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ORIGINAL RESEARCH

Induced Sputum Transcriptomics Profile and Serum C3 are Associated with Asthma Severity

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Rational: Asthma severity assessment is essential for asthma management. Transcriptomics contributes substantially to asthma pathogenesis. Then, this study aimed to explore asthma severity-associated transcriptomics profile and promising biomarkers for asthma severity prediction.

Methods: In discovery cohort, induced sputum cells from 3 non-severe and 3 severe asthma patients were collected and analyzed using RNA-seq. Multivariate analysis was performed to explore asthma severity-associated transcriptomics profile and differential expressed genes (DEGs). The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were used for pathway enrichment analysis. Subsequently, based on the previous study and clinical experience, the mRNA expressions of 6 overlapped asthma severity-associated DEGs and *C3* in induced sputum cells and serum C3 were verified in validation cohort.

Results: Distinct asthma severity-associated transcriptomics profile was identified in induced sputum cells in discovery cohort. Then, 345 DEGs were found, of which 38 terms and 32 pathways were enriched using GO and KEGG, respectively. In validation cohort, the mRNA expressions of *ZNF331, CD163, MACC1, ADAMTS2*, and *C3* were increased, and *RYR1* and *NRXN3* were decreased in induced sputum cells in severe asthma. Meanwhile, the AUC of ROC was 0.890 for serum C3 in asthma severity prediction, with the best cut-off of 1.272 g/L.

Conclusion: Collectively, this study provides the first identification of the association between induced sputum cells transcriptomics profile and asthma severity, indicating the potential value of transcriptomics for asthma management. The study also reveals the promising value of serum C3 for predicting asthma severity in clinical practice.

Keywords: asthma severity, induced sputum, RNA-seq, serum C3, ACT scores, FeNO

Introduction

Asthma, one of the most common chronic airway inflammatory diseases, is characterized by reversible expiratory airflow limitation and recurrent respiratory presentations, such as wheezing, cough, short of breath, and chest tightness.[1,2] The global prevalence of asthma was proximately 1%~18% in different regions and areas, affecting about 300 million people.[2,3] Meanwhile, asthma is also a highly heterogeneous disease with diverse severity and multiple subtypes.[2,4] Recently, a handful of studies showed that transcriptomics is essential for the etiology, pathogenesis, and diversity of asthma.[5–7] In a clinical study, Zeng et al revealed that a noticeable blood transcriptomics profile was associated with severe asthma patients, who were featured with eosinophil-low/symptom-high and more oral corticosteroids.[6] Meanwhile, they also found that compared to non-severe asthma, 4,162 DEGs (2,057 upregulated genes and 2,105 downregulated genes) in severe asthma were identified. In an animal

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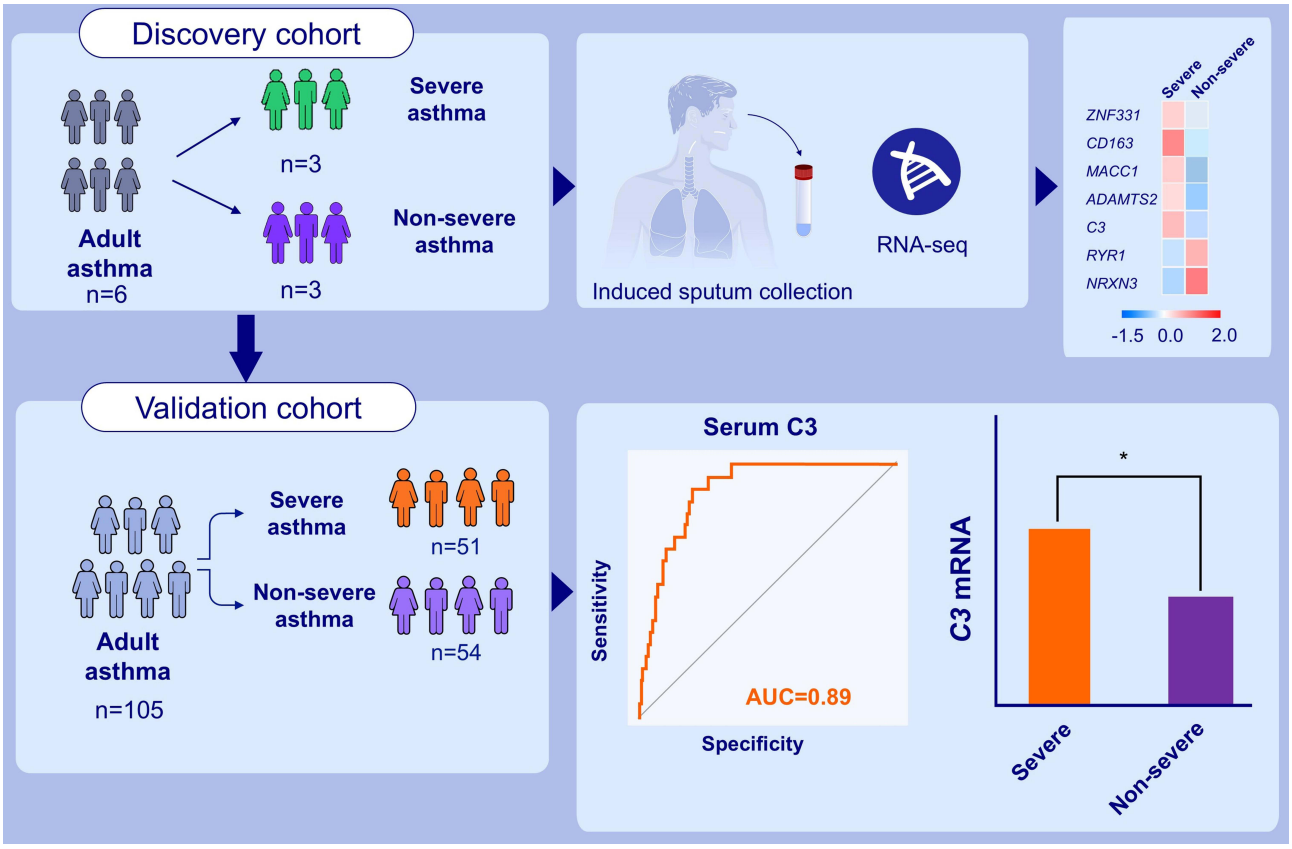
Graphical Abstract



study, Zhang et al showed that lung transcriptomics profile was markedly altered in ovalbumin (OVA)-induced asthmatic mice, which identified 4,233 asthma status-associated DEGs, including 2,099 upregulated genes and 2,134 downregulated genes.[7]

