Distinct Type 2-high Inflammation Associated Molecular Phenotypes of Chronic Rhinosinusitis with Nasal Polyps with Comorbid Asthma

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Ming Wang
Beijing Institute of Otolaryngology

Xiangting Bu
Beijing Institute of Otolaryngology

Ge Luan
Beijing Institute of Otolaryngology

Liqing Lin
Beijing Institute of Otolaryngology

Yang Wang
Beijing Institute of Otolaryngology

Jianmin Jin
Beijing Tongren Hospital

Luo Zhang
✉ dr.luozhang@139.com Corresponding Author
ORCID: https://orcid.org/0000-0002-0910-9884

Chengshuo Wang
Beijing Tongren Hospital

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Abstract

Background

Patients with chronic rhinosinusitis with nasal polyps (CRSwNP) and comorbid asthma have more severe disease and are difficult to treat. However, the phenotypes especially the molecular phenotypes of CRSwNP with comorbid asthma (CRSwNP+AS) are not clear. This study aimed to investigate the molecular phenotypes associated with CRSwNP+AS.

Methods

Nasal tissues from patients with CRSwNP+AS, CRSwNP-alone and control subjects were assessed with infiltrated inflammatory cells and concentrations of total IgE, and performed whole-transcriptome sequencing. Differentially expressed mRNAs (DE-mRNAs) and IncRNAs (DE-IncRNAs) and their associated pathways were analyzed. The correlations between type 2 cytokines and local eosinophils, tissue IgE, and transcriptome signatures were evaluated.

Results

More local eosinophils infiltration and higher levels of total IgE were found in nasal tissues from CRSwNP+AS than from CRSwNP-alone. RNA sequencing analysis identified 1988 common DE-mRNAs, and 176 common DE-IncRNAs shared by CRSwNP+AS versus control and CRSwNP-alone versus control. Weighted gene coexpression network analysis (WGCNA) identified LINC01146 as hub IncRNA dysregulated in both subtypes of CRSwNP. We identified 968 DE-mRNAs and 312 DE-IncRNAs between CRSwNP+AS and CRSwNP-alone. Both pathway enrichment analysis and WGCNA indicated that the phenotypic traits of CRSwNP+AS were mainly associated with higher activities of arachidonic acid metabolism, Th2 cytokines related pathway and fibrinolysis pathway, and oppositely lower activities of IL-17 signaling pathway. We further showed that the expression of Th2 cytokines, IL5 and IL13, were positively correlated with local eosinophils infiltration, tissue IgE level, and the expression of DE-mRNAs that related to arachidonic acid metabolism. Moreover, WGCNA identified HK3-006 as hub IncRNA in yellow module that most positively correlated with phenotypic traits of CRSwNP+AS.

Conclusions

Patients with CRSwNP+AS have distinct type 2-high inflammation and its associated transcriptome...
signatures in nasal tissues compared to patients with CRSwNP alone.

Background
Chronic rhinosinusitis (CRS) is characterized by chronic inflammation of the sinonasal tissue and affects 5%-15% of the general population [1]. CRS with nasal polyps (CRSwNP) accounts for approximately 20% of all CRS and has greater severity of clinical disease [2]. Asthma is one of the most common chronic inflammatory disorders of the lower airway worldwide with increasing morbidity. Studies have reported that up to 60% of CRSwNP patients have comorbid asthma (CRSwNP + AS), which is one of the most challenging subtypes to treat [3]. Patients with CRSwNP + AS have greater disease severity, higher recurrence rates of nasal polyps after surgery, poorer asthma control and higher costs [4–6].

There is evidence that eosinophilic CRSwNP tends to have comorbid asthma more frequently [7]. Formation of IgE which is independent of the presence of allergy in nasal polyp tissue is also associated with asthmatic condition in patients with CRSwNP [8]. However, there is still no clear explanation for the association between CRSwNP and asthma. And likewise, the pathogenetic mechanisms leading to CRSwNP and asthma are uncertain. The united airway concept suggests that the upper and lower airway inflammation share common pathogenetic mechanisms and influence each other [9, 10]. Similar features of inflammatory pattern, disruptive epithelial barrier and airway remodeling were found in CRSwNP and asthma [11–13]. Understanding the molecular relationship between CRSwNP and comorbid asthma may help to reveal the mechanisms that underlie airway chronic inflammation.

