Original Research

Differential expression profile of plasma exosomal microRNAs in chronic rhinosinusitis with nasal polyps

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Impact Statement

Plasma exosomes can contribute to inflammation, immune responses, and infection by delivering their miRNA contents, which has not been studied in sufficient detail in people with chronic rhinosinusitis. We identified the differentially expressed plasma exosomal miRNAs and corresponding target gene functions in this study, which could provide theoretical support for the pathophysiological mechanisms underlying chronic rhinosinusitis.

Abstract

Exosomes mediate inflammation and immune responses. The aim of the study was to examine the expression profiles of plasma exosomal microRNAs (miRNAs) and analyze their target gene functions in participants with chronic rhinosinusitis with nasal polyps (CRSwNP). We measured plasma exosomal miRNAs in five patients with CRSwNP and five controls. Transcripts per million (TPM) was used to assess miRNAs expression and the Benjamini–Hochberg procedure was employed for multiple comparisons correction. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene ontology (GO) analyses revealed biological annotation and functional prediction of target genes. Compared with controls, we found that 159 exosomal miRNAs were differentially expressed by miRNA sequencing in CRSwNP. The top three upregulated miRNAs were novel_miR_677, novel_miR_1037, and novel_miR_79, while the top three downregulated miRNAs were novel_miR_192, novel_miR_1022, and novel_miR_4. The target functions in the GO and KEGG analyses included axon guidance, extracellular matrix (ECM)–receptor interaction, protein digestion and absorption, the calcium, the Hippo, the Notch, the ErbB, the cAMP signaling pathway, and focal adhesion. This study describes the dissection of plasma exosomal miRNA profiling in CRSwNP. Our findings may provide a certain basis for further mechanism research and exploration of diagnostic values.

Keywords: Plasma exosomes, microRNA, chronic rhinosinusitis

Experimental Biology and Medicine **2022; 247: 1039–1046. DOI: 10.1177/15353702221090184**

Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous inflammatory disease. The main symptoms include nasal congestion, facial pain, headache, and decreased sense of smell.[1] At present, CRS has seriously affected the quality of life and caused a great economic burden.[2,3] Chronic rhinosinusitis with nasal polyps (CRSwNP) is an important phenotype of CRS, characterized by accumulation and increase of eosinophils, subepithelial edema, basement membrane thickening, and fibrosis.[4] However, the mechanisms underlying the various clinical manifestations caused by chronic inflammation and tissue remodeling are complex and unclear.

MicroRNAs (miRNAs) are a kind of small, non-coding RNAs that are transcribed from endogenous genic loci in the nucleus by RNA polymerase II. Mature miRNAs are approximately 21–24 nucleotides in length which post-transcriptionally regulate gene expression, primarily by binding to the target mRNAs, resulting in degradation or inhibition of protein translation.[5,6] miRNAs are associated with the pathophysiological process of CRS. For example, compared with CRS without nasal polyps (CRSsNP) and controls, miR-21 is found to significantly increased and regulate transforming growth factor (TGF)-β1-induced epithelial–mesenchymal transition in CRSwNP.[7] Up-regulated expression of miR-125b enhances interferon (IFN) expression and contributes to tissue eosinophilia in CRSwNP.[8]

Exosomes are a subset of extracellular vesicles (EVs) with a diameter range of ~40–160 nm, and they are generated by all cells. Exosomes can contribute to inflammation, immune responses, and infection by delivering their protein and miRNA contents as well as influencing signaling pathways in recipient cells. Exosomal miRNAs can be transported in plasma, mediate intercellular communication, and

ISSN 1535-3702

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affect various aspects of cell biology in disease, which favor their use as diagnostic biomarkers or therapeutic agents.[9] However, the characterization of exosomal miRNAs in the plasma of CRSwNP remains unclear. The purpose of this study was to identify exosomal miRNA expression profiles and further infer the potential functions of differentially abundant miRNAs.

Materials and methods

Blood sample collection

We collected all blood samples from five controls (with deviation of nasal septum, without CRS symptoms, sinonasal opacification on computerized tomography (CT) scan, allergy, or asthma) and five patients with CRSwNP (with clinical and pathological evidence). None of the participants took any antibiotics, systemic or intranasal corticosteroids, or anti-leukotrienes for three months before the blood samples were collected. This study was approved by the Ethics Committee of Beijing Tongren Hospital (TRECKY2020-117), and written informed consent was obtained from all participants.

Exosome isolation

To remove cellular debris and cells, we thawed frozen plasma specimens and cryogenically centrifuge specimens (below 4°C) at 5000*g* for 20 min. We put supernatant through a 0.45 μm membrane. Plasma was used for exosome isolation with an exosome isolation kit (Abace Biotechnology, Beijing, China). Briefly, the size exclusion column was washed and equilibrated with 15 mL phosphate-buffered saline (PBS). We loaded the plasma (1.0 mL) onto the column and allowed to enter the bed. Twelve 1.0 mL fractions were collected using PBS. The first several fractions were discarded, and the remaining fractions (3–4 mL) containing exosomes were further concentrated using a spin column. The purified exosomes were refrigerated at −80°C.

