

Differential expression of miR-4492 and IL-10 is involved in chronic rhinosinusitis with nasal polyps

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Abstract. Chronic rhinosinusitis (CRS) is one of the most common types of respiratory disease and affects a large proportion of the population worldwide. The clinical differences between CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) facilitate studies on the development of polyps. In the present study, next-generation sequencing was performed to identify differentially expressed microRNAs (miRNAs/miRs) in CRSwNP vs. CRSsNP tissues and subsequently validated the expression of selected genes using reverse transcription-quantitative polymerase chain reaction analysis. In total, 6 miRNAs were identified to be downregulated in the CRSwNP samples compared with those in the CRSsNP samples. The predicted targets of these miRNAs were determined to be enriched in a number of signaling pathways, including the ErbB, Ras, cyclic adenosine monophosphate and Janus kinase (Jak)/signal transducer and activator of transcription (STAT) pathways. The expression of miR-4492 and that of its targets predicted by a bioinformatics analysis, tumor necrosis factor α (TNF- α) and interleukin (IL)-10, was validated in 96 clinical specimens. miR-4492 was downregulated and IL-10 was upregulated in CRSwNP vs. CRSsNP tissues, and an inverse correlation between miR-4492 and IL-10 was determined in CRS tissues; however no difference was identified in the expression of TNF- α between the different groups. The present results indicate that the miR-4492/IL-10 interaction in the Jak/STAT signaling pathway may be one of the key mechanisms in CRSwNP.

Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous disease of the sinuses and upper airways that affects a large proportion of the population worldwide (1,2). CRS is classified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP), and is typically characterized by two or more of the following symptoms: Nasal obstruction, drainage, loss of sense of smell, headaches and facial pain or pressure (3,4). CRSsNP is associated with the symptoms of headache and facial pain or pressure, whereas CRSwNP is more closely associated with the symptoms of nasal obstruction and loss of sense of smell (5-7). In the clinic, CRSwNP is frequently resistant to medical therapy, and therefore, management of patients with CRSwNP is costly. Furthermore, a proportion of patients with CRSwNP do not benefit from topical corticosteroids, long-term systemic glucocorticosteroids and repeated sinus surgery (8-10). Thus, a better understanding of the reason why CRSwNP is not controlled by current approaches and the underlying molecular mechanisms is required.

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNAs that are 21-24 nucleotides in length and have key roles in the pathogenesis of numerous diseases. miRNAs either suppress target transcripts or inhibit translation in order to post-transcriptionally regulate protein expression (11-13). Aberrant miRNA expression is a common feature of a diverse range of diseases (14,15). Previous studies indicate a distinct expression pattern of inflammatory and re-modeling mediators in CRSwNP (8). With the development of high-throughput gene detection techniques and bioinformatics, a new era of miRNA investigation has rapidly developed for numerous types of allergic airway disease, including allergic rhinitis (16) and asthma (17,18). However, the genome-scale miRNA expression pattern of CRSwNP remains to be fully elucidated.

In the present study, the different miRNA expression patterns of CRSwNP and CRSsNP were investigated using next-generation sequencing, and the expression of a candidate miRNA and its predicted targets was subsequently validated in CRSwNP using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

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Materials and methods

Patients. A total of 100 patients requiring endoscopic sinus surgery were recruited at The First Affiliated Hospital of Xinjiang Medical University (Urumqi, China) between October 2016 and February 2017, including 61 patients with CRSwNP (38 male and 23 female patients; median age, 44 years old; age range, 23–63 years old) and 39 patients with CRSsNP (27 male and 12 female patients; median age, 41 years old; age range, 20–65 years old). All subjects met the criteria for CRS as defined by the European position paper on rhinosinusitis and NPs 2012 (10). An additional 35 patients (22 male and 13 female patients; median age, 39 years old; age range, 19–57 years old) that required septoplasty and did not have any inflammatory nasal disease were enrolled as control subjects. Patients that were pregnant or dependent on systemic steroid therapy were excluded. During surgery, ethmoidal mucosa or polyp tissues were collected from the patients with CRS and inferior turbinate tissues were obtained from the control patients. The specimens from 5 of the patients with CRSwNP, 4 of the patients with CRSsNP and 4 of the control patients were used for miRNA sequencing. Subsequently, the RNA of 26 of the patients was excluded due to low quality (Fig. S1). Finally, the RNA of 96 of the patients was used in the RT-qPCR validation assay.

