Sequence (5' to 3')
HDC-F	CGAAGAGTTTAAGCCAGCATGA
HDC-R	AACCACAGAAGCTGCCTGTCTAC
EGR1-F	ACTGAGTAGGCGGCGATTTT
EGR1-R	ATCCCATGGGCAATAAAGCG
DEFA4-F	TTGGGAACCTGCCTCATTGG
DEFA4-R	TTGGACAGCAGAACGTTAATCG
LTF-F	GCTCCACCAACAGGCTAAATTT
LTF-R	AAGGTTTTTGGTTTCAGACTGGAA
G0S2-F	CCACAAGCATCCACCAAAGG
G0S2-R	ATCCTTCCTCCCTAGTGCAA
IL4-F	AACGGCTCGACAGGAACCT
IL4-R	CTCTGGTTGGCTTCCTTCACA
TFF3-F	TCAGCTTTTCTGTCCCTTTGC
TFF3-R	CGTTAAGACATCAGGCTCCAGAT
FCER1A-F	ACTCAGCAGCAGGTCACATT
FCER1A-R	TGGCTTAGGATGTGGGTTTCAG
CAMP-F	GATTTGCCCTGCTGGGTGAT
CAMP-R	CCTGGGTACAAGATTCCGCA
CTSG-F	TGGATAAGGACAACAATGAGAAGCT
CTSG-R	CCCCGAGAAGAAGAGTCAGTCA
GAPDH-F	AGTTGCTCATACCGTGCTGCTA
GAPDH-R	CAATCAATTATAGAGGTGGCTTTAACAC

Table 2 Characteristics of individuals in each group.

	HC (n = 5)	CA (n = 5)	CVA (n = 5)
Age (years)	32.4 ± 8.26	46 ± 11.37	41.2 ± 11.23
Sex (Female/Male)	5/0	5/0	5/0
BMI	20.2 ± 2.1	23.14 ± 0.55	21.2 ± 2.26
Course of disease (year)	/	8 ± 11.38	1.8 ± 1.6
Allergic rhinitis	No	Yes	Yes
Smoking history	No	No	No
FEV1/FVC	95.2 ± 4.03	88.78 ± 5.54	89.95 ± 6.53
FEV1%/Pred	116.5 ± 7.72	107.58 ± 5.96	110.06 ± 5.12
Elevated WBC ($\geq 10 \times 10^9/L$ or $\leq 4 \times 10^9/L$)	0	0	0
Elevated percentage of EOS in blood ($\geq 5\%$)	0	1	0
Elevated FeNO (ppb)	/	20(16.5)	24(26.5)
Elevated IgE (≥ 100 IU/ml)	0	1	0
ICS	0	4	3
OCS	0	0	0

*Data are *n* or mean ± SD or median(IQR), BMI: body mass index; WBC: white blood cell; EOS: eosinophils; FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity; FeNO: fraction of exhaled nitric oxide; All subjects in HC group did not complete FeNO analysis. ICS: inhaled corticosteroids; OCS: oral corticosteroids. HC: healthy control group; CA: classical asthma group; CVA: cough variant asthma group.

Table 3 Top 15 enriched pathways between the HC and CA groups.

Pathway ID	Description	Genes	Enrich factor
hsa04512	ECM-receptor interaction	GP6 ITGB5 TNXB GP1BA GP5 LAMA3 COL4A4 VWF THBS1	6.18
hsa00260	Glycine, serine and threonine metabolism	ALAS2 SARDH PSPH	5.47
hsa05143	African trypanosomiasis	FASLG LAMA3 HPR	5.00
hsa05322	Systemic lupus erythematosus	HIST1H4H ELANE HIST2H4B HIST1H2AA HIST1H4B HIST1H2AB HIST1H4I CTSG HIST1H3E HIST1H3J HIST1H4D	4.65
hsa00071	Fatty acid degradation	CPT1B CPT1C ADH1C	4.07
hsa00860	Porphyrin and chlorophyll metabolism	CBLB UGT2B17 ALAS2	4.07
hsa04530	Tight junction	CTTN PPP2R2D RAB13 ASH1L MYL2 TJP1 CLDN5 JAM3	3.51
hsa05414	Dilated cardiomyopathy	MYL2 ITGB5 TPM1 TTN ADCY2	3.24
hsa05410	Hypertrophic cardiomyopathy (HCM)	MYL2 ITGB5 TPM1 TTN	2.81
hsa05219	Bladder cancer	MMP9 THBS1	2.78
hsa05120	Epithelial cell signaling in Helicobacter pylori infection	GIT1 TJP1 JAM3	2.57
hsa04670	Leukocyte transendothelial migration	ESAM MYL2 CLDN5 MMP9 JAM3	2.49
hsa03018	RNA degradation	CNOT4 PABPC1L CNOT7	2.47
hsa04510	Focal adhesion	PDGFRB MYL2 ITGB5 TNXB LAMA3 COL4A4 VWF THBS1	2.33
hsa05144	Malaria	CCL2 THBS1	2.29

* HC: healthy control group; CA: classical asthma group.