Exosome identification

Nanoparticle analysis. We used isolated exosome fractions in nanoparticle tracking analysis. Particle diameter and concentration were estimated by ZetaView PMX110 (Particle Metrix, Germany). The sample was diluted to yield particle concentrations in the range of 1×10^7/mL to 1×10^9/mL. Videos 60 s long were recorded for each sample using a shutter speed of 30 ms. After capture, the videos were analyzed using ZetaView Software 8.02.28.

Western blot. The presence of exosomal markers (CD9, CD63, TSG101, or Alix) was tested using the western blotting. Briefly, exosomes were lysed in sodium dodecyl sulfate and heated at 100°C for 10 min. Lysates were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels electrophoresis and transferred to nitrocellulose blotting membrane (GE Healthcare, USA). We blocked the membranes with 4% non-fat milk. Then, we incubated the membranes with primary antibodies at 20°C for 2 h. Next, we added the goat antimouse IgG (H + L) horseradish peroxidase (HRP) conjugate secondary antibody for 1 h at 20°C. We developed blots with enhanced chemiluminescence (ECL) detection reagents.

Electron microscopy. We used transmission electron microscopy to observe the morphology of the exosomes. First, we added 10 μL exosomes to a copper grid with a pipette and after 1 min of sedimentation, we removed the excess solution by filter paper. Then, we dripped 10 μL phosphotungstic acid onto the copper grid and blotted out after 1 min of sedimentation. Following this, the sample was dried at room temperature and examined using a transmission electron microscope (FEI Company, USA) operated at 80 kV.

RNA extraction and sequencing. We extracted the total RNA from exosome fractions using the Qiagen miRNeasy Mini Kit and monitored RNA degradation and contamination. RNA concentration and purity were tested using the Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA) and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

A total amount of each RNA sample was 2.5 ng, which was used as input material. To generate the sequencing libraries, we used the NEBNext® Small RNA Sample Library Prep Kit for IlluminaR (NEB, USA). The 3′ SR adaptor and 5′ SR adaptor were ligated to the 3′ and 5′ ends of small RNA, respectively, using T4 RNA Ligase. To prevent the formation of adaptor-dimer, the SR RT Primer was hybridized to excessive 3′ SR adaptor. We used M-MuLV reverse transcriptase to synthesize first-strand cDNA and accomplished polymerase chain reaction amplification for the library enrichment. The library preparations were sequenced on an Illumina platform after cluster generation.

We removed raw reads with adaptors and poly N >10% in order to get clean data. In addition, reads with lengths <15 nt or >30 nt were discarded. We calculated the GC contents, Q20 and Q30, to verify the data quality, and used high-quality data for downstream analysis.

Data analysis. Using Bowtie software,[10] clean data were aligned to reference databases (Silva, GtRnab, Rfam, and Repbase) to filter out other RNA types and repeats. The unannotated reads were mapped to the human genome sequence, and the mapped reads were used to identify novel miRNAs and known miRNAs in miRBase (version 22.0).

We used transcripts per million (TPM) to estimate the expression levels of miRNAs.[11] Differentially expressed miRNAs were analyzed using the DESeq R package,[12] and corresponding differentially expressed target genes were predicted using miRanda and Targetscan.[13,14] The Benjamini–Hochberg procedure was employed for correction of multiple comparisons. Differentially expressed transcripts were set at a false discovery rate (FDR) <0.05 and |log2 fold change| >0.58 and were considered for further analyses. The identification of the influenced pathways and the target genes' main functions was performed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses.

Results

Participant characteristics

Statistical analysis was performed to compare the categorical (chi-square test) and the continuous data (analysis of variance [ANOVA]). Statistical significance was set at $P < 0.05$. Significant differences were not observed with regard to sex,