Total RNA extraction and complementary (c)DNA synthesis. Total RNA was extracted from each specimen using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Small RNAs with 18–30 nt fragments were enriched using denaturing polyacrylamide gel electrophoresis (15% gel). According to the manufacturer's protocol, the fragments were ligated to 5' and 3' adaptors and reverse-transcribed into cDNA using a Mir-X™ miRNA First-Strand Synthesis kit (cat. no. 638313; Takara Bio Inc.) after purification. The quality of these RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). For mRNA quantification, total RNA was subjected to reverse transcription using a PrimeScript™ 1st Strand cDNA Synthesis Kit (cat. no. 6110A; Takara Bio Inc.). Only samples meeting the criteria of optical density ratio at 260/280 nm ≥ 1.8 and RNA integrity number ≥ 8.0 were used for all of the analyses. The RNA integrity of each sample was also assessed using a denaturing agarose gel in order to determine the intensity ratio of the 28S and 18S ribosomal RNA bands.

miRNA sequencing (miRNA-seq). Following RT of the RNA into cDNA, the cDNA was subjected to deep sequencing, which was performed by Genesky Biotechnologies. The small RNA libraries were evaluated using FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). After removing adaptor and low-quality sequences, the remaining high-quality sequences were analyzed with the miRBase (19) (<http://www.mirbase.org/>) and Rfam (20) (<http://rfam.xfam.org/>) databases.

Differentially expressed gene (DEG) screening. The R package edgeR (<http://www.bioconductor.org/packages/2.4/bioc/html/edgeR.html>) was used to identify DEGs, with the cut-off values of \log_2 fold change (FC) ≥ 1 and adjusted P-value of < 0.05 .

2-Dimensional (2D) hierarchical clustering analysis. A gene expression matrix of the DEGs was prepared and analyzed with the pheatmap package (<http://CRAN.R-project.org/package=pheatmap>) in order to perform unsupervised clustering in R after normalization.

Target prediction and functional enrichment. The miWalk database version 2.0 (mirwalk.uni-hd.de/) was used for predicting miRNA-target interactions (21). Subsequently, all of the predicted targets were subjected to gene ontology (GO) (22) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (23) pathway enrichment analysis in WebGestalt (<http://www.webgestalt.org/>), with a criterion of $P < 0.01$.

qPCR. In order to investigate the expression of miR-4492, interleukin (IL)-10 and tumor necrosis factor α (TNF- α) in the specimens, qPCR was performed using SYBR Advantage qPCR Premix (cat. no. 638322; Takara Bio Inc.) on an ABI 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH served as a housekeeping gene for standardization of protein-coding gene expression, and U6 served as the reference for standardization of miRNA expression. All assays were performed at least in triplicate and gene expression was quantified using the $2^{-\Delta\Delta C_q}$ method (24). The sequences of the primer pairs were as follows: miR-4492 forward, 5'-CTGGGCGCGCGCCAAA-3'; IL-10 forward, 5'-TCAAGGCGCATGTGA ACTCC-3' and reverse, 5'-CCA CGGCCTTGCTCTTGT-3'; TNF- α forward, 5'-TTCTGC CTGCTGCACTTTGG-3' and reverse, 5'-TGTCAC TCGGGG TTCGAGAAG-3'. The universal miRNA reverse primer was in the Mir-X miRNA First-Strand Synthesis kit.

Statistical analysis. Statistical analysis was performed with SPSS software version 16.0 (SPSS Inc.). All of the assays were performed at least in triplicate and values are expressed in bar charts as the mean \pm standard error of the mean. Significant differences between groups were analyzed using Student's t-test or one-way analysis of variance followed by Duncan's multiple-comparisons test. $P < 0.05$ was considered to indicate a statistically significant difference. Pearson correlation analysis was performed in order to assess the co-expression of miR-4492 and IL-10 in the 96 specimens.

Results

Identification of differentially expressed miRNAs in CRSwNP. A total of 6 DEGs (6 downregulated miRNAs) were identified between CRSwNP and CRSsNP. One DEG (1 downregulated miRNA) was identified between CRSwNP and the control, and no DEGs were identified between CRSsNP and the control. The details of the DEGs are presented in Table I. Subsequently, the expression data of six DEGs were subjected to 2D hierarchical clustering analysis. The distinctive clusters for CRSwNP and CRSsNP were segregated for all of the CRS specimens (Fig. 1), revealing differences in the miRNA expression profiles of the two subgroups of CRS.

Gene enrichment and functional annotation of the predicted targets of the DEGs. A total of 4,395 predicted target genes of the six DEGs were obtained from the miWalk database.

Table I. Details of aberrantly expressed miRNAs.