CRSwNP is a heterogeneous inflammatory condition with different endotypes [14]. The majority of white patients with CRSwNP in western countries have a type 2 pattern of inflammation characterized by pronounced eosinophilia and high levels of interleukin-4 (IL-4), IL-5 and IL-13 cytokines [15]. Nevertheless, Chinese patients with CRSwNP have less type 2 inflammation and show more T-helper type 1 (Th1)/Th17 inflammation [16, 17]. In view of the previous studies on epidemiology and clinical characteristics, CRSwNP + AS can be considered a subtype of CRSwNP [18–20]. This study aimed to investigate the characteristics of type 2 inflammation and molecular phenotypes associated with
CRSwNP + AS by whole-transcriptome sequencing. Distinct type 2-high inflammation and its associated transcriptome signatures indicated by coding mRNAs and long non-coding RNAs (lncRNAs) were found in patients with CRSwNP + AS compared to patients with CRSwNP-alone.

Materials And Methods

Subjects
A total of 195 subjects, including 65 CRSwNP patients with comorbid asthma (CRSwNP + AS), 99 patients with CRSwNP-alone and 31 healthy control subjects were enrolled from the Rhinology Department of Beijing TongRen Hospital. Patients with CRSwNP were diagnosed according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines. The diagnosis of comorbid asthma was based on the Global Initiative for Asthma 2014 guidelines. Patients undergoing septoplasty because of anatomic variations and without other sinonasal diseases were recruited as control subjects. All subjects were aged 18 to 70 years. Subjects with immunodeficiency, fungal sinusitis, coagulation disorder, neoplasia and pregnancy were excluded. The Ethics Committee of Beijing Tongren Hospital approved this study, and all subjects signed informed consent forms.

Nasal tissue samples were collected from the inferior turbinate of control subjects and the nasal polyps of CRSwNP patients during surgery. The tissue samples were processed for staining with hematoxylin and eosin, RNA sequencing and ELISA as previously described [21].

Histological evaluation of polyp tissue
Nasal polyp tissues were immediately formalin fixed after surgery, and then dehydrated, embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) and processed for histological evaluation. All sections were examined by optical microscopy at × 400 magnification. The absolute numbers and percentages of infiltrating inflammatory cells including eosinophils, neutrophils, plasma cells, and lymphocytes were assessed by two independent pathologists, who were blinded to the study design. For each section, the inflammatory cells were recorded as mean data of six nonoverlapping regions.

Assessment of total IgE in nasal tissues
Concentrations of total IgE in nasal tissues were assayed by using Human IgE ELISA Kit (Arigo Biolaboratories Corporation, Taiwan). Briefly, fresh nasal tissues were placed into RIPA lysis buffer
with 1% protease inhibitor cocktail (Thermo Fisher Scientific) and homogenized using a standard bench-top homogenizer (Qiagen, Valencia, CA). The supernatants were collected and used for IgE analysis after centrifugation. The total IgE assay was conducted according to the manufacturer’s instructions. All samples were tested in duplicate.

**RNA isolation and RNA sequencing**

Nasal tissue samples of CRSwNP + AS (n = 10), CRSwNP-alone (n = 10), and control (n = 9) were randomly selected for whole-transcriptome sequencing. The nasal tissue samples were freshly preserved in RNAlater solution (Qiagen, Hilden, Germany) after surgery. Total RNA was extracted and purified with an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The quantity and quality of the isolated RNA was determined with NanoDrop 2000 Spectrophotometer (Thermo Fischer Scientific) and 2100 TapeStation Automated Electrophoresis System (Agilent Technologies), and samples with an RNA integrity number of greater than 8.0 were chosen for sequencing. Ribosomal RNA was removed and sequencing libraries were prepared using the rRNA-depleted RNA by NEBNext UltraTM Directional RNA Library Prep Kit (New England Biolabs, USA) following manufacturer’s instructions. RNA sequencing was performed on the Illumina Hiseq platform and 150 bp paired-end reads were generated by Novogene Bioinformatics Technology Cooperation (Beijing, China).

**RNA sequencing data analysis**

Adapters and low-quality tail were trimmed from reads prior to read alignment. Clean sequence reads were aligned to the human genome with Hisat2 (v2.0.5). Cufflinks (v2.2.1) was used to assemble transcripts, estimate the abundance of these transcripts, and detect differential expression among samples. For mRNA analyses, the reference genome build GRCh37 was chosen as the annotation references. For lncRNA analyses, the GENCODE v19 database was chosen as the annotation references. Fragments per kilo-base of exon per million fragments mapped (FPKM) of both lncRNAs and mRNAs in each sample was calculated based on the length of the fragments and reads count mapped to this fragment. Differential expression analysis was performed using Cuffdiff software (v2.2.1). An adjusted $P < 0.05$ plus fold change $> 2$ was used as the cut-off for significantly
differentially expressed mRNAs (DE-mRNAs) and IncRNAs (DE-IncRNAs).