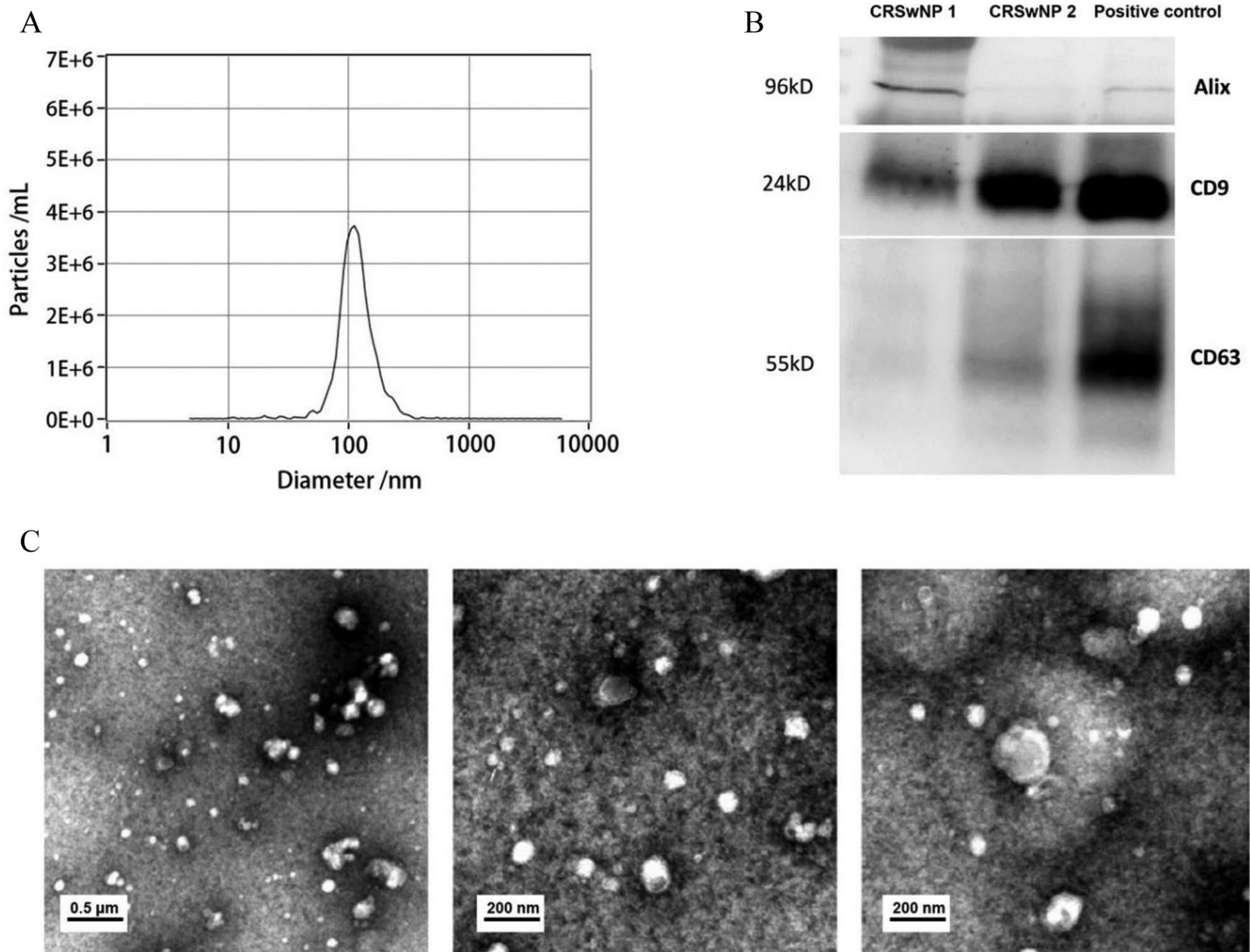

Figure 1. (A) Nanoparticle tracking analysis indicated that mean diameter of EVs was 110.1 nm. (B) CD9, CD63, and Alix proteins are the exosomal markers in the western blot. Positive control: HEK293T cell lysates. (C) Transmission electron microscopic images of extracted exosomes revealed cup-shaped structures with a diameter of about 40–160 nm.

age, smoking status, or concomitant asthma between the CRSwNP and control groups. Compared with the controls, eosinophils in the peripheral blood, prior sinus operation history, and serum total IgE were higher in the CRSwNP cohort (Supplementary Table 1).

Identification of plasma exosomes

The characterization of exosomes was based on the presence of exosomal markers, morphology, size, and concentration. Nanoparticle tracking results demonstrated that the median diameter and the concentration of isolated EVs were 110.1 nm and 3.6E+7 particles/mL, respectively (Figure 1(A)). The markers for exosomes, such as CD9, CD63, and Alix, were detected by the western blotting (Figure 1(B)). Furthermore, transmission electron microscopy revealed membrane-bound spherical structures of ~40–160 nm in diameter, which are the typical characteristics of exosomes (Figure 1(C)).

Overview of exosomal miRNA sequencing

A total of 207,810,565 raw reads (109,665,938 for CRSwNP and 98,144,627 for the control) were generated, and 151,219,246 clean reads (79,174,959 for CRSwNP and 72,044,287 for the control) were obtained after quality control. After discarding other types of non-coding RNA, we mapped the residual reads to the human reference genome, and the mapping rates were approximately 11.55%. Ultimately, we identified 1692 known miRNAs and 1068 novel miRNAs.

Differential expression analysis: CRSwNP versus the control

We estimated miRNA expression levels using TPM. Compared to the control, a total of 159 significantly dysregulated miRNA transcripts, including 93 upregulated and 66 downregulated transcripts, were differentially expressed in CRSwNP (Supplementary Table 2). Differentially expressed exosomal miRNAs were visualized using a volcano plot and heatmap (Figures 2 and 3). Based on the log2 fold change value, novel_miR_677, novel_miR_1037, and novel_miR_79 were the top three upregulated miRNAs, while novel_miR_192, novel_miR_1022, and novel_miR_4 were the top three downregulated miRNAs (Table 1).