Gene names	CRSwNP vs. CRSsNP		CRSwNP vs. NC		CRSsNP vs. NC	
	logFC	FDR	logFC	FDR	logFC	FDR
hsa-miR-144-3p	-3.065465067	0.002555901	-1.820765547	0.821307633	0.857082137	0.996058565
hsa-miR-144-5p	-3.160003078	0.003750538	-1.835835772	0.821307633	0.856915634	0.996058565
hsa-miR-486-5p	-2.135055827	0.013129379	-1.258258512	0.939213466	0.713031482	0.996058565
hsa-miR-200a-3p	-2.187602496	0.013129379	-0.284138738	0.998572989	1.406274811	0.996058565
hsa-miR-451a	-2.09844785	0.015772606	-1.231243617	0.998572989	0.720894	0.996058565
hsa-miR-4492	-1.745117094	0.019422342	-1.513875624	0.012444032	0.284184	0.996058565

miR, microRNA; hsa, *Homo sapiens*; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; FDR, false discovery rate; FC, fold change.

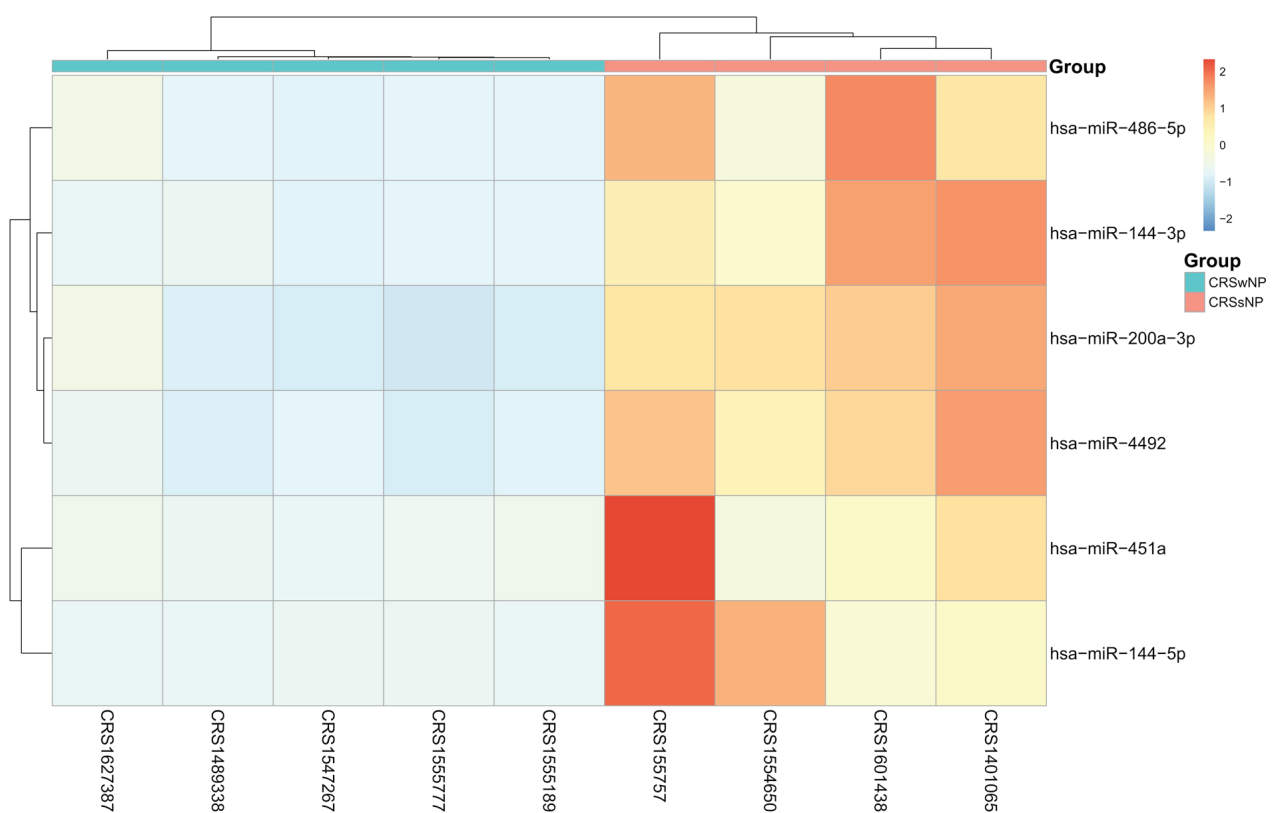


Figure 1. Heat map of the differentially expressed miRs in the different groups. The lateral axis represents the differentially expressed miRs, and the longitudinal axis represents the samples. Red indicates upregulation, blue indicates downregulation, pink indicates CRSwNP specimens and cerulean indicates CRSsNP specimens. miR, microRNA; hsa, *Homo sapiens*; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps.

Among these genes, a diverse range of GO terms were significantly enriched in the following three categories: Biological process (BP), cellular component (CC) and molecular function (MF). The top 5 enriched terms in each category are provided in Fig. 2, of which establishment of protein localization, endosome and purine ribonucleotide binding were the most significant terms in each group, respectively. The KEGG pathway enrichment analysis identified a total of 26 pathways as being enriched by the DEGs (Fig. 3), several of which are associated with the ErbB, Ras, cyclic adenosine monophosphate and Janus kinase (Jak)/signal transducer and activator of transcription (STAT) signaling pathways. These

pathways may therefore be involved in the development of CRSwNP.