Table 4 Top 15 enriched pathways between the HC and CVA groups.

Pathway ID	Description	Genes	Enrich factor
hsa05310	Asthma	HLA-DQA2 IL4 MS4A2	27.16
hsa05020	Prion diseases	C1QB C1QA	15.59
hsa05150	Staphylococcus aureus infection	C1QB HLA-DQA2 C1QA	15.04
hsa05330	Allograft rejection	HLA-DQA2 IL4	14.39
hsa04672	Intestinal immune network for IgA production	HLA-DQA2 IL4	11.46
hsa05320	Autoimmune thyroid disease	HLA-DQA2 IL4	10.40
hsa05322	Systemic lupus erythematosus	C1QB HLA-DQA2 C1QA HIST4H4	8.14
hsa04610	Complement and coagulation cascades	C1QB C1QA	8.14
hsa05140	Leishmaniasis	HLA-DQA2 IL4	7.69
hsa04664	Fc epsilon RI signaling pathway	IL4 MS4A2	7.11
hsa04640	Hematopoietic cell lineage	IL5RA IL4	6.38
hsa04630	Jak-STAT signaling pathway	IL5RA CLC IL4	5.43
hsa05142	Chagas disease (American trypanosomiasis)	C1QB C1QA	5.40
hsa04060	Cytokine-cytokine receptor interaction	IL5RA CCR3 CLC IL4	4.24
hsa04145	Phagosome	HLA-DQA2 TUBB2A	3.60

*HC: healthy control group; CVA: cough variant asthma group.

Table 5 Top 15 enriched pathways between the CA and CVA groups.

Pathway ID	Description	Genes	Enrich factor
hsa00770	Pantothenate and CoA biosynthesis	ENPP3 VNN1	20.47
hsa05310	Asthma	FCER1A IL4 MS4A2	15.84
hsa05020	Prion diseases	C1QB EGR1	9.10
hsa04512	ECM-receptor interaction	ITGA2B GP6 ITGB5 GP5	7.70
hsa05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	ACTN3 ITGA2B ITGB5	6.64
hsa04664	Fc epsilon RI signaling pathway	FCER1A IL4 MS4A2	6.22
hsa05322	Systemic lupus erythematosus	ACTN3 ELANE C1QB CTSG HIST1H3J	5.93
hsa05410	Hypertrophic cardiomyopathy (HCM)	ITGA2B ITGB5 TTN	5.92
hsa04640	Hematopoietic cell lineage	ITGA2B GP5 IL4	5.58
hsa05414	Dilated cardiomyopathy	ITGA2B ITGB5 TTN	5.46
hsa05140	Leishmaniasis	FOS IL4	4.49
hsa04520	Adherens junction	ACTN3 WASF3	4.49
hsa04510	Focal adhesion	ACTN3 ITGA2B PARVB ITGB5	3.27
hsa05142	Chagas disease (American trypanosomiasis)	C1QB FOS	3.15
hsa05146	Amoebiasis	ACTN3 CTSG	3.09

*CA: classical asthma group; CVA: cough variant asthma group.

Table 6 Comparison of the relative expression of candidate genes in groups.

Gene	Group		
	HC (M ±SD) for $2^{-\Delta C_t}$	CA (M ±SD) for $2^{-\Delta C_t}$	CVA (M ±SD) for $2^{-\Delta C_t}$
CTSG	7.59E-04 ± 6.68E-04	9.50E-03 ± 1.04E-02	1.56E-03 ± 1.64E-03
DEFA4	4.88E-03 ± 3.95E-03	6.68E-02 ± 7.27E-02	5.00E-03 ± 3.63E-03
EGR1	1.88E-01 ± 3.22E-01	6.22E-03 ± 3.30E-03	4.25E-02 ± 4.39E-02
FCER1A	7.75E-02 ± 2.47E-02	5.29E-02 ± 2.66E-02	1.45E-01 ± 5.26E-02
CAMP	1.66E-02 ± 1.39E-02	2.90E-01 ± 2.85E-01	2.93E-02 ± 1.73E-02
GOS2	6.60E-02 ± 1.16E-01	1.14E-03 ± 4.15E-04	1.62E-02 ± 1.61E-02
HDC	8.89E-03 ± 3.40E-03	7.44E-03 ± 5.29E-03	2.86E-02 ± 1.47E-02
IL4	3.75E-04 ± 3.28E-04	3.44E-04 ± 2.21E-04	1.66E-03 ± 8.63E-04
LTF	8.95E-03 ± 9.84E-03	1.29E-01 ± 1.23E-01	1.56E-02 ± 1.03E-02
TFF3	1.88E-04 ± 1.61E-04	3.46E-03 ± 3.36E-03	4.69E-04 ± 2.97E-04

*HC: healthy control group; CA: classical asthma group; CVA: cough variant asthma.