Functional annotation

To investigate the potential functions of differentially expressed miRNAs, the corresponding differentially

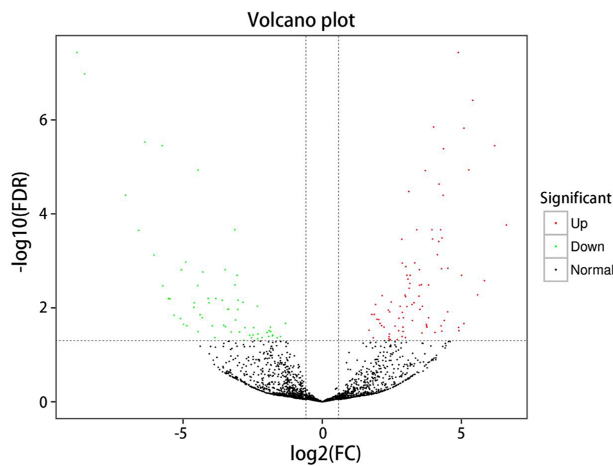

Figure 2. Volcano plot of the differentially expressed plasma exosomal microRNAs. X-axis: log2 (fold change); Y-axis: –1 × log10(FDR). Red plots: upregulated microRNAs, and green plots: downregulated microRNAs. (A color version of this figure is available in the online journal.)

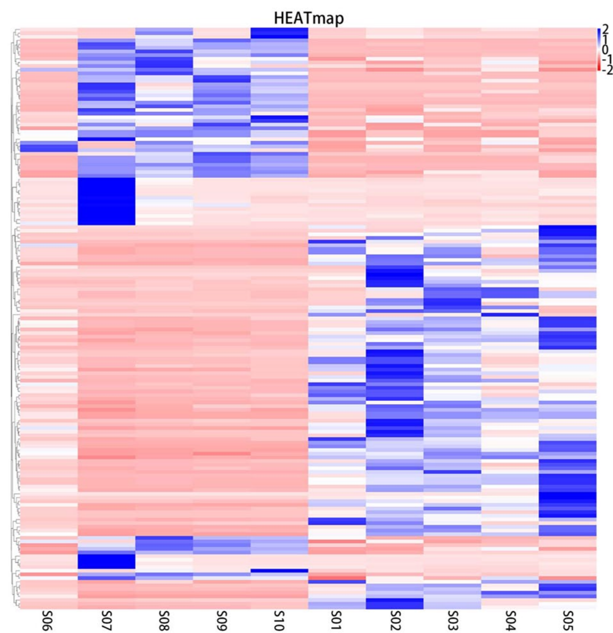

Figure 3. Heatmap demonstrated cluster analysis of the differentially expressed plasma exosomal microRNAs in CRSwNP relative to control (S01–S05: CRSwNP; S06–S10: control; the red and the blue markings indicated increased and decreased expression).

expressed target genes were predicted and KEGG enrichment and GO analyses of target genes were conducted.

We selected the top 10 significantly classification terms in GO analysis (Figure 4). Differentially expressed target genes were significantly enriched in biological processes related to small GTPase-mediated signal transduction, extracellular matrix (ECM) and structure organization, RAS protein signal transduction, and regulation of cell morphogenesis. Similarly, molecular function analysis revealed that target genes might be involved in ECM structural constituent, beta-catenin binding, small GTPase binding, and the activity of a series of factors. The results of cellular components indicated that proteins encoded by target genes might be located in the ECM and synaptic structures.

Table 1. The top 10 upregulated and downregulated microRNAs.

#ID	FDR	log2FC	Regulated
novel_miR_677	0.000171555	6.611790353	Up
novel_miR_1037	3.56E–06	6.1941173	Up
novel_miR_79	0.002634108	5.827962978	Up
novel_miR_357	0.005275369	5.580370764	Up
novel_miR_1058	3.83E–07	5.405788925	Up
novel_miR_757	1.16E–05	5.266442155	Up
novel_miR_228	1.49E–06	5.090450229	Up
novel_miR_551	1.49E–06	5.090450229	Up
novel_miR_320	0.021534125	5.089104788	Up
novel_miR_839	0.002030259	5.00337913	Up
hsa-miR-410-3p	0.006431983	–5.470173336	Down
novel_miR_667	0.006282554	–5.519595619	Down
novel_miR_594	0.003361495	–5.725469669	Down
novel_miR_376	3.56E–06	–5.752499435	Down
hsa-miR-9-3p	0.000747169	–6.040720019	Down
novel_miR_595	2.97E–06	–6.368067536	Down
novel_miR_923	0.000223302	–6.593320495	Down
novel_miR_4	4.03E–05	–7.060304947	Down
novel_miR_1022	1.06E–07	–8.539769376	Down
novel_miR_192	3.66E–08	–8.812188036	Down

FDR: false discovery rate; log2FC: log2 fold change.