Validation of the miR-4492 expression profile in CRS. RNA samples from 122 patients were prepared for RT-qPCR. A total of 26 samples were removed due to a lack of RNA integrity (Fig. S1). The remaining 96 RNA samples all passed the quality control checks for RNA integrity and purity. As presented in Fig. 4, the expression levels of miR-4492 were significantly reduced in the CRSwNP samples compared with those in the CRSsNP samples ($P=3.17 \times 10^{-5}$). Similarly, miR-4492 was also markedly downregulated in the CRSwNP group compared

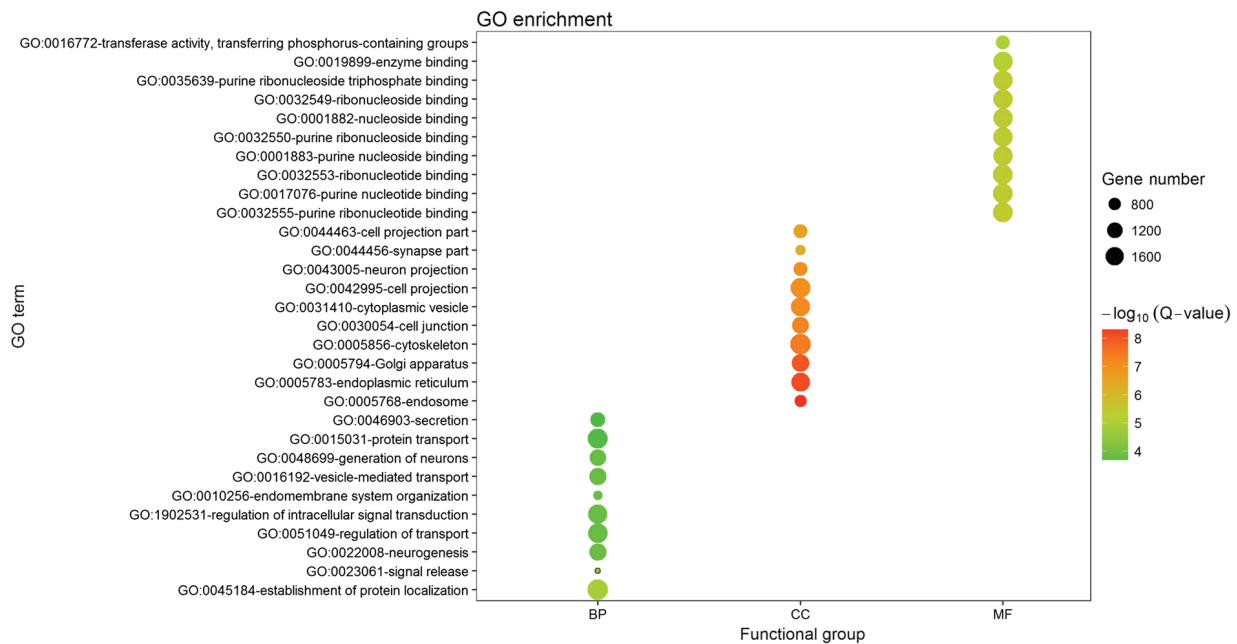


Figure 2. Visualization of GO enrichment for the predicted targets of the DEGs in CRSwNP. The detailed GO enrichment information is presented in a bubble diagram. The Y-axis represents the GO terms and the X-axis represents the categories BP, CC and MF. The number of genes enriched in a GO term is represented by the size of the node. The significance of the GO terms is represented by the colour, with red indicating the highest significance. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function.

with the control group ($P=2.86 \times 10^{-3}$). The difference between the miR-4492 expression levels of the CRSsNP group and the control group was not significant.

IL-10 and TNF- α expression in CRS. In order to investigate the targets of miR-4492, the protein-coding target genes were predicted using a Bioinformatics analysis. From the list of predicted targets of miR-4492, IL-10 and TNF- α were selected and their expression in the 96 samples was detected (Fig. 5). While the difference between the CRSsNP samples and the control samples was not significant, the expression levels of IL-10 were elevated in the CRSwNP samples compared with those in the CRSsNP samples ($P=8.76 \times 10^{-4}$) and the control samples ($P=1.74 \times 10^{-4}$). In addition, a negative correlation between the expression of miR-4492 and IL-10 expression was determined among the 96 specimens (Fig. 6). However, no significant correlation of miR-4492 with TNF- α expression was observed.