Induced sputum is a commonly used technic for identifying the subtypes and airway inflammatory endotypes in lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis.[5,8–10] Apparently, induced sputum can more directly and precisely reflect the pathological alterations of lower airway and lung parenchyma than blood sample.[11] Compared to spontaneous sputum, induced sputum has less contamination and confounders, such as saliva, which is more accurate.[5,12] Currently, the cellular profile of induced sputum is used to differentiate the phenotype of asthma.[2] Some studies revealed that sputum omics are promising and prospective tools for the diagnosis and assessment of airway diseases, particularly asthma and COPD.[5,13] Liu et al demonstrated that different inflammatory phenotypes of asthma showed distinct metabolomics profiles in induced sputum.[13] Simultaneously, in our previous study, a significant difference in metabolomics profile between moderate COPD and severe COPD was found in induced sputum.[5]

Since transcriptomics plays a key role in asthma, and induced sputum is a suitable and accessible sample for the effective and accurate assessment of asthma severity with high safety and tolerability in clinical practice. Consequently, the main purpose of this study was to explore the role of transcriptomics profile of induced sputum in the determination of asthma severity and to identify the potential biomarkers which can accurately predict asthma severity.

Materials and Methods

Study Design and Sites

This observational study was carried out at Respiratory Medicine and Critical Care Medicine of Suining Central Hospital and Respiratory Medicine and Critical Care Medicine of Zhongshan Hospital of Fudan University, from April 2019 to

November 2023. The study protocol was approved by the Research Ethics Committees of Zhongshan Hospital of Fudan University (No. B2018-196R). Meanwhile, this study was conducted according to the Declaration of Helsinki. Informed consent was obtained from all the patients by the responsible physician or an appropriately trained staff member. The patients provided written informed consent to participate in this study. Meanwhile, the past history of all asthma patients was obtained from our hospital databases and reviewed by the responsible physician.

Inclusion and Exclusion Criteria

The inclusion criterion was adult patient with asthma, based on the Global Initiative for Asthma (GINA) guideline.[2] Exclusion criteria were as follows: age <18 years old, respiratory tract infection in the past 4 weeks, exacerbation of asthma in the past 4 weeks, other respiratory diseases [including chronic obstructive pulmonary disease (COPD), bronchiectasis, pneumoconiosis, active pulmonary tuberculosis (TB), interstitial lung diseases (ILDs), etc.], other chronic diseases [including diabetes, hypertension, coronary artery disease (CAD), etc.], immunocompromised status [organ transplants, immunosuppressive agents use within the last 4 weeks, and human immunodeficiency virus (HIV) infection], history of malignant diseases, renal dysfunction, liver dysfunction, individuals with pregnancy, and the patients were unable to perform the required measurements (Figure 1).

Definitions

According to the GINA, the asthma diagnosis was established by the pulmonologists.[2] Severe asthma was defined as asthma that requires high-dose treatment with inhaled corticosteroids (ICS) and additional controllers, such as long-acting inhaled β2 agonists (LABA) and montelukast, or by oral corticosteroid treatment to prevent it from becoming "uncontrolled", or asthma that remains "uncontrolled" despite these treatments.[2,14,15] Allergic rhinitis and other allergic