The KEGG results demonstrated that a series of potential pathways could be affected by dysregulated exosomal miRNAs. As illustrated in Figure 5, 59 pathways were significantly enriched and the top 10 enriched pathways were as follows: regulation of the actin cytoskeleton, axon guidance, protein digestion and absorption, ECM–receptor interaction, the Hippo, the Notch, the ErbB, the cAMP, the calcium signaling pathway, and focal adhesion. The exosomal miRNAs and corresponding target genes that were attributed to the top 10 enriched pathways are listed in Supplementary Table 3, and the regulated association between exosomal miRNAs and pathways is shown in Figure 6.

Discussion

CRS is a multifactorial disorder with diverse clinical phenotypes, pathologies, and pathophysiological mechanisms. Exosomes may play a role in CRS pathogenesis by regulating complex intracellular pathways and there is already evidence of an association between exosomes and CRS. For example, nasal lavage fluid-derived exosomes from patients with CRSwNP contained disintegrin, metalloprotease 10, and miRNA-22-3p, which were able to promote angiogenesis and vascular permeability.[15] In another study, mucus-derived exosomes in CRSwNP were enriched with P-glycoprotein, and the intercellular transfer of exosomal P-glycoprotein resulted in a pro-inflammatory response.[16] The coagulation and fibrinolysis pathways, which are implicated in the etiopathogenesis of CRSwNP, associated proteins in mucus-derived exosomes in CRSwNP exhibited strong correlation with the matched proteins within tissue.[17] Another study demonstrated that 80 differentially regulated exosomal proteins overlapped with the matched nasal polyps tissue