Discussion

CRS is an inflammatory upper airway disease with a high prevalence worldwide (1). Although CRSwNP is not the most common form of CRS, there is a higher proportion of patients with CRSwNP in tertiary care populations that have failed under medical management (25). A considerable number of studies have confirmed that miRNAs are involved in a diverse range of biological functions, including cell proliferation, differentiation, apoptosis, stress responses and signal transduction, and abnormal miRNA expression is considered to be a common feature of various diseases (26,27). The key role of miRNAs in inflammation is demonstrated by studies on the association of miRNA dysregulation with excessive inflammation and

aberrant immune responses, including pulmonary fibrosis and asthma (17,18). More recently, several studies have identified that marked changes in miRNA expression, which is tightly regulated, serve essential roles in the sustained and exaggerated inflammation of CRS (26-30). In the present study, next-generation sequencing was performed in order to detect key miRNAs in CRSwNP. Compared to the CRSsNP group, six differentially expressed miRNAs, namely *Homo sapiens* (hsa)-miR-200a-3p, hsa-miR-144-5p, hsa-miR-144-3p, hsa-miR-486-5p, hsa-miR-4492 and hsa-miR-451a, were identified in the CRSwNP samples. Downregulation of miR-200 family members as part of the epithelial to mesenchymal transition has been previously implicated in cancer development and progression (31). Furthermore, previous studies have confirmed the involvement of miR-200 family members in inflammatory and immune responses (32). Downregulation of exosome miR-200 has been detected in the bronchoalveolar lavage fluid of patients with asthma (33). A recent study indicated that miR-144 is an important regulator of innate immune responses, and its major and minor products are involved in colorectal polyposis, which involves inflammatory polyps at a different anatomic site (34). Panganiban *et al* (35) detected the expression of 420 plasma miRNAs in healthy individuals and patients with allergic rhinitis (AR) and asthma using RT-qPCR. They revealed that miR-144 was commonly dysregulated in patients with AR and asthma, suggesting that miR-144 is a promising plasma biomarker in these diseases. Furthermore, a previous study suggested that miR-541a, another miRNA that was identified as aberrant in the present study, is also an asthma-associated miRNA (36). It is widely accepted that CRS and asthma are closely associated in numerous aspects and they have similar pathogenic mechanisms and therapeutic principles. Based on the above, it may be possible to speculate