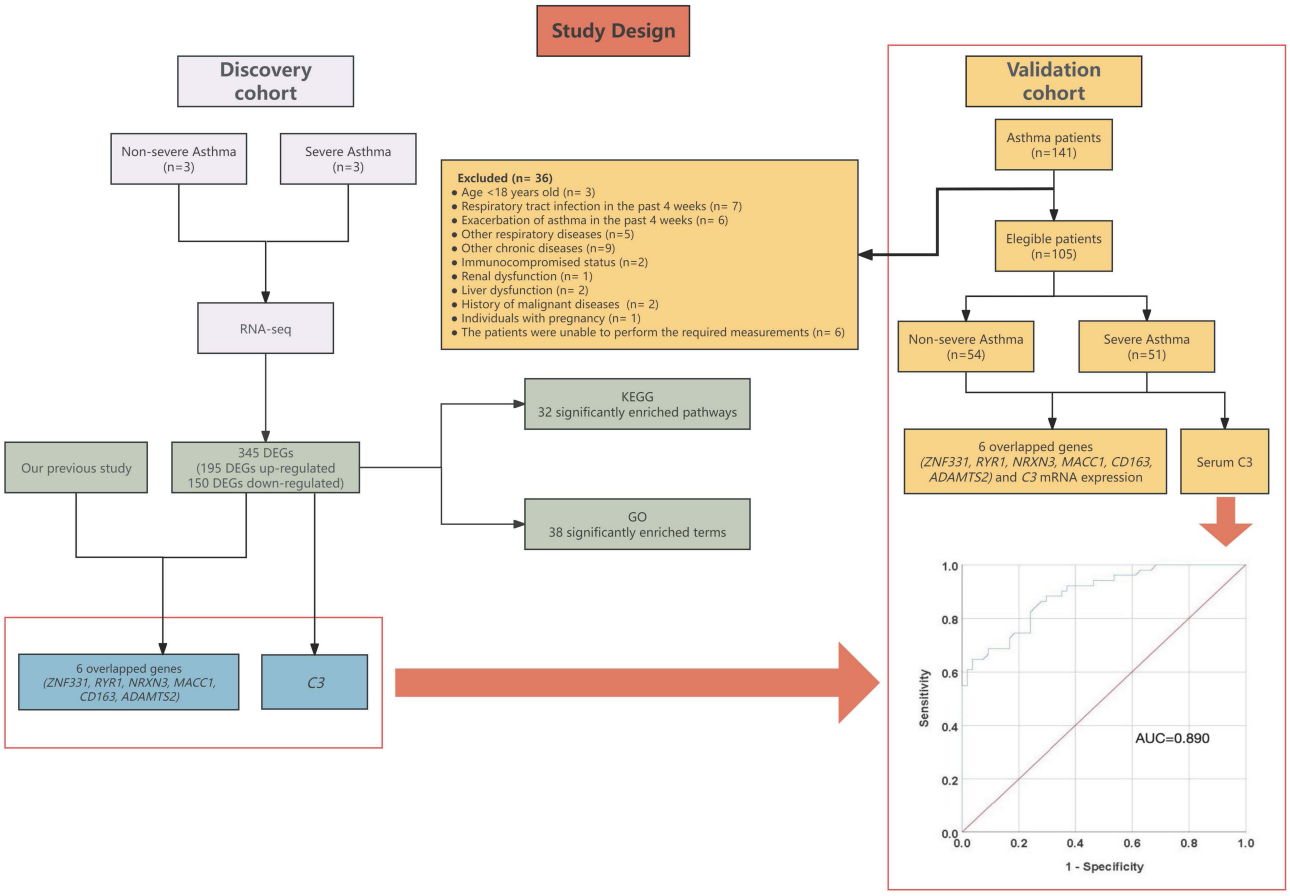


Figure 1 Summary of study design and major findings.

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diseases were defined as having a physician's diagnosis. Ex-smoker was defined as quitting smoking more than 6 months before data collection.[16–18] Fractional exhaled nitric oxide (FeNO) was measured by Nano Coulomb expiration Analyzer (Sunvou CA2122, Wuxi, China). Total serum IgE were measured by ImmunoCAP fluoroenzyme immunoassay (Phadia ThermoFisher Scientific).

Data Collection

Demographics, lung function tests, FeNO, ACT scores, current therapy (GINA treatment steps), blood routine, and serum IgE data were all collected. All patients underwent chest high-resolution CT (HRCT) scan within 48 hours after enrolling in the study, which was used to exclude most other lung diseases.

Quantification of Serum C3

According to our previous studies[19–21] and the manufacturer's instructions, the level of serum C3 was measured by ELISA (ab108822, Abcam, UK).

Induced Sputum Collection

According to our previous study,[5] induced sputum was collected within 12 hours after enrolling in the study. In brief, 3% NaCl solution was nebulized to induce sputum. After the cells in sputum were collected, they were stored in liquid nitrogen for further analysis.

Induced Sputum Cells RNA-Seq Analysis

According to previous reports,[22,23] the cells in induced sputum were assayed using RNA-seq. Briefly, cells were harvested, and RNA was extracted with the Trizol reagent (Invitrogen, USA). The Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE) and 2100 Bioanalyzer (Agilent, Santa Clara, CA) were used for precise RNA quantification and integrity assessment. Then, poly-A oligo (dT) beads were used to purify mRNA. According to the manufacturer's instruction, the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Massachusetts, USA) was employed to prepare RNA libraries. Meanwhile, cDNA concentration was quantified with the Qubit dsDNA HS Analysis Kit (Invitrogen, Eugene, OR, USA). Lastly, the samples were submitted for an in-depth pair-end sequencing of 2 × 150bp on the Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA). Next, TopHat2 was employed for conducting sequence alignment analysis, meticulously controlling the alignment position, direction, and quality of each read or pair-end read.[24] Subsequently, the Cufflinks were utilized for the assembly and splicing of the aligned results.[25] The assembled transcripts were then aligned with known genome annotations to ensure precision. The RSEM software was then applied to procure Read Counts for each transcript with the genome annotation file.[26] Ultimately, through the conversion of Read Counts into FPKM values, standardized transcript expression levels were obtained, ensuring the accuracy of our transcriptome data for subsequent analysis.

Differentially expressed (DE) genes were identified using DESeq.[26] Meanwhile, the genes with FDR adjusted P-value (q-value) <0.05 and $\left|\log2FC\right|>2.32$ were considered significantly different. KEGG pathway ("clusterProfiler" package 3.8.0) and GO functional enrichment ("clusterProfiler" package 3.8.0) analyses were performed using R software (version 4.1.2).

Quantitative PCR (qPCR)

The mRNA expression of *ZNF331, RYR1, NRXN3, MACC1, CD163, ADAMTS2*, and *C3* in induced sputum cells were measured by qPCR. Meanwhile, GAPDH was used as an internal reference. The primers were synthesized by Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA synthesis was carried out using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA) with the isolated RNA. QPCR was performed using aliquots of cDNA equivalent to 5 ng of total RNA samples. The mRNA expression levels of the target genes were normalized to GAPDH expression in each sample. QPCR analyses were conducted using the CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with SYBR Green I PCR reagents (QIAGEN, Germany). Changes in the expression of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method,[20,27] where $\Delta\Delta Ct = (Ct_{target}\text{-}Ct_{GAPDH})_{sample}\text{-}(Ct_{target}\text{-}Ct_{GAPDH})_{control}$. The primer sequences were listed in Table S1.

Clinical Data Analysis

Clinical data were analyzed using R software (version 4.1.2). Categorical data were expressed as the frequencies. Continuous data with normal distribution were presented by mean ± standard deviation (SD). Then, continuous variables with abnormal distribution were presented as median and interquartile range [Mean (Q1, Q3)]. Categorical variables were analyzed by chi-square test. Continuous variables with normal distribution were analyzed by Student *t* test. Ordinal variables and continuous variables without normal distribution were analyzed by Mann–Whitney *U*-test. The data distribution was examined by Kolmogorov–Smirnov test. The Spearman rank correlation coefficient was used for the correlation analyses. $P < 0.05$ was considered to be statistically significant.