Pathway analysis of DE-mRNAs and function prediction of DE-IncRNAs

DE-mRNAs were loaded into Enrichr (https://amp.pharm.mssm.edu/Enrichr/) [22] for pathway enrichment analysis. P < 0.05 was considered to be statistically significant. The top significantly enriched KEGG and Biocarta pathways with high Combined Score, provided by Enrichr, were presented in the results section.

In order to predict DE-IncRNA functions, we applied weighted gene coexpression network analysis (WGCNA) [23] to construct a coexpression network between DE-IncRNAs and their highly correlated DE-mRNAs according to the Pearson correlation coefficient between their normalized expression levels. Briefly, network construction and module detection were performed using the “blockwiseModules” function in the WGCNA package. A coexpression similarity matrix was calculated by computing Pearson correlations between all gene pairs, and then transformed into an adjacency matrix using a soft threshold power (β) equal to 18. A dynamic tree cut algorithm was used to detect groups of highly correlated genes. Modules were defined as the branches cutoff of the tree and each module was labeled in unique colors, which grey color contains probes not assigned to any module.

The module eigengenes were utilized to represent each module, which was calculated via the first principal component. Using the module eigengenes, the relationship between module and tissue type (CRSwNP-alone and CRSwNP + AS) was estimated.

Enrichr was used to perform the functional enrichment analysis for each module. As above description, the top significantly enriched pathways were presented. In interesting modules that related to the disease condition, top hub genes with high connectivity and in which edges with weight above a threshold of 0.1 were identified and visualized by cytoscape network.

Statistical analysis

All data were presented as medians and interquartile range (IQR) except for age which was presented as mean ± SDs. Data analysis was performed using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, Calif). All parametric variants were analyzed by using Student t tests, and nonparametric variants were analyzed by using Mann-Whitney U tests. The χ² or Fisher exact test was used for
qualitative data. Relationships between variables were evaluated by using Spearman correlation analysis. Differences were considered significant at P value < 0.05.

Results

Demographic and clinical characteristics of the subjects

Demographic and clinical characteristics of all participants enrolled in this study were presented in Additional file 1: Table S1. There was no significant difference with regard to age, gender and smoker status between 3 groups. Peripheral blood eosinophils and total IgE were increased in both subtypes of CRSwNP patients compared to control subjects. The differences between CRSwNP + AS group and CRSwNP-alone group were statistically determined. Atopy and recurrence were significantly more frequent in patients with CRSwNP + AS than in patients with CRSwNP-alone. Comorbid asthmatic conditions led to a decreased forced exhalation volume in one second (FEV1) / forced vital capacity (FVC) ratio and an increased fractioned exhaled nitric oxide (FeNO) for CRSwNP patients. Moreover, CRSwNP patients with comorbid asthma had a significant higher percentage of eosinophils (6.45%) and total IgE (143.00 kU/l) in peripheral blood compared to CRSwNP-alone group (3.00% and 53.90 kU/l; Fig. 1a and b).

Local features of nasal tissue in different patient group

To identify the local inflammatory patterns of nasal tissue in CRSwNP patients with and without comorbid asthma, the infiltrating eosinophils, neutrophils, plasma cells, and lymphocytes were assessed. Tissue sections stained with H&E demonstrated that patients with CRSwNP + AS had significant more eosinophils in nasal polyp tissues than patients with CRSwNP-alone (Fig. 1c-e). Given the relationship between IgE and Type 2 immune response, we further examined the total IgE levels in nasal tissues. The results showed significant higher IgE levels in nasal tissues from CRSwNP + AS compared to CRSwNP-alone and control group (Fig. 1f). Moreover, Spearman correlation test showed that percentage of local eosinophils was positively correlated with concentration of tissue IgE (Additional file 1: Figure S1).