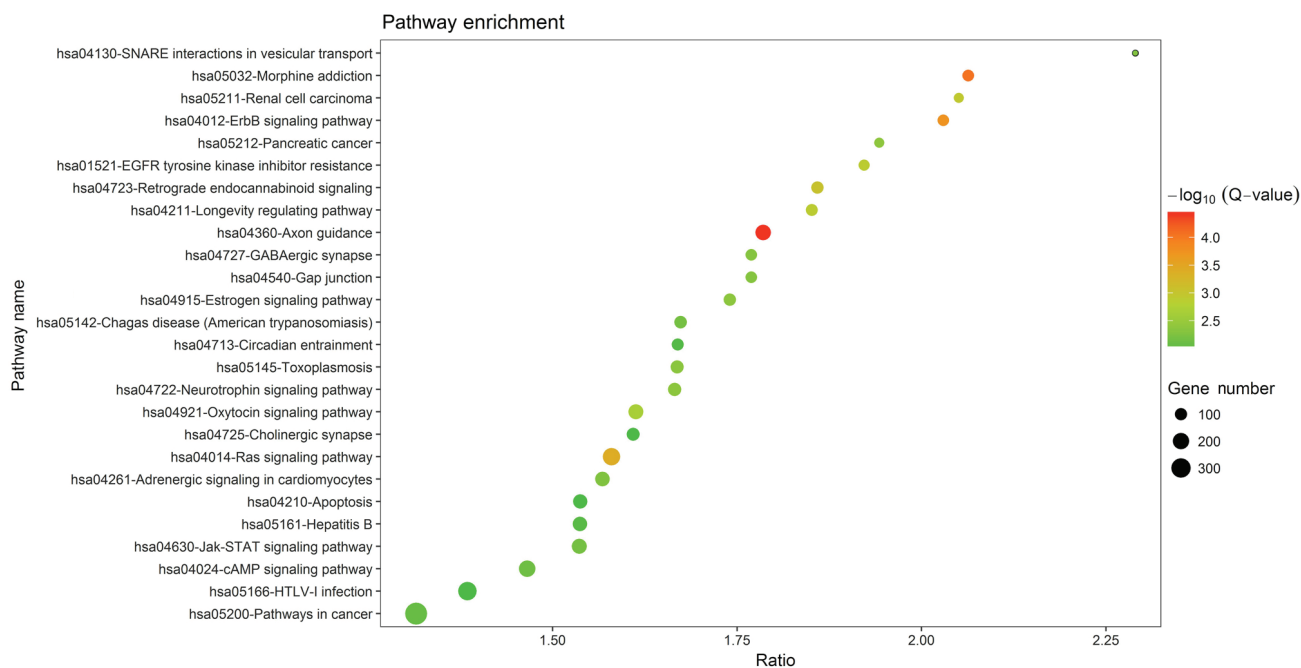


Figure 3. Visualization of KEGG enrichment for the predicted targets of the differentially expressed miRs in chronic rhinosinusitis with nasal polyps vs. chronic rhinosinusitis with nasal polyps. The detailed KEGG enrichment information is presented as a bubble diagram. The Y-axis represents the KEGG pathways and the X-axis represents the enrichment factor (gene ratio). The number of genes classified into the pathway is represented by the size of the node. The significance of the pathway is represented by the colour, with red indicating the highest significance. hsa, *Homo sapiens*; KEGG, Kyoto Encyclopedia of Genes and Genomes; Jak, Janus kinase; STAT, signal transducer and activator of transcription; cAMP, cyclic adenosine monophosphate; hTLV, human T-lymphocyte virus; EGFR, epidermal growth factor receptor.

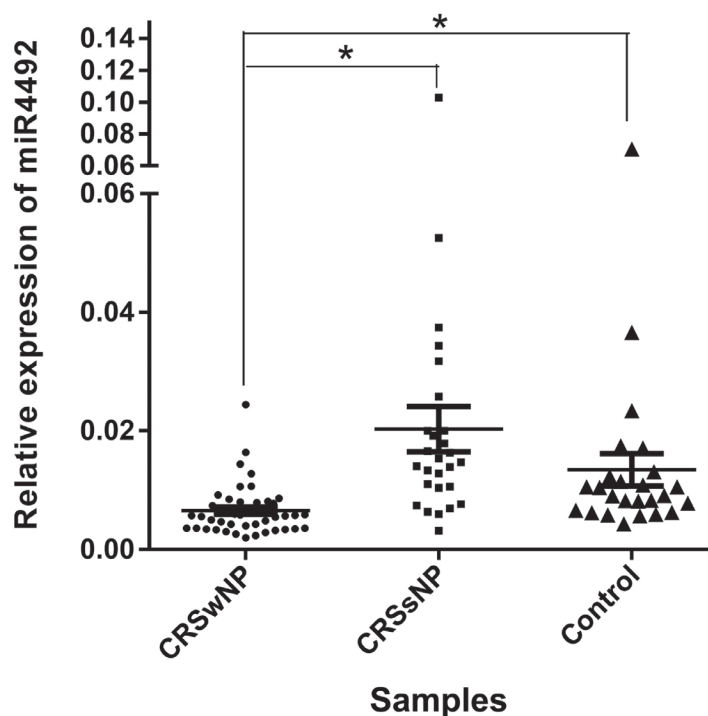


Figure 4. Differential expression of miR-4492 in patients with CRS. Reverse transcription polymerase chain reaction analysis of miR-4492 was performed and the expression levels were quantified relative to those of U6. miR-4492 expression in the nasal polyps was reduced to 32.3% of that in the CRSsNP group. * $P < 0.05$. miR, microRNA; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps.

on a concordant function of miR-200a, miR-144 and miR-451a in these two diseases. miR-485 has also been demonstrated to be reduced in CRSwNP, as detected using a microarray-based

approach (29). The differential expression of miR-4492 has been identified in a number of conditions, including responses to viral infection (37,38), necrosis (39), Hirschsprung's