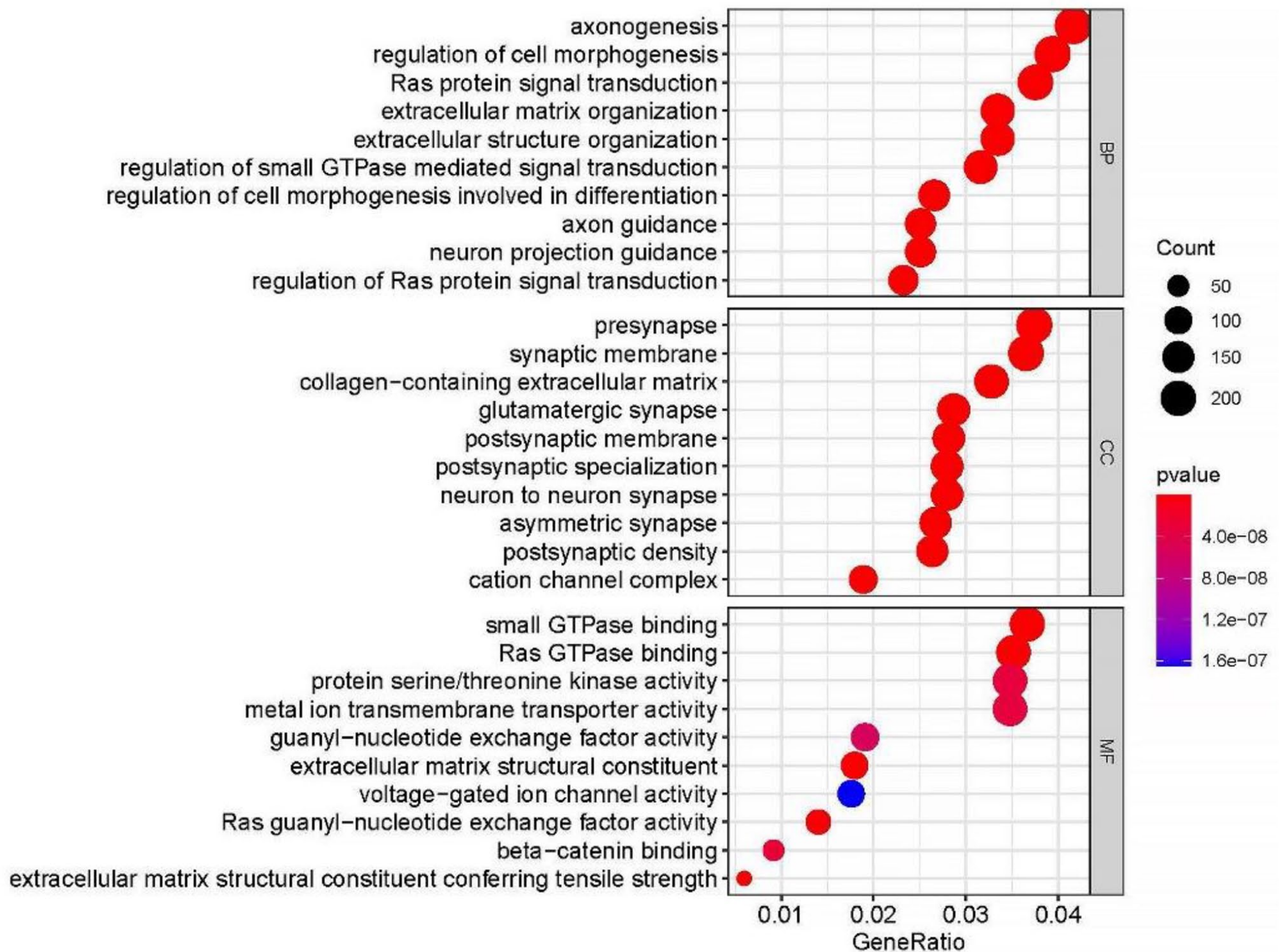

Figure 4. GO annotation of the predicted target genes. (A color version of this figure is available in the online journal.)

proteome, and some of these proteins had predictive value for the disease.[18] All of these studies support the idea that exosomal analysis could be a promising method for investigating chronic sinonasal inflammation.

Although circulating miRNAs are specifically expressed in asthmatic, which could act as an non-invasive biomarkers for the diagnosis and characterization of the disease,[19] the changes in exosomal miRNA content in the plasma of CRSwNP have not yet been described. In this study, we isolated exosomal miRNAs from plasma and identified 1068 novel miRNAs and 1692 known miRNAs in CRSwNP and controls using RNA sequencing. We then detected the differentially expressed miRNAs between them. A total of 93 upregulated and 66 downregulated miRNAs and target genes were identified. Some of these circulating exosomal miRNAs are consistent with other findings that are based on the nasal mucosa, while others that affect the expression of multiple genes associated with chronic inflammation have not yet been studied. Xia *et al.*[20] analyzed the seven differentially expressed miRNAs associated with inflammation in the mucosal tissue of CRS patients and found that, compared to controls, miRNA-125b and miRNA-92a were dysregulated in CRS. Similarly, Zhang *et al.*[8] explored the miRNA expression profile in the sinonasal mucosa of CRS and found that, compared to controls, miRNA-125b, miRNA-136, and miRNA-486-5p were differentially expressed in CRSwNP. Another study demonstrated that eosinophil trafficking is regulated by endothelial miRNA-1 in the context of allergic airway inflammation and could be downregulated by interleukin (IL)-13. Serum miRNA-1 levels were inversely correlated with sinonasal tissue eosinophilia in patients with CRS.[21]

KEGG enrichment and GO analyses were performed to detect the functions of 159 dysregulated miRNAs. The results demonstrated that a few exosomal miRNAs could regulate functionally related genes and pathways associated with CRS. Mucosal innervation and neurons could affect the mucosal microenvironment and protective responses via the axon response, and proteins associated with the axonal guidance signaling pathway and neuronal growth were suppressed in CRSwNP.[22] We found that exosomal miRNAs, such as miRNA-6850-5p, miRNA 598-5p, and other novel miRNAs, were upregulated in CRSwNP and were able to suppress gene expression under axon guidance. Ten dysregulated miRNAs post-transcriptionally regulate gene expression in the Hippo signaling pathway. The epithelial cell proliferation and remodeling in nasal polyps are associated with abnormal upregulation of the Hippo pathway components.[23] β-catenin is one of the GO terms for gene function and may control cell–cell adhesion. More importantly,