The gene expression levels of FCER1A, HDC and IL4 in the CVA group were upregulated compared with those in the CA group ($P < 0.05$). Compared with those in the HC group, the expression levels of the three genes were also upregulated in the CVA group ($P < 0.05$).

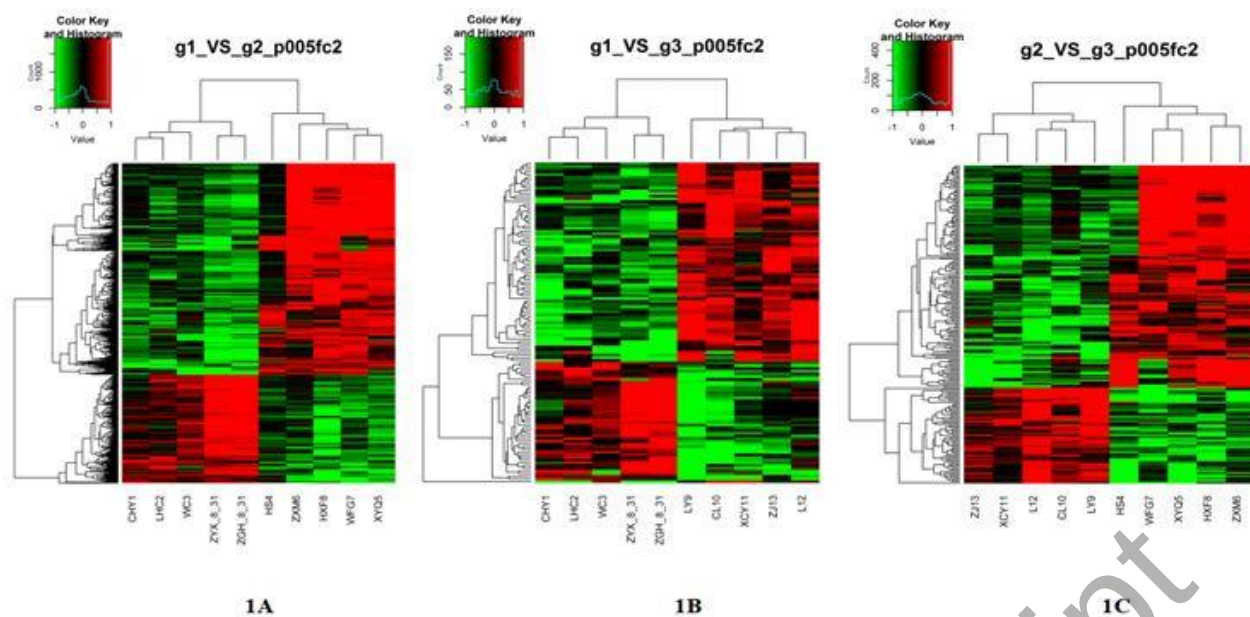


Figure 1 Heatmap of the groups Gene expression heatmap of the groups. (1 A) Heatmap between the HC group and the CA group. (1B) Heatmap between the HC group and the CVA group. (1 C) Heatmap between the CA group and the CVA group. Green indicates low expression, and red indicates high expression. Samples in group g1 (HC, healthy control): CHY1, LHC2, WC3, ZYX.8_31, ZGH.8_31. g2 (CA, classical asthma): HS4, ZXM6, HXF8, WFG7, XYQ5. g3 (CVA, cough variant asthma): LY9, CL10, XCY11, ZJ13, L12.