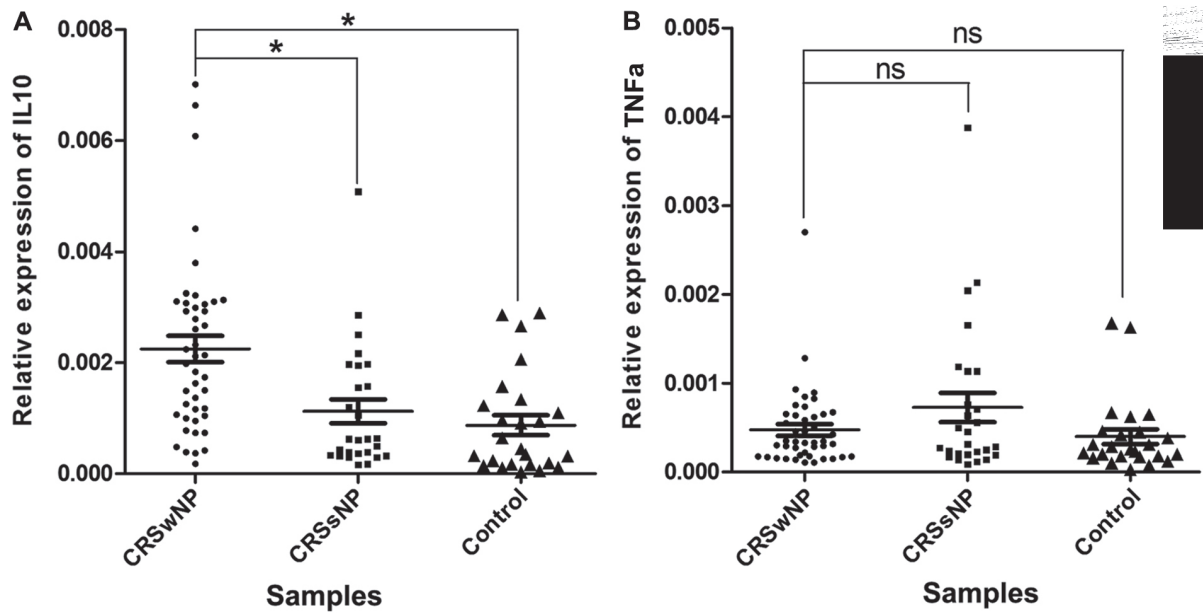


Figure 5. Differential expression of IL10 in patients with CRS. Reverse transcription- polymerase chain reaction of IL10 and TNFa was performed and the expression levels of IL10 and TNFa were quantified relative to those of GAPDH. (A) IL10 in the nasal polyps was upregulated 2.0-fold of that in the CRSsNP group. (B) There was no significant difference in the expression levels of TNFa between the different groups. *P<0.05. ns, no significance; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; TNFa, tumor necrosis factor; IL, interleukin.

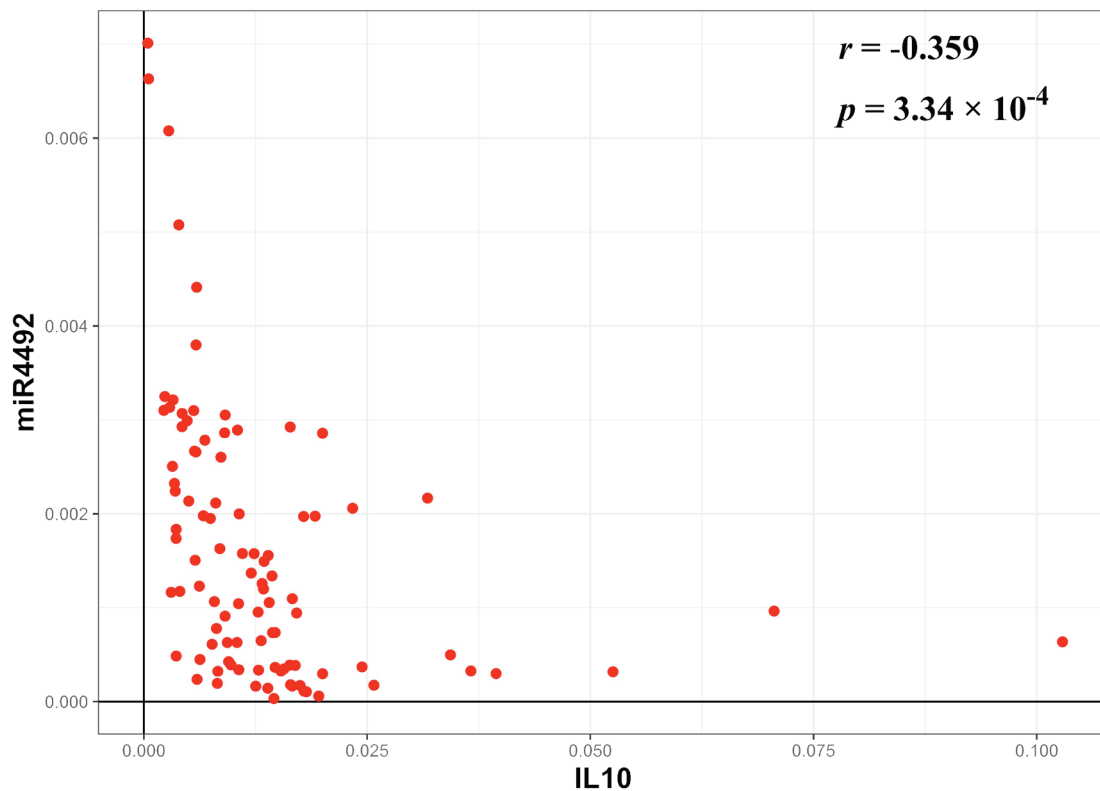


Figure 6. Correlation of microRNA-4492 and interleukin-10 expression in the 96 samples. Pearson correlation analysis was performed to assess the correlation between miRNA4492 and IL10 expression. *r*, Pearson correlation coefficient; *P*, P-value. P<0.05 served as statistically significant.

disease (40) and breast cancer (41). However, the exact functions and mechanisms require further investigation.