Results

Demographics and Laboratory Parameters of Asthma Patients in Discovery Cohort and Validation Cohort

In the discovery cohort, 3 patients with severe asthma and 3 patients with non-severe asthma were included. Compared to non-severe asthma patients, severe asthma patients had lower ACT scores, higher level of GINA steps, FeNO, blood EOS counts, EOS%, and serum IgE (Table 1). In validation cohort, 141 asthma patients were recruited. Ultimately, 51 patients with severe asthma and 54 patients with non-severe asthma were included (Figure 1). Compared to non-severe asthma

Table 1 Demographic Characteristics of Asthma Patients

	Discovery Cohort				**Validation Cohort**			
	Non-Severe Asthma (n=3)	**Severe Asthma (n=3)**	**Statistical Values**	***P***	**Non-Severe Asthma (n = 54)**	**Severe Asthma (n = 51)**	**Statistical Values**	***P***
Sex [male, n (%)]	2 (66.7%)	2 (66.7%)	0.000	1.000	22 (40.7%)	14 (27.5%)	2.056	0.152
Age (years)	43.67 ± 10.50	45.33 ± 8.74	−0.211	0.843	45.65 ± 14.37	48.63 ± 13.16	−1.106	0.271
Body mass index (BMI)	24.54 ± 1.93	24.43 ± 1.03	0.082	0.939	23.85 ± 3.99	24.01 ± 3.49	−0.217	0.828
Smoking status			0.000	1.000			−0.841	0.400
Non-smoking	3 (100%)	3 (100%)			42 (77.8%)	42 (82.4%)		
Ex-smoking	0 (0%)	0 (0%)			3 (5.6%)	7 (13.7%)		
Current-smoking	0 (0%)	0 (0%)			9 (16.7%)	2 (3.9%)		
Drinking	0 (0%)	0 (0%)	0.000	1.000	19 (35.2%)	11 (21.6%)	2.383	0.123
Allergic rhinitis	1 (33.33%)	3 (66.67%)	−3.000	0.083	19 (35.2%)	34 (66.7%)	−10.399	0.001
Other allergic diseases [n (%)]	3 (100%)	1 (33.3%)	3.000	0.083	7 (13.0%)	5 (9.80%)	0.259	0.611
Lung function								
FEV1%pre	82.31 ± 22.22	42.49 ± 20.19	2.297	0.083	88.45 (82.53, 98.82)*	80.3 (73.1, 93.75)*	2.783	0.005
FEV1/FVC%	64.82 ± 10.91	45.76 ± 13.46	1.906	0.129	81.02 ± 7.65	78.34 ± 7.49	1.81	0.073
FeNO (ppb)	22.00 ± 4.36	50.33 ± 14.36	−3.269	0.031	30 (18.75, 45.75)*	48 (29, 64)*	−3.248	0.001
ACT score	22.33 ± 1.53	15.00 ± 2.00	5.047	0.007	20 (20, 22)*	15 (13, 17)*	8.96	0.000
GINA step [n (%)]			−2.236	0.000			−9.162	0.000
1	0 (0%)	0 (0%)			0 (0%)	0 (0%)		
2	0 (0%)	0 (0%)			35 (64.8%)	0 (0%)		
3	3 (100%)	0 (0%)			19 (35.2%)	0 (0%)		
4	0 (0%)	0 (0%)			0 (0%)	26 (51.0%)		
5	0 (0%)	3 (100.00%)			0 (0%)	25 (49.0%)		
Blood routine								
WBC ($\times 10^9$/L)	7.65 ± 0.77	8.67 ± 0.49	−1.943	0.124	-	-	-	-
EOS ($\times 10^9$/L)	0.23 ± 0.05	0.50 ± 0.10	−4.061	0.015	-	-	-	-
EOS%	3.03 ± 0.40	5.80 ± 1.31	−3.501	0.025	-	-	-	-
Serum IgE (IU/mL)	76.67 ± 25.11	225.67 ± 57.32	−4.124	0.015	-	-	-	-

Notes: *Data presented as Median (Q1, Q3).

Abbreviations: FVC: Forced expiratory volume; FEV1: forced expiratory volume in one second; FeNO: Fractional exhaled nitric oxide; ACT: Asthma control test, EOS: Eosinophils; WBC: White blood cells.

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patients, the incidence of allergic rhinitis, FeNO level, and GINA treatment steps were higher, FEV1%pre and ACT scores were lower in severe asthma patients (Table 1).

Identification of Asthma Severity-Associated Differential Expressed Genes (DEGs) in Induced Sputum Cells in Discovery Cohort

In discovery cohort, 28,331 genes were identified using RNA-seq in induced sputum cells. Firstly, PCA score plot showed distinct dissimilarities in the induced sputum cells transcriptomics profiles between severe asthma and non-severe asthma (Figure 2A). Secondly, 345 asthma severity-associated DEGs in induced sputum cells were found (Table S2). Among them, 195 genes were up-regulated, and 150 genes were down-regulated in severe asthma compared to non-severe asthma (Figure 2B, Table S2). Meanwhile, the heat map was used to classify the up-regulated and down-regulated DEGs in patients with severe asthma compared to those with non-severe asthma (Figure S1).

Pathway Analysis of DEGs in Discovery Cohort

GO function analysis (Figure 3A) and KEGG pathway analysis (Figure 3B) were used to categorize and describe the biological functions of genes and gene products. Then, a total of 38 significantly enriched terms were identified by GO analysis (q value <0.05) (Table S3), including Inflammatory response, T cell costimulation, G-protein coupled receptor signaling pathway, complement receptor mediated signaling pathway, etc. Then, a total of 32 significantly enriched pathways were revealed by KEGG analysis (q value <0.05) (Table S4), encompassing Asthma, Antigen processing and presentation, Th1 and Th2 cell differentiation, IL-17 signaling pathway, etc.

The Overlapped Genes Between Asthma Severity-Associated DEGs in Induced Sputum Cells and Asthma Severity-Associated DMPs Annotated Genes in Nasal Epithelium

To further select severe asthma-associated genes and the potential biomarkers for asthma severity, the overlapped genes between 345 asthma severity-associated DEGs in induced sputum cells in current study and 397 asthma severity-associated differentially methylated positions (DMPs) annotated genes in nasal epithelium in our previous study were explored (Figure S2).[4] Then, 6 overlapped genes were identified, which were *ZNF331, RYR1, NRXN3, MACC1, CD163*, and *ADAMTS2*.