Whole transcriptome profiling of nasal tissues from CRSwNP + AS and CRSwNP-alone

To identify the gene expression profile of CRSwNP with and without comorbid asthma, RNA sequencing was performed on nasal tissue from 3 groups (control, CRSwNP-alone and CRSwNP + AS),
with both DE-mRNAs and DE-IncRNAs were assessed. As showed in Additional file 1: Figure S2, there were 5218 DE-mRNAs and 2949 DE-IncRNAs between CRSwNP-alone and control, and 2512 DE-mRNAs and 464 DE-IncRNAs between CRSwNP + AS and control. In addition, 968 DE-mRNAs and 312 DE-IncRNAs were identified between CRSwNP + AS and CRSwNP-alone.

Common dysregulated genes shared by CRSwNP + AS and CRSwNP-alone

As showed in Fig. 2a, we identified 1988 common DE-mRNAs shared by CRSwNP-alone and CRSwNP + AS respectively compared to control. Common DE-mRNAs were performed pathway enrichment analysis using Enrichr. The top enriched KEGG pathways were associated with cytokine-cytokine receptor interaction, chemokine signaling pathway, staphylococcus aureus infection, asthma, and cell adhesion molecules, likewise, the top enriched BioCarta pathways were related to eicosanoid metabolism, the co-stimulatory signal during T-cell activation and IL-10 anti-inflammatory signaling pathway (Fig. 2b).

There were 176 common DE-IncRNAs shared by CRSwNP + AS versus control and CRSwNP-alone versus control (Fig. 2c). WGCNA was applied to explore the potential functions of common DE-IncRNAs. A hierarchical clustering tree was constructed, resulting in 9 modules each of which was labeled with a distinct color as shown in the Fig. 2e. The size of these modules ranged from 41 to 539 genes with the largest one labeled with turquoise (Additional file 1: Figure S3). There were 45 IncRNAs and 494 mRNAs highly coexpressed in the turquoise module. Pathway enrichment analysis showed that genes of turquoise module were mainly associated with cytokine-cytokine receptor interaction, asthma, T cell receptor signaling pathway, staphylococcus aureus infection, chemokine signaling pathway, and Th1, Th2, Th17 cell differentiation (Fig. 2d).

The top 50 hub genes with high connectivity and edge weigh were identified, and visualized by cytoscape network in Fig. 2f. Of these top hub genes, there is only one IncRNA, named LINC01146, which significantly up-regulated in both CRSwNP-alone and CRSwNP + AS. Then, the coexpressed mRNAs of LINC01146 were performed pathway analysis to predict function. LINC01146 was mostly associated with T cell receptor signaling pathway, natural killer cell mediated cytotoxicity, Fc gamma R-mediated phagocytosis, and Th1 and Th2 cell differentiation (Additional file 1: Figure S4).
Distinct transcriptome signatures in nasal tissue of CRSwNP + AS

Compared to CRSwNP-alone, 212 mRNAs were down-regulated whereas 756 mRNAs were up-regulated in nasal tissues of CRSwNP + AS (Fig. 3a). Top 50 significant DE-mRNAs were presented in Additional file 1: Table S2. Enrichr pathway analysis showed that arachidonic acid metabolism, ECM-receptor interaction, IL-17 signaling pathway, GATA3 participate in activating Th2 cytokine genes, and fibrinolysis pathway were the top significant pathways enriched by DE-mRNAs (Fig. 3b).

We further checked the detailed expression of genes in arachidonic acid metabolism which plays an important role in airway inflammatory conditions. As showed in Fig. 3c, compared to CRSwNP-alone, 14 of 16 DE-mRNAs related to arachidonic acid metabolism were up-regulated in CRSwNP + AS. The up-regulated DE-mRNAs of arachidonic acid metabolism including PTGS1, TBXAS1, ALOX15B, PLA2G4D, LTC4S, GGT1, GGT5, GPX6, PLA2G4B, PLA2G6, CYP4F8, JMJD7-PLA2G4B, PTGIS and GPX3, which indicated an enhanced activity of arachidonic acid metabolism and a severe inflammatory condition.

Severe type 2 inflammation in nasal tissues of CRSwNP + AS

As indicated by above transcriptome data, Th1, Th2 and Th17 related signaling pathways might be differentiated between CRSwNP-alone and CRSwNP + AS. Thus, we investigated the expression of critical cytokines and their receptors that indicated the activities of different inflammatory endotypes: (1) IFNG and IFNG receptor (IFNGR1 and IFNGR2) for Th1 activity (type 1 inflammation); (2) IL5, IL4, IL13 and their receptors (IL5RA, IL5RB, IL4R, IL13RA1 and IL13RA2) for Th2 activity (type 2 inflammation); (3) IL17A and IL17A receptor (IL17RA and IL17RC) for Th17 activity; (4) TNF, IL1B, IL6 and their receptors (TNFRSF1A, TNFRSF1B, IL1R1, IL1R2 and IL6R) for proinflammatory action.