The mechanisms underlying the inflammation are different between the two types of CRS. CRSsNP involves more

T helper type 1 cell (Th1) polarization and collagen deposition, whereas CRSwNP is characterized by more Th2 polarization and tissue eosinophilia (42,43). It has been reported that miRNA is involved in the regulation of innate immune cells

that are recruited to infiltrate the inflamed tissue, as well as the polarization of Th2 cells (44). Although relatively few studies have assessed the miRNAs involved in CRS, a recent study indicated that miRNAs are associated with the inflammatory pattern of this disease (45). Previous studies have revealed that miR150-5p, miR-125b and miR-124 promote the development of CRS via regulating the immune response to inflammation (27,30,46). Xia *et al* (26) demonstrated that 7 miRNAs were differentially expressed in CRSwNP by using RT-qPCR, and the expression was correlated with the occurrence of NPs. Although these miRNAs were not among the DEGs identified in the present study, the disparity may be attributed to a number of reasons. First, different detecting platforms may partly account for the difference, as the RNA-seq data do not have complete genome coverage. In addition, in the present study, a relatively small number of samples were used for RNA-seq. Furthermore, the distinctive genetic backgrounds of the individuals and the tissue heterogeneity characteristic of this disease may have also affected the results. Therefore, a validation analysis of these DEGs was necessary in the present study. Of note, miR-4492, was the only differentially expressed miRNA identified between the CRSwNP samples and the control samples, and was downregulated in the polyps. Hence, the expression of miR-4492 was validated in the 96 specimens with acceptable RNA quality using RT-qPCR. The RT-qPCR analysis confirmed the downregulation of this miR in NP. Therefore, despite the lack of previous studies that support the involvement of this miRNA in NPs, the present results suggest that miR-4492 may have important roles in NPs according to the results of the present study.

Deregulation of a number of cytokines, including IL-1, IL-2, IL-10 and TNF- α , has been detected in a large number of diseases, including cancer, autoimmune diseases and inflammatory diseases. Increasing evidence suggests that cytokines are key mediators of the inflammation and immune response associated with NP, among which TNF- α and IL-10 are two predicted targets of miR-4492. Therefore, these two molecules were selected as candidates for validation in the present study. TNF- α is one of the most important pro-inflammatory cytokines produced by macrophages and T lymphocytes. Previous studies of Parkinson's disease demonstrate the importance of upregulated TNF- α in immune-mediated initiation of inflammation (47,48). Although differential expression of TNF- α has been reported in CRS with and without NP in previous studies, differences in the expression of TNF- α were not detected in the present study using RT-qPCR. A possible explanation for this discrepancy may be the heterogeneity of individuals from different regions. IL-10 is a pleiotropic cytokine with important immunoregulatory functions (49). Dysregulation of IL-10 is involved in the immunopathologic response to infection and the development of numerous autoimmune diseases (50). In the present study, elevated levels of IL-10 were detected in the CRSwNP samples, and IL-10 expression was negatively correlated with the expression of miR-4492 in CRS. Consistent with this, a previous study using clinical specimens demonstrated that increased expression levels of IL-10 have a pivotal role in the pathogenesis of CRSwNP (51). In a previous study by our group, dysregulation of miR-4492, miR-200-3p, TNF- α and IL-10 was observed in paraffin-embedded CRSwNP

samples using RNA-seq and RT-qPCR (52), whereas differences in the expression of TNF- α were not detected in the present study. Differences in sample type and sample size may at least partly account for this discrepancy. In addition, Luo *et al* (53) indicated that miR-19a has a crucial role in nasal polyposis through the suppression of IL-10 in peripheral dendritic cells. Furthermore, in KEGG pathway analysis, IL-10 was enriched in the Jak/STAT signaling pathway. Combined with the results of the present study, it is indicated that hsa-miR-4492 may serve roles in nasal polyposis via the hsa-miR-4492/IL-10 interaction in the Jak/STAT signaling pathway (52). However, the present study has a number of limitations, including the relatively small number of samples for miRNA sequencing and lack of a functional validation assay. Therefore, further studies are required in order to elucidate the interactional mechanism of this pathway in CRSwNP.

In conclusion, the present study demonstrated that miR-4492 is downregulated and IL-10 is upregulated in NPs, and suggests that the involvement of the hsa-miR-4492/IL-10-axis in the Jak/STAT signaling pathway may be a key mechanism in CRSwNP.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL and JYo conceived and designed the study. LL, JF, DZ, YW, JYa and RH performed the data analysis. LL wrote the manuscript. LL and JYo edited the manuscript. All authors reviewed the manuscript and approved the final version.

Ethics approval and informed consent

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Urumqi, China; permit no. 20160114-05) and it was performed with written informed consent from the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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