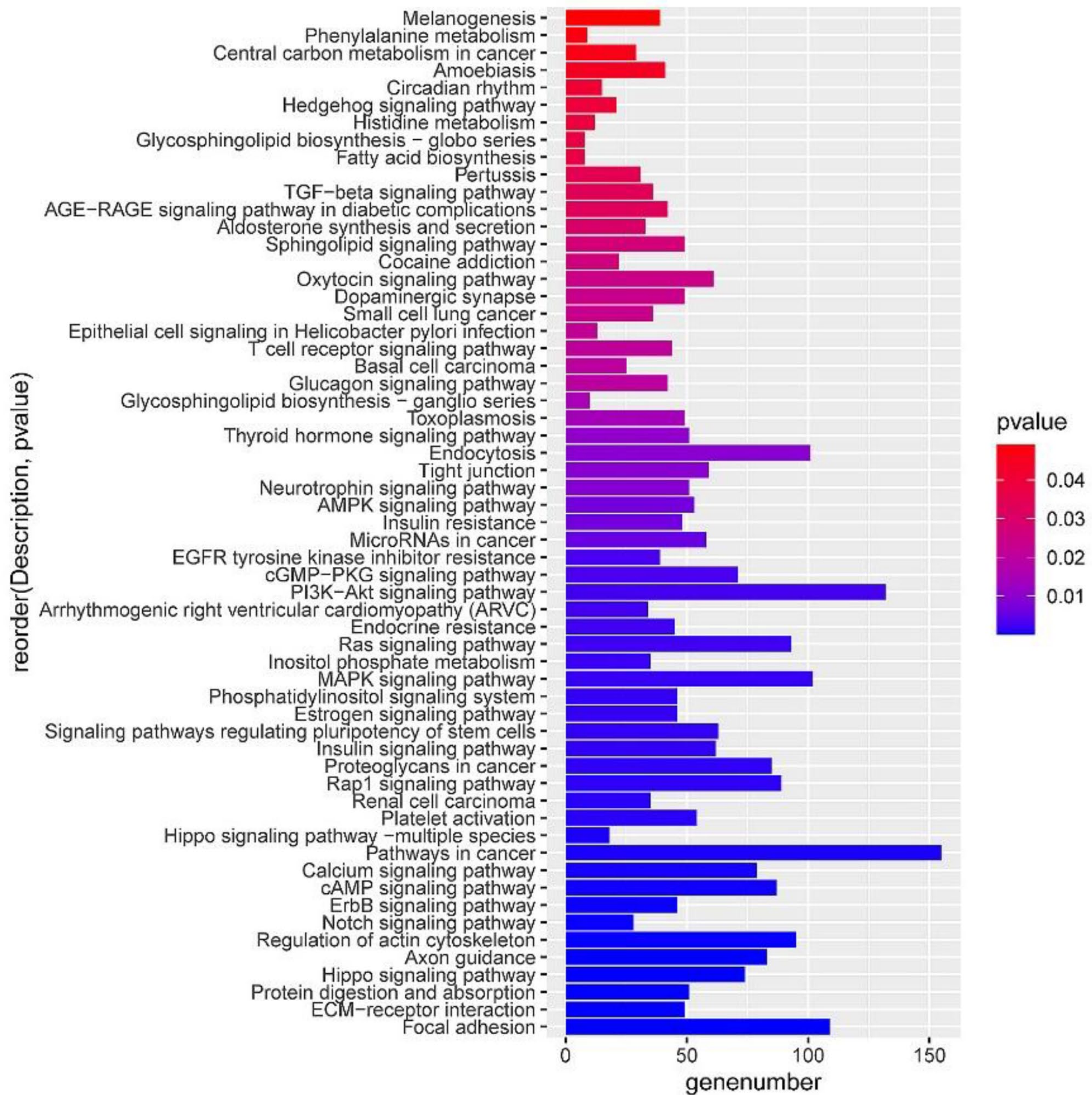

Figure 5. KEGG pathway enrichment analysis of the predicted target genes. (A color version of this figure is available in the online journal.)

β-catenin is a significant downstream regulator of the Wnt pathway. A recent study about the mechanism of epithelial to mesenchymal transition (EMT) in nasal polyps indicated that the Wnt/β-catenin pathway is activated in epithelium and can lead to the stimulation of EMT.[24] EMT is characterized by epithelial cells lose polarity, cell–cell adhesion decreases, and the cytoskeleton becomes reorganized. Chronic EMT can accelerate nasal polyp formation.[25,26]

MiRNAs isolated from blood might be important regulators of Th2 cytokines and have utility as biomarkers in upper airway inflammation.[19,27] Therefore, differential exosomal miRNAs from plasma in patients with CRS are unlikely to be mere epiphenomena. Depending on the regulatory networks of miRNA-pathway interactions, differentially expressed miRNAs may play a regulatory role in pathological processes. In addition, miRNAs can serve as anti-inflammatory agents, and it is also possible that upregulated miRNAs in polyps are secondarily to chronic inflammation and cannot promote pathogenic of the disease. Most cells can produce miRNAs and secreted it into exosomes, which can enter the circulation and communicate with target cells over long distances. It is possible that the abnormal uptake of exosomal miRNAs between the blood and nose impairs the ability of anti-inflammatory substances and stimulates inflammation. These areas have not been well explored in CRS and require further study.

We determined the differences in exosomal miRNA expression between CRSwNP and controls. These data could be further used to explore their role in the expression