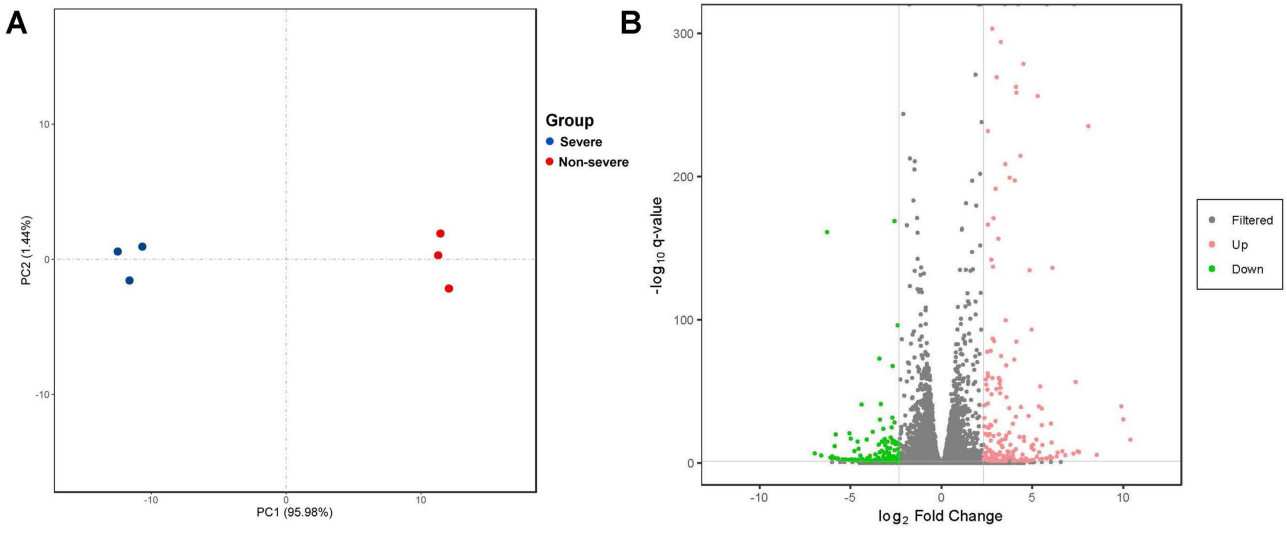

Figure 2 Principal component analysis (PCA) plot and volcano plot results derived from transcriptomics profiles of discovery cohort. (**A**) PCA score plot; (**B**) Volcano plot. The down-regulated and up-regulated DEGs in severe asthma patients compared to non-severe asthma patients are marked in green and red, respectively. X-axis: log_2 fold change of DEGs; Y-axis: fold change of –log10 q value determined by Student's *t* test.

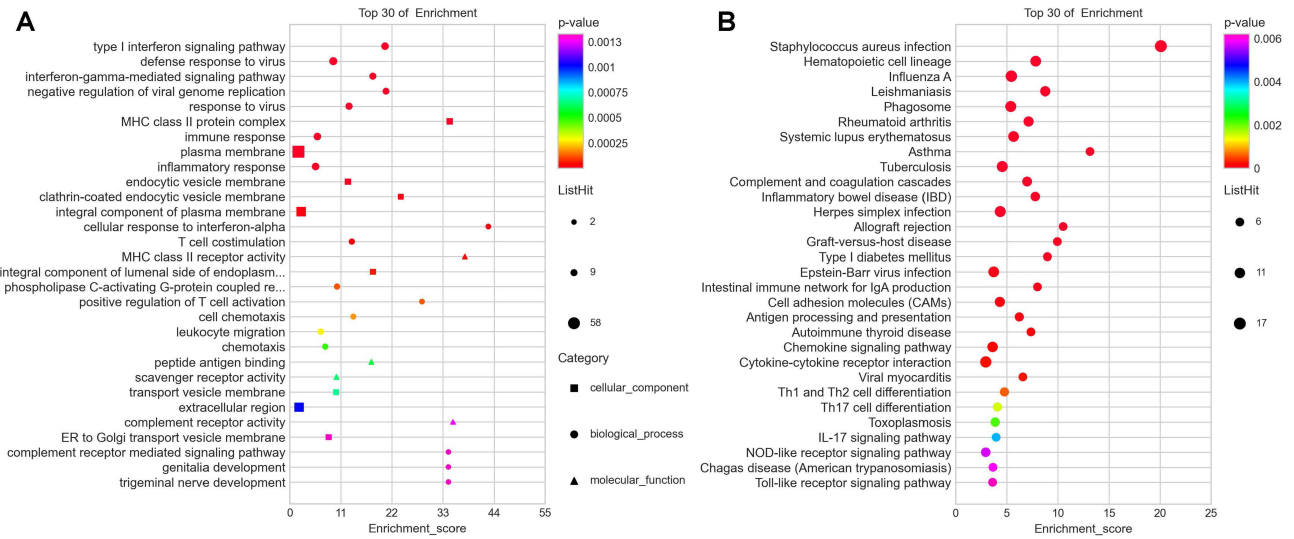

Figure 3 Genes enrichment analysis of 345 asthma severity-associated DEGs in discovery cohort. (**A**) GO pathways. Different geometrical shapes represent GO functional categories. The Y-axis represents GO enrichment terms. (**B**) KEGG pathways. The Y-axis represents different KEGG pathway terms. In both (**A**) and (**B**), geometrical size indicates the number of list hits and color reflects p-values. The X-axis represents enrichment score.

The Selected Genes Expression in Induced Sputum Cells in Validation Cohort

Simultaneously, to verify the results of RNA-seq in the discovery cohort, the mRNA expressions of these above mentioned 6 genes and *C3* in induced sputum cells were measured in validation cohort. Compared to non-severe asthma, the expressions of *ZNF331, CD163, MACC1, ADAMTS2*, and *C3* were significantly increased, and *RYR1* and *NRXN3* were noticeably reduced in severe asthma (Figure 4A–G).

Predictive Value of Serum C3 for Predicting Asthma Severity in Validation Cohort

It is found that C3 plays a key role in pathogenesis and development of asthma.[28] Of note, *C3* was one of asthma severity-associated DEGs in discovery cohort (Table S2). Thus, serum C3 was measured in validation cohort. The level of serum C3 in severe asthma patients was markedly higher than in non-severe asthma patients (Figure 4H). For serum C3 in asthma severity prediction, the AUC of ROC was 0.890 (Figure 5A). The Youden index was 0.610, with the best cut-off concentration of 1.272 g/L (Figure 5B).

The Correlations Between Serum C3 and Lung Function, ACT Scores, FeNO, and GINA Steps in Validation Cohort

The correlations between serum C3 and FeNO, ACT score, lung functions, and GINA steps were explored using Spearman correlation in validation cohort. The data showed that serum C3 was significantly positively correlated with FeNO and GINA steps, and noticeably negatively correlated with ACT scores (Table 2).