As showed in Fig. 3d and Additional file 1: Table S3, the enhanced expression of cytokines IL5 and IL13, and receptors IL5RA and IL5RB indicated augmented type 2 inflammation in both CRSwNP-alone and CRSwNP + AS compared to control. Furthermore, CRSwNP + AS had higher expressed IL4, IL5 and IL13, whereas conversely lower expressed IL17A than CRSwNP-alone. Together with the findings that more infiltrating eosinophils and higher concentration of tissue IgE in CRSwNP + AS, there were clearly more severe type 2 inflammation in nasal tissues of CRSwNP + AS than that of CRSwNP-alone.
We further investigated the correlations between cytokines and above inflammatory indicators. As showed in Fig. 4, both the expression of ILS and IL13 were positively correlated with percentage of local eosinophils, concentration of tissue IgE and the expression of LTC4S which reflected the imbalanced arachidonic acid metabolism. By contrast, the expression of IL17A was negatively correlated with tissue IgE and the expression of LTC4S.

LncRNA signatures in nasal tissue of CRSwNP + AS
We identified 229 up-regulated and 83 down-regulated DE-IncRNAs in nasal tissues of CRSwNP + AS compared to CRSwNP-alone (Fig. 5a). Top 50 significant DE-IncRNAs were presented in Additional file 1: Table S4. A coexpression network was constructed based on the expression of DE-IncRNAs and DE-mRNAs by using WGCNA. Finally, we got 7 modules as can be seen from the color-band underneath the cluster tree (Additional file 1: Figure S5). The relationship between each module and tissue type (CRSwNP + AS) was estimated by using the module eigengenes. As showed in Fig. 5b, all the 7 modules were significantly correlated with tissue type changes, among which blue module was most negatively correlated with the phenotypic traits of CRSwNP + AS ($r = -0.73, P = 2 \times 10^{-4}$), whereas yellow module was the most positive correlation ($r = 0.66, P = 0.001$).

There were 31 IncRNAs and 94 mRNAs in blue module, whereas only one IncRNA (HK3-006) and 63 mRNAs in yellow module. Pathway enrichment analysis indicated that blue module was mainly associated with IL-17 signaling pathway and cytokine-cytokine receptor interaction (Fig. 5c), whereas yellow module was mainly related to asthma, arachidonic acid metabolism, and signaling pathways such as calcium and JAK-STAT signaling pathway (Fig. 5d). Top 50 hub genes respectively from blue module and yellow module were presented in Fig. 5e and f.

Discussion
Both CRSwNP and asthma are airway inflammatory disorders and have serious effects on quality of life. A great proportion of CRSwNP patients have comorbid asthmatic conditions, which is one of the most challenging phenotypes to treat [3, 12]. However, the phenotypes especially the molecular phenotypes of CRSwNP + AS are not clear. To our knowledge, this is the first study investigating the molecular phenotypes of CRSwNP + AS compared to CRSwNP-alone by whole-transcriptome RNA
sequencing. Our study identified distinct type 2-high inflammation patterns and their associated transcriptome features in CRSwNP + AS compared to CRSwNP-alone, which will be helpful in understanding the underlying mechanisms and developing specific molecular biomarkers and personalized therapeutic strategies.

Our findings indicated that patients with CRSwNP + AS had more frequent atopy, increased chance for recurrence, more local eosinophils infiltration and higher level of tissue IgE compared to CRSwNP-alone. We previously showed that high proportion of eosinophils in nasal tissue acted as a reliable prognostic indicator for CRSwNP recurrence [24]. Thus, the local features of nasal tissue were closely associated with the clinical characteristics. Together with the recent finding that asthma is a dominant factor increased the chance of recurrence in CRSwNP [6], comorbid asthmatic conditions might be a strong indicator for CRSwNP recurrence.

Airway type 2 inflammation is mainly mediated by eosinophils, mast cells, Th2 cells, ILC2s and IgE-producing B cells, and high production of cytokines IL-5, IL-4 and IL-13 [25]. More than 80% of Western white patients with CRSwNP are characterized by type 2 inflammation, while less than 50% of CRSwNP cases in East Asia countries show features of type 2 reaction [26, 27]. Our findings that patients with CRSwNP + AS had more severe type 2 inflammation than patients with CRSwNP-alone might more valuable to Chinese CRSwNP patients which characterized by Th2/Th1/Th17 mixed pattern of immune response.