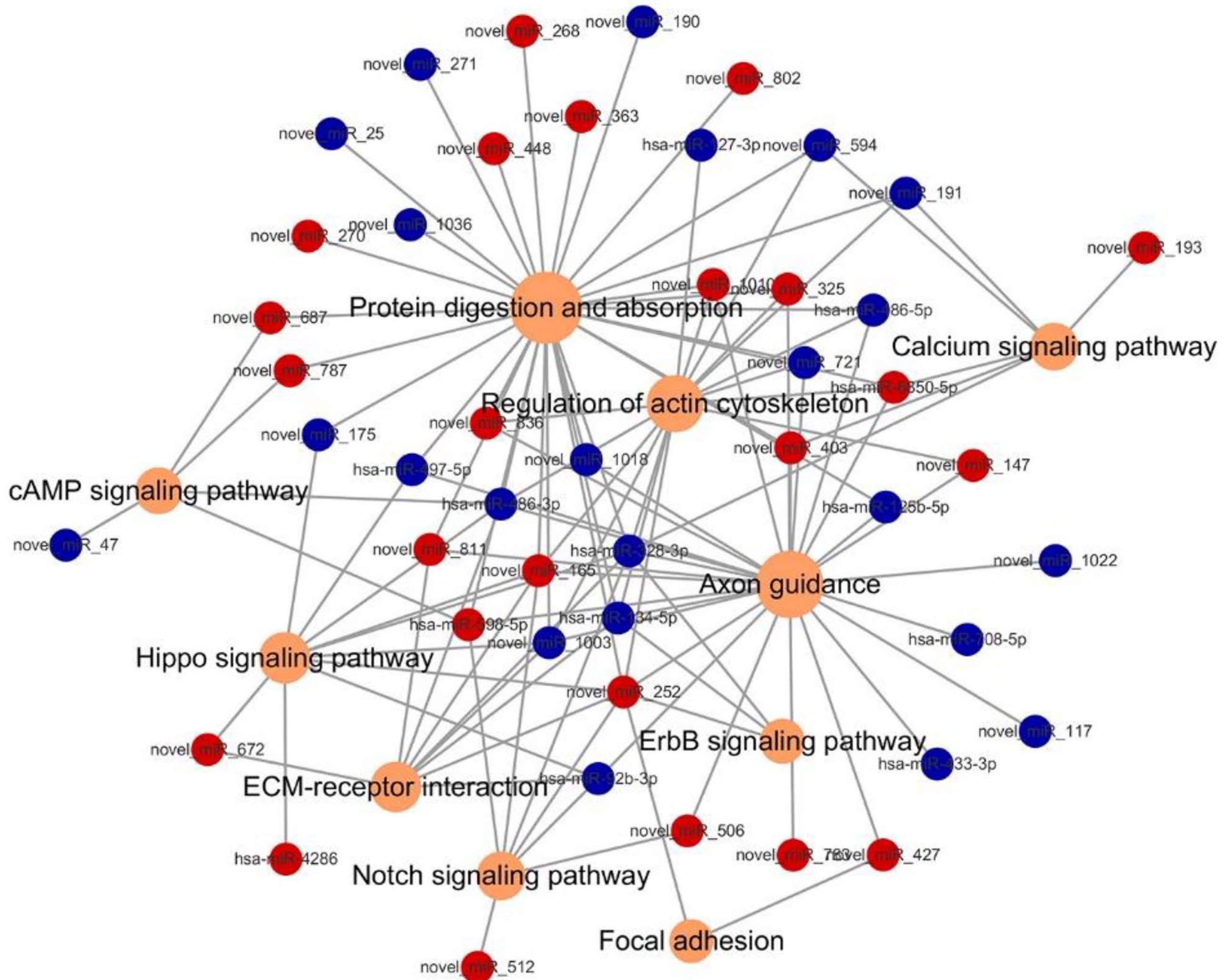

Figure 6. The interactive network constructed by the top 10 enriched pathways and dysregulated microRNAs. Red nodes represented upregulated microRNAs, and blue nodes represented downregulated microRNAs. (A color version of this figure is available in the online journal.)

of inflammatory cytokines in various cell lines, the differentiation, proliferation, and survival of immune cells, epithelial cells, and mesenchymal stem cells in CRS, or to explore the effect of exosomal miRNA expression after treatment. Specific miRNAs in the serum could be used for less invasive liquid examination, with potential for the detection of CRS or long-term sampling to observe disease progression, for example, as a biomarker of the persistence and recurrence of chronic rhinosinusitis after treatment. Because exosomal miRNAs can effectively suppress gene expression in recipient cells, the therapeutic potential of exosomes is being actively explored. Intravenously injected exosomes delivered RNA to recipient cells may have potential utility in the treatment of cancer or neurological disease.[28,29] Engineering exosomes to deliver a specific miRNA payload to nasal structural and immune cells may be developed for CRS.

The relatively small sample size is a limitation of this study, which highlights the heterogeneity of the disease. Further studies are required to assess a larger sample size in a validated clinical trial. The results concerning these miRNAs could be validated through quantitative polymerase chain reaction (qPCR) as part of future exploration of the underlying mechanisms.

Conclusions

In this study, we elucidated the exosomal miRNA profile in the plasma of patients with CRSwNP using RNA sequencing. Our findings further expand our knowledge on exosomal miRNA biology and may contribute to the understanding of their regulatory therapeutic targets in pathogenesis. In addition, exosomal miRNAs could be used as potential biomarkers or in therapeutic strategies in the future.

AUTHORS' CONTRIBUTIONS

S.H. and J.W. participated in the study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, and original draft preparation. D.H., Y.L., T.W., H.W., and Y.P. participated in analysis and interpretation of data, and statistical analysis. H.Z. participated in the study concept and design, analysis and interpretation of data, and drafting and critical revision of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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

ETHICAL APPROVAL

This study was approved by the Ethics Committee of Beijing Tongren Hospital (TRECKY2020-117).

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the priming scientific research foundation for the junior researcher in Beijing Tongren Hospital, Capital Medical University.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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(Received November 21, 2021, Accepted March 6, 2022)