Discussion

In this study, 3 severe asthma patients and 3 non-severe asthma patients were enrolled in the discovery cohort. Meanwhile, 51 severe asthma patients and 54 non-severe asthma patients were included in the validation cohort. In the discovery cohort, transcriptomics in induced sputum cells were analyzed using RNA-seq. Then, we found that 345 DEGs were associated with asthma severity. Furthermore, among these DEGs, 38 terms were enriched using GO, and 32 pathways were enriched by KEGG. Of note, *C3* was identified as one of asthma severity-associated DEGs, which plays a pivotal role in asthma pathogenesis and development.[28,29] Next, 6 overlapped genes, *ZNF331, RYR1, NRXN3, MACC1, CD163*, and *ADAMTS2*, were revealed between asthma severity-associated DEGs in induced sputum cells and asthma severity-associated DMPs annotated genes in nasal epithelium in our previous study.[4] Subsequently, in the validation cohort, we found that the mRNA expressions of *ZNF331, CD163, MACC1, ADAMTS2*, and *C3* were markedly up-

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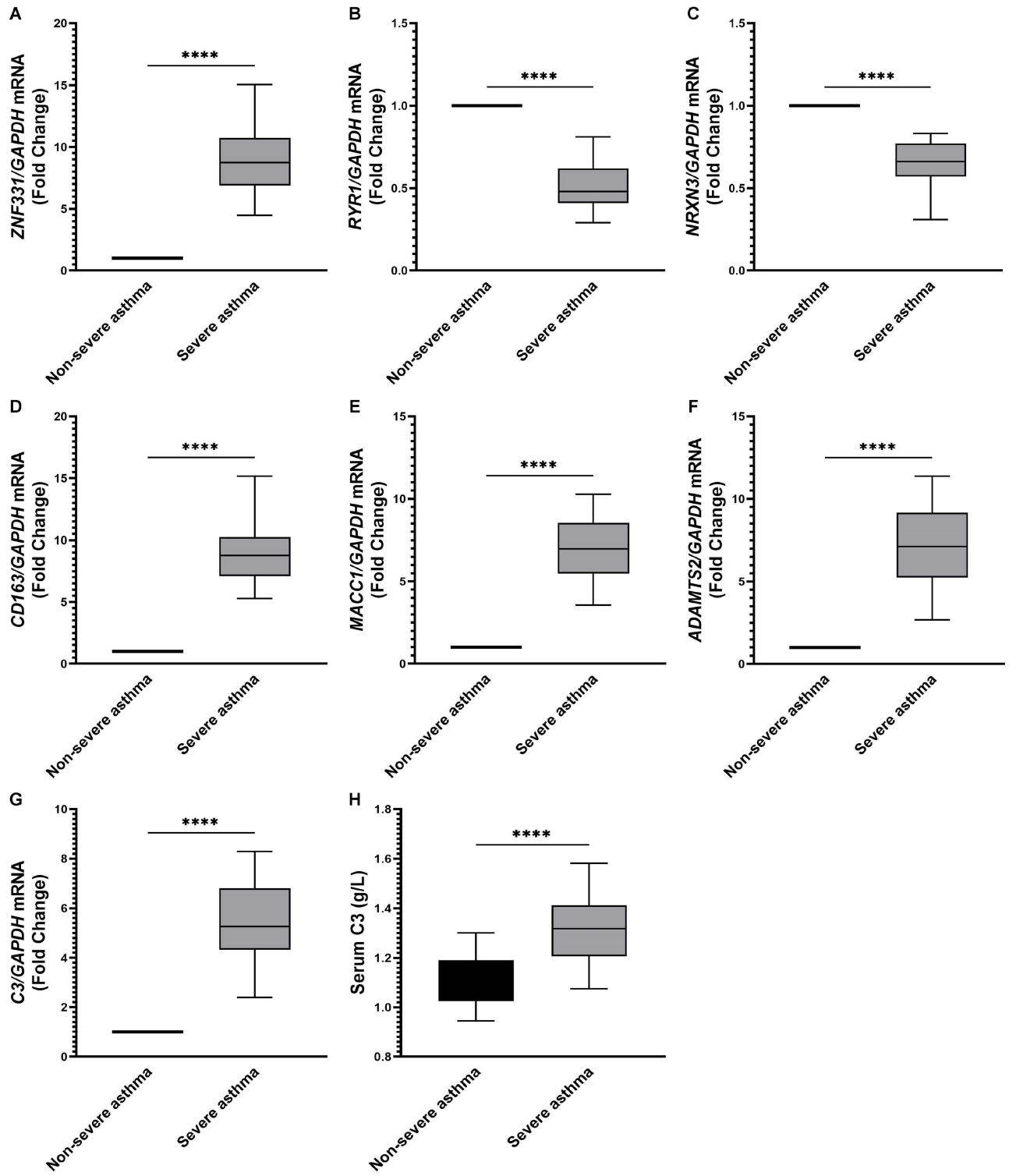
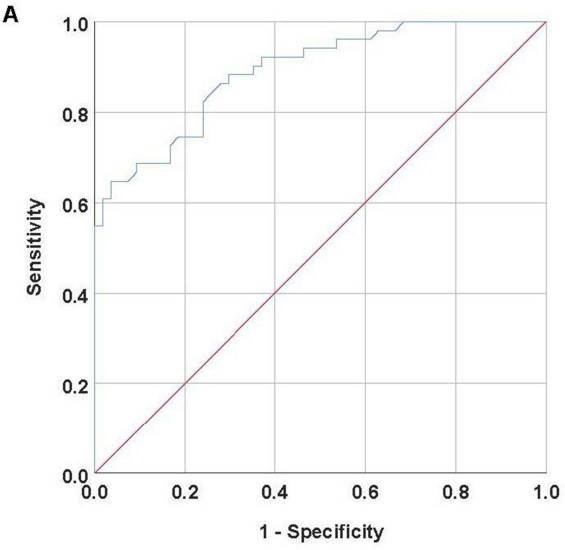


Figure 4 The mRNA expression of *ZNF331*, *RYR1*, *NRXN3*, *MACC1*, *CD163*, *ADAMTS2*, and *C3* in induced sputum cells and serum C3 in validation cohort. (**A**) *ZNF331* mRNA; (**B**) *RYR1* mRNA; (**C**) *NRXN3* mRNA; (**D**) *MACC1* mRNA; (**E**) *CD163* mRNA; (**F**) *ADAMTS2* mRNA; (**G**) *C3* mRNA; (**H**) Serum C3 level. **** $P < 0.001$.

regulated, and *RYR1* and *NRXN3* were noticeably down-regulated in severe asthma compared to those with non-severe asthma in induced sputum cells. Additionally, serum C3 that exhibited promising value for the prediction of asthma severity was revealed. We also found that the level of serum C3 was significantly positively correlated with FeNO and GINA steps, and noticeably negatively correlated with ACT scores.