Our RNA sequencing data provides valuable information for exploring the general molecular mechanisms underlying the pathogenesis of CRSwNP. Regardless of the subtypes of CRSwNP, genes and pathways that most likely contribute to the pathogenesis of CRSwNP might be mainly associated with cytokine and chemokine signaling pathway, staphylococcus aureus infection, eicosanoid metabolism and cell adhesion molecules, which were similar to Peng’s findings [28]. The well-known genes or biomarkers closely related to CRSwNP such as CLC, POSTN, CCL18, IL13, TSLP and BPIFA1 were also nicely identified as top DE-mRNAs in both CRSwNP-alone group and CRSwNP + AS group. The transcriptome signatures of CRSwNP + AS were characterized by groups of differentially expressed genes and their enriched pathways, compared to CRSwNP-alone. We showed that CRSwNP
+ AS were mainly associated with higher activities of arachidonic acid metabolism, Th2 signaling pathway and fibrinolysis pathway, and lower activity of IL-17 signaling pathway. Beyond that, top significant DE-mRNAs between CRSwNP + AS and CRSwNP-alone also provided important information. In line with the high concentration of tissue IgE in CRSwNP + AS, increased mRNA expression of constant region of heavy chain of IgE (IGHE) was also confirmed by RNA sequencing. Some recently identified biomarkers of CRSwNP such as CST1 [29], were found to be differentially expressed between CRSwNP + AS and CRSwNP-alone. Importantly, as top significant DE-mRNAs, several genes that closely associated with the pathogenesis of asthma such as ITLN1, KCNA3 and CCR10 [30–32] were up-regulated in nasal tissue of CRSwNP + AS compared to CRSwNP-alone. Therefore, the gene expression pattern of upper airway could be influenced by lower airway conditions, which is consistent with the united airway concept.

It has been well demonstrated that alterations in the arachidonic acid pathway play an important role in airway inflammatory conditions like rhinosinusitis, nasal polyps, allergic rhinitis, and asthma [33]. We found a generally enhanced activity of arachidonic acid metabolism in CRSwNP + AS, indicated by the up-regulated expression of PLA2 (PLA2G4A, PLA2G4B, PLA2G4D and PLA2G6) which promoted the release of membrane-bound arachidonic acid [34]. The imbalanced synthesis of eicosanoid which characterized by increased synthesis of cysteinyl leukotrienes (CysLTs) is correlated with the inflammatory pattern and severity of the airway inflammation [33, 35]. Consistent with this, we showed an increased expression of LTC4S which promoted the biosynthesis of CysLTs, indicated a severe inflammatory condition in CRSwNP + AS. The increased expression of PTGS1 and PTGIS may accelerate arachidonic acid conversion to prostacyclins (PGI2) and prostaglandins (PGD2, PGE2) which related to an aggravated airway inflammation and asthmatic conditions [36, 37]. Moreover, the increased expression of ALOX15B in CRSwNP + AS might have an active proinflammatory role [38]. A quantitative metabolomic approach is needed to further profile arachidonic acid metabolites in different subtypes of CRSwNP.

Th2 and Th17 signaling pathways are two major regulators and negatively interrelated in patients with CRSwNP [17]. Our RNA sequencing data revealed higher activities of Th2 signaling pathway and
lower activities of IL-17 signaling pathway in CRSwNP + AS than CRSwNP-alone. IL-4, IL-5 and IL-13 are typical Th2 cytokines which reflect the severity of type 2 inflammation, whereas IL-17 is a typical Th17 cytokine. We further showed that the expression of IL5 and IL13 are positively correlated with other indicators of type 2 inflammation (local eosinophils and tissue IgE). Genes that related to arachidonic acid metabolism were also correlated with the expression of type 2 cytokines, which suggested the crucial roles of type 2 inflammation in CRSwNP + AS.

LncRNAs play important roles in various biological processes and are emerging as biomarkers and potential therapeutic targets of human chronic diseases [39-40]. However, very few studies have reported the involvement of IncRNAs in chronic nasal inflammation. Yue et al. found that linc00632 was down-regulated in nasal tissues of allergic rhinitis patients and inhibits IL-13 induced inflammatory cytokine and mucus production [41]. Wang et al. showed that IncRNA XLOC_010280 might regulate the expression of CCL18 and eosinophilic inflammation in eosinophilic CRSwNP [42]. In the present study, the whole-transcriptome sequencing revealed all the dysregulated lncRNAs in both subtypes of CRSwNP, which provided a candidate reservoir for lncRNAs research in CRSwNP.