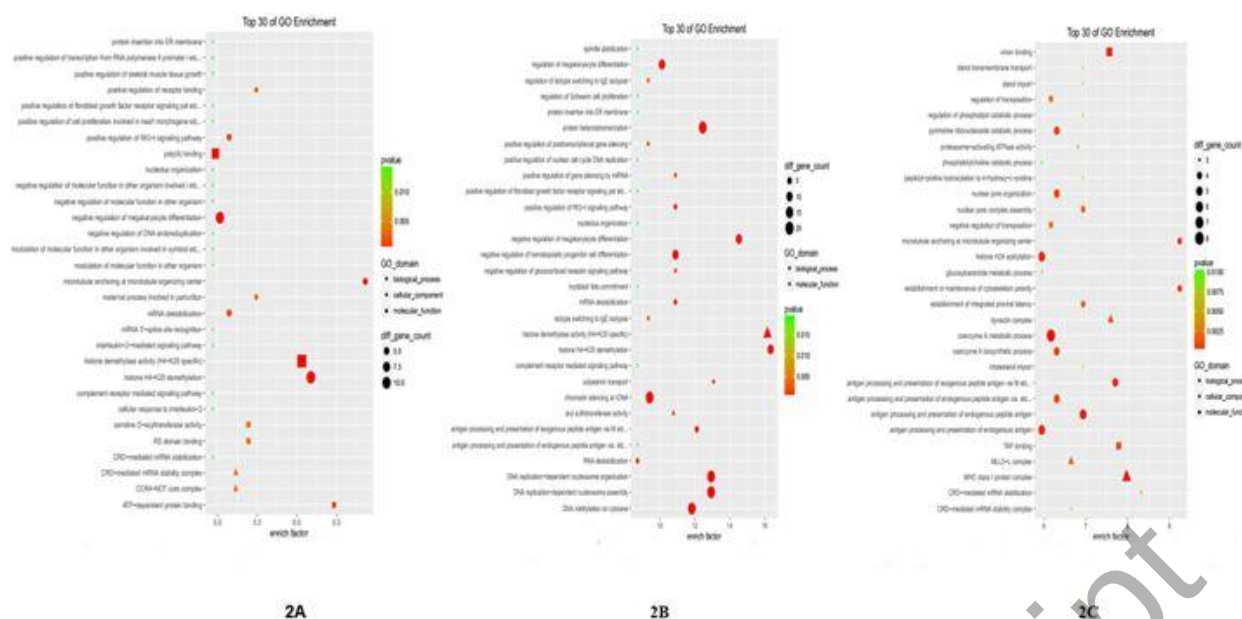


Figure 2 Top 30 GO enrichment terms between groups (2 A) Top 30 GO enrichment terms between the HC group and the CA group, (2B) Top 30 GO enrichment terms between the HC group and the CVA group, (2 C) Top 30 GO enrichment terms between the CA group and the CVA group. HC: healthy control group; CA: classical asthma group; CVA: cough variant asthma group.

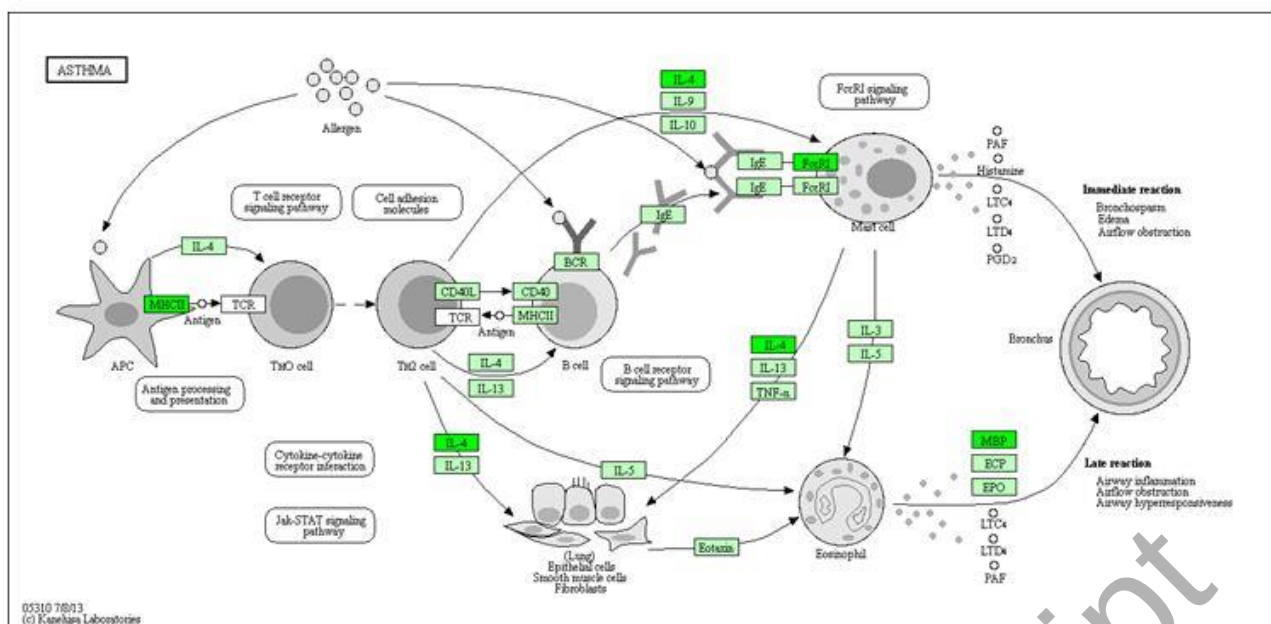


Figure 3 Changes of KEGG pathway of ‘asthma’ between the CA and the CVA group Green represents downregulated genes. Light green represents the genes are present in this species. CA: classical asthma group; CVA: cough variant asthma group.