A



B

The sensitivity and specificity of serum C3 in prediction of severe asthma

	Sensitivity	Specificity	Youden index	Cut-off value
Serum C3 (g/L)	0.647	0.963	0.610	1.272

Figure 5 The ROC curve of serum C3 in predicting asthma severity in validation cohort. (**A**) ROC curve; (**B**) Sensitivity, specificity, Youden index, and best cut-off value.

Asthma is one of most globally prevalent airway disorders, and it has a substantial impact on quality of life. It is estimated that 6%–7% of adults had asthma, affecting around 334 million people worldwide.[1,3] Recent years, the prevalence of asthma has continued to increase in China.[30] In the national cross-sectional China Pulmonary Health (CPH) study, Huang et al found that the overall prevalence of asthma was 4.2% (95% CI 3.1–5.6%),[30] which affected 45.7 million Chinese adults. Additionally, asthma is chronic disease with a highly heterogeneity and a wide range of severity. Then, it is critical for severity evaluation in asthma management. Mounting evidence revealed that transcriptomics contributed substantially to different aspects of asthma.[6,31–36] In a bioinformatic study, DNA methylation (DNAm) and gene expression data of bronchial epithelial cells (BECs) from Gene Expression Omnibus (GEO) database were further analyzed.[32] It was found that a cluster of genes, encompassing *INAGL1, SERPINE1, TLR5, SLC9A3* and *CD9*, were associated with asthma severity. Meanwhile, they also identified that a group of genes, such as *INAGL1, TLR5*, and *CD9*, were related to lung function. In a randomized, controlled, single-blind, multicenter study in US, 301 adult patients with severe asthma were enrolled.[6] A unique blood transcriptomics prolife of type-2 (T2) cytokine-low symptom-high asthma was revealed, which encompassed 627 DEGs. Cao et al developed a 73 DEGs-composed RNA-seq-based transcriptomic risk score (RSRS) system to identify asthmatics from healthy subjects.[31] Then, based on Connectivity map analysis, it was found that mepacrine and dactolisib were potential drugs, and *PAK1, GSR, RBM15* and *TNFRSF12A* were promising therapeutic target for asthma. Nevertheless, the association between the transcriptomics

Table 2 The Correlations Between Serum C3 and FeNO, ACT Score, FEV1%, FEV1/FVC%, and GINA Steps in Validation Cohort

	FeNO (ppb)	ACT score	FEV1%	FEV1/FVC%	GINA Steps
Serum C3 (g/L)					
R	0.383	−0.582	−0.057	−0.102	0.580
P	<0.001	<0.001	0.564	0.299	<0.001

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profiles of induced sputum and asthma severity has not been previously studied. This study aimed to explore the DEGs in induced sputum cells between non-severe asthma and severe asthma in adults.

Induced sputum is a reliable and repeatable approach, which can directly reflect lung pathophysiological alterations.[5,37] It has been widely used in the diagnosis, severity evaluation, and drug response monitor of several lung diseases, particularly COPD, asthma, and chronic cough.[5,12,38–40] In our previous study, we found that 573 differential metabolites and glycerophospholipid metabolism pathway in induced sputum were associated with COPD severity.[5] In current study, in discovery cohort, our RNA-seq results showed that 345 DEGs were associated with asthma severity in induced sputum cells. Meanwhile, 32 enriched pathways were identified by KEGG. Some pathways, such as Asthma, Antigen processing and presentation,[41] Th1 and Th2 cell differentiation,[42] Th17 cell differentiation,[43] IL-17 signaling pathway,[44] Toll-like receptor signaling pathway,[45,46] etc, are essential for asthma. Simultaneously, 38 enriched terms were revealed by GO analysis. The significantly enriched terms-associated with asthma included immune response, inflammatory response, T cell costimulation, complement receptor mediated signaling pathway, phospholipase C-activating G-protein coupled receptor signaling pathway, etc.

Mounting evidence proved that airway epithelium was much more than a simple air-conducting structure, more importantly, played a hub role in the initiation and control of the immune responses to different types of environmental exposures contributing to asthma pathogenesis.[47,48] Recently, a handful of studies confirmed that abnormal epigenetic mechanisms in airway epithelium, such as DNA methylation (DNAm), histone modifications, non-coding RNAs (ncRNAs), etc., were critical for the different aspects of asthma.[47–49] Briefly, when an exposure (eg, allergens) occurs, the underlying genetic sequence does not change in airway epithelial cells. Thus, epigenetic regulators, which can modify gene expression, are essential for responding to these exposures. While regulation of gene expression is inherently a complex process, studies have linked changes in DNAm, histone modifications, and changes in ncRNAs to exposures and airway epithelial cells in asthma.[49] Mounting evidence showed that nasal epithelium is a biologically relevant proxy and useful surrogate for lower airway epithelium in asthma.[47,50] Recently, in a well-designed clinical asthma omics study in African-American children, asthma severity-associated DNAm and transcriptomics profiles in nasal epithelium were identified by its comprehensive data analysis.[4] It was found that 816 differentially methylated positions (DMPs) in nasal epithelium were associated with asthma severity. Furthermore, 398 genes were annotated by these DMPs. Subsequently, based on these findings,[4] we identified that 6 genes were both asthma severity-associated DEGs in induced sputum cells and asthma severity-associated DMPs annotated genes in nasal epithelium, which encompassed *ZNF331, RYR1, NRXN3, MACC1, CD163*, and *ADAMTS2*.