WGCNA is an effective method of multigene analysis to construct coexpression network, and has successfully been applied for studying mRNAs and IncRNAs to distinguish dysfunctional regulatory subnetworks, select out potentially key genes and predict IncRNA functions [43, 44]. We applied WGCNA to predict functions of DE-IncRNAs and identify hub genes. Common dysregulated IncRNAs in CRSwNP-alone and CRSwNP + AS have very similar predictive functions to common DE-mRNAs, as indicated by genes in the maximal module. Furthermore, we identified LINC01146 as the only one top hub IncRNA which may play key roles in the pathogenesis of CRSwNP. LINC01146 was found to be dysregulated in hepatocellular carcinoma with unverified functions [45]. Further study is needed to validate the function of common dysregulated IncRNAs in CRSwNP.

The key IncRNAs associated with CRSwNP + AS had also been identified. HK3-006 is the only one top hub IncRNA in the module that most positively correlated with phenotypic traits of CRSwNP + AS. HK3-006 may be related to asthma pathway and arachidonic acid metabolism as predicted by its highly coexpressed mRNAs. Several top hub IncRNAs were identified in the module that most negatively
correlated with phenotypic traits of CRSwNP + AS. Among them, a new identified IncRNA LINC686 might be most likely associated with IL-17 signaling pathway. Although growing studies show that IncRNAs are involving in the regulation of cytokine signaling and inflammation [46-47], our understanding of IncRNA functions is just at the beginning.

There are some limitations in our study. First, this study did not subgroup CRSwNP + AS and CRSwNP-alone. Both CRSwNP and asthma are heterogeneous disease. One recent study showed that patients with CRSwNP + AS can be grouped into 3 subtypes with different inflammatory status by clinical phenotypes [19]. Thus, future studies to investigate the clinical and molecular phenotypes of subgroups of CRSwNP + AS are needed. Second, we did not evaluate lower airway inflammation such as assessment of inflammatory cells and cytokines in bronchial biopsy or induced sputum, which might be helpful in understanding the association between CRSwNP and asthma. Third, the detection of critical cytokines based on the secreted protein levels should be conducted and verified. Finally, this study performed RNA sequencing on heterogeneous tissue with multiple cell types. Single-cell RNA sequencing allows the analysis of transcriptome from individual cells in nasal tissues [48], which might provide more information for understanding the function of different cell types.

Conclusions
The present study demonstrated that CRSwNP patients with comorbid asthma had more severe type 2 inflammation and type 2 inflammation associated transcriptome signatures indicated by expression profile of mRNAs and IncRNAs in nasal tissue compared to patients with CRSwNP-alone. These findings indicated the molecular mechanisms in the pathogenesis of CRSwNP and highlighted the roles of type 2 inflammation related molecules in CRSwNP with comorbid asthma. This study also provided information for future validation and use of these genes as potential biomarkers and therapeutic targets for CRSwNP.

Abbreviations
CRS: Chronic rhinosinusitis; CRSwNP: chronic rhinosinusitis with nasal polyps; CRSwNP+AS: Chronic Rhinosinusitis with Nasal Polyps with Comorbid Asthma; IL: interleukin; Th1: T-helper type 1; IncRNA: long non-coding RNA; DE: differentially expressed; FPKM: Fragments per kilo-base of exon per million
fragments mapped; WGCNA: weighted gene coexpression network analysis.

Declarations

Author contributions

M.W., C.W. and L.Z. designed the study. M.W., X.T., G.L., L.Q. and Y.W. performed the experiments. J.M. and C.W. contributed to discussions. M.W. analyzed the data and wrote the manuscript. C.W. and L.Z. critically revised the manuscript. All authors reviewed the manuscript.

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Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and materials

The complete dataset is included in this manuscript.

Ethics approval and consent to participate

The Ethics Committee of Beijing Tongren Hospital approved this study, and all subjects signed informed consent forms.

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Figures
Detection of eosinophils and total IgE in blood and nasal tissues from patients with CRSwNP.