ZNF331, encoding a zinc finger protein, contains Kruppel-associated box (KRAB) domain and plays a critical role in the regulation of transcriptional regulation.[51,52] In bronchial alveolar lavage (BAL) cells, Guan et al found that *ZNF331* was one of overlapped genes between non-severe asthma-associated DEGs and severe asthma-associated DEGs, indicating that *ZNF331* is essential for the pathogenesis and development of asthma.[52] *CD163* is a maker of macrophage activation in many inflammatory diseases, such as COPD,[53] asthma,[54] type 2 diabetes mellitus (T2DM),[55] etc. It was found that CD163-positive macrophages in lung were markedly enhanced in fatal asthma compared to non-asthma participants.[54] Furthermore, their results showed that airway inflammation, airway hyperresponsiveness (AHR), and the levels of IFN-γ and IL-5 in BALF were attenuated in asthmatic mice with CD163-deficient compared to those with wildtype. It is reported that *RYR1* contributed substantially to regulating airway smooth muscle cells (ASMCs) contraction.[56] Savoia et al identified that calcineurin (CaN) mediated ASMC Ca(2+) sparks specifically through RyR1 in a murine model of asthma.[56] *NRXN3*, encoding a membrane protein in the nervous system, is essential for synaptic connections and plasticity.[57] Qi et al revealed that nasal epithelial DNAm of *NRXN3* was associated with asthma in children.[58] MACC1 is a regulator of multiple signal pathways, such as HGF/c-Met pathway, Akt pathway, TWIST1/2 pathway, MAPK pathway, etc.[58,59] In a retrospective study, GWAS data of asthma patients from UK Biobank were analyzed.[60] It is reported that *MACC1* was associated with asthma onset. ADAMTS2, a member of zinc metalloendopeptidases, has a wide biological function through the regulation of extracellular matrix (ECM) component metabolism.[61,62] Rastogi et al revealed that DNA methylation and mRNA expression of *ADAMTS2* was linked to pulmonary function and Th cell activation in obese children with asthma.[63]

Of note, our data revealed that *C3* was one of asthma severity-associated DEGs. Furthermore, serum C3 is also a widely used routine testing item in clinical practice, which is economic, convenient, and repetitive. Number of studies showed that C3 played a major part in several aspects of asthma.[28,29] In the Copenhagen General Population Study (CGPS), a prospective study of the general population, 101,029 individuals were included.[29] Of these, 1238 individuals

were hospitalized due to asthma during follow-up, which median time was 6 years (range 0–11 years). Furthermore, they found that increased plasma C3 was positively correlated with the cumulative incidence of asthma hospitalizations and linked to increased risk of allergic asthma, reduced lung functions, a higher percentage of anti-asthma medication use, low physical activity during leisure time and high levels of inflammatory biomarkers. Meanwhile, in a pilot study in Sweden, Kokelj et al showed that C3 in small airway lining fluid was noticeably increased in inadequately controlled asthma compared to well-controlled asthma.[28] Therefore, the mRNA expression of 6 overlapped genes as well as *C3* was verified in validation cohort. Consistent with the results in discovery cohort, the mRNA expressions of *ZNF331, CD163, MACC1, ADAMTS2*, and *C3* were markedly raised, and *RYR1* and *NRXN3* were noticeably decreased in severe asthma compared to those with non-severe asthma in induced sputum cells. These results indicate that these 7 genes play potential roles in the regulation of asthma severity; thus, further research is warranted to investigate their underlying mechanism in the pathogenesis of asthma.

Concurrently, based on our findings in discovery cohort, the previous studies[28,29] as well as our clinical experience, the role of serum C3 in the prediction of asthma severity was investigated in the validation cohort. Compared to non-severe asthma patients, serum C3 in severe asthma patients significantly increased. ROC curve revealed that serum C3 had a strong specificity (0.963) and a reasonable sensitivity (0.647) for the prediction of asthma severity (AUC=0.890, Youden index=0.610). Furthermore, our data identified that 1.272 g/L was the best cut-off value. Subsequently, our results also revealed the significant positive correlations between serum C3 and FeNO and GINA steps, meanwhile, the significant negative correlations between serum C3 and ACT scores. Therefore, our results suggest that serum C3 is a promising marker to distinguish severe asthma from non-severe asthma. Consequently, future study with a larger population is required to verify these findings.

In the current study, discovery cohort was included to explore asthma severity-associated transcriptomics profile using RNA-seq. Furthermore, data from our previous relevant DNA methylation (DNAm)/RNA-seq study were obtained to screen genes as well as support the relevance of our findings. Secondly, these results were verified in validation cohort. The clinical value of serum C3 in asthma severity prediction was identified in validation cohort. Thirdly, relatively comprehensive data, such as clinical features, lung function, and laboratory parameters, etc, were recorded and collected. Of note, chest HRCT was performed in each patient, which effectively excluded other lung disease and reduced confounders. Lastly, induced sputum directly represents asthmatic lung pathological alterations. Then, these were the major strengths and values of our study. The limitations of this study included several aspects. The limited sample size in discovery cohort is one of the major weaknesses of this study. The differences in cell types and their proportions were not investigated. Furthermore, due to the limitation of bulk RNA-seq, the transcriptomic profiles of different cell types in induced sputum still need further investigation. Additionally, the predictive values of serum C3 for asthma severity should also be replicated in other ethnic groups and populations.

Conclusions

Collectively, a unique transcriptomics profile of severe asthma was revealed in induced sputum. Based on our previous relevant study and other studies, *ZNF331, RYR1, NRXN3, MACC1, CD163, ADAMTS2*, and *C3* were further identified, which was verified and replicated in validation cohort. Consistent with the results in discovery cohort, the mRNA expression of *ZNF331, CD163, MACC1, ADAMTS2*, and *C3* were up-regulated, and *RYR1* and *NRXN3* were down-regulated in severe asthma in the validation cohort. These results imply that these 7 genes are essential for the progression of asthma, which are potential biomarkers and therapeutic targets of asthma. Simultaneously, our data also found that serum C3 had a good discriminating capability (AUC=0.890) to predict asthma severity. Then, the best cut-off was 1.272 g/L. This result indicates that serum C3 is a promising biomarker for asthma severity assessment. However, these findings should be validated in larger population and in other ethnic groups in future.

Data Sharing Statement

Due to the respect to and the protection of patient privacy, the data generated and/or analyzed in this study are not publicly available. However, they are available from the corresponding authors on reasonable request.

Ethics Approval

The study protocol was approved by the Research Ethics Committees of Zhongshan Hospital of Fudan University (No. B2018-196R).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest.

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