(A-B) Blood eosinophils and total IgE was detected in CRSwNP patients with asthma (CRSwNP+AS), CRSwNP-alone and control (Ctrl) subjects. (C-D) Representative images of...
nasal polyp tissues stained with hematoxylin and eosin from patients with CRSwNP-alone and CRSwNP+AS. Red arrows point out eosinophils. (E) The percentage of infiltrating eosinophils, neutrophils, plasma cells and lymphocytes were assessed in nasal tissues of CRSwNP-alone (n=24) and CRSwNP+AS (n=25). (F) Concentrations of total IgE in nasal tissues of Ctrl (n=10), CRSwNP-alone (n=14) and CRSwNP+AS (n=17) were assayed by using Human IgE ELISA Kit. Data are presented as medians and interquartile range (IQR). * P<0.05, ** P<0.01, Mann-Whitney U tests.
Figure 2

Common dysregulated genes shared by CRSwNP+AS and CRSwNP-alone. Nasal tissue samples of CRSwNP+AS (n=10), CRSwNP-alone (n=10), and control (n=9) were performed whole-transcriptome sequencing. (A) Venn diagrams depicting DE-mRNAs of CRSwNP+AS versus control and CRSwNP-alone versus control. The number of DE-mRNAs is marked in the corresponding areas. (B) The 1988 common DE-mRNAs shared by CRSwNP+AS versus control and CRSwNP-alone versus control were performed pathway enrichment analyses using Enrichr. Top 15 significantly enriched KEGG pathways (blue column) and top 5 significantly enriched BioCarta pathways (turquoise column) were depicted. P<0.05 were considered statistically significant. (C) DE-lncRNAs of CRSwNP+AS versus control and CRSwNP-alone versus control. (D-E) Common DE-lncRNAs expression based modules identified by weighted gene coexpression network analysis (WGCNA). WGCNA was applied to explore the potential functions of 176 common DE-lncRNAs, based on a coexpression network of DE-lncRNAs and DE-mRNAs. Branches of the dendrogram obtained by hierarchical clustering of adjacency based similarity result in 9 modules which is labeled with a distinct color (E). Top 15 KEGG pathways (blue column) and top 5 BioCarta pathways (turquoise column) were significantly enriched by genes in turquoise module (D). (F) Top 50 hub genes of turquoise module were visualized by cytoscape network. mRNAs or lncRNAs with high connectivity and in which edges with weight above a threshold of 0.1 were identified as hub genes. The red nodes denote lncRNAs, and the green nodes denote mRNAs.
Differentially expressed genes and pathways between CRSwNP+AS and CRSwNP-alone. (A) Volcano plots illustrating DE-mRNAs of CRSwNP+AS versus CRSwNP-alone identified by RNA sequencing. (B) Top 15 KEGG pathways (blue column) and top 5 BioCarta pathways (turquoise column) significantly enriched by DE-mRNAs. P<0.05 were considered statistically significant. (C) The expression of arachidonic acid metabolism related DE-mRNAs between CRSwNP+AS and CRSwNP-alone. The color coding of heat maps represents the gene expression level normalized to Ctrl group, which calculated based on fragments per kilo-base of exon per million fragments mapped (FPKM). Yellow box indicated the up-regulated genes in CRSwNP+AS group. (D) The expression of critical cytokines and their
receptors that indicated the activity of different inflammatory endotypes. Yellow star represents significantly differential expression of genes between CRSwNP+AS and CRSwNP-alone.

Figure 4

Correlations between cytokines and inflammatory indicators. Spearman correlation analysis was performed between cytokines expression (IL5, IL13, IL17A) and percentage of local eosinophils, concentration of tissue IgE and LTC4S expression.
Differentially expressed IncRNAs and pathway analysis between CRSwNP+AS and CRSwNP-alone. (A) Volcano plots illustrating DE-IncRNAs of CRSwNP+AS versus CRSwNP-alone identified by RNA sequencing. (B) The correlation between modules and phenotype of CRSwNP+AS. Seven modules were identified by WGCNA based on expression of DE-mRNAs and DE-IncRNAs of CRSwNP+AS versus CRSwNP-alone. Pearson’s correlation coefficient between each module and phenotype of CRSwNP+AS and their associated P values were
labeled in the corresponding modules. The red and green colors show a strong positive and negative correlation, respectively. (C-D) All or top 10 KEGG pathways significantly enriched by genes in blue module (C) and yellow module (D). P <0.05 were considered statistically significant. (E-F) Top 50 hub genes in blue module (E) and yellow module (F) were visualized by cytoscape network. mRNAs or IncRNAs with high connectivity and in which edges with weight above a threshold of 0.1 were identified as hub genes. The red nodes denote IncRNAs, and the green nodes denote mRNAs.

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