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ORIGINAL RESEARCH

miR-6089 Alleviates Inflammation and Cell Apoptosis Through Modulating the TLR4 Pathway in Mite-Sensitized Allergic Rhinitis

Chang-Yu Qiu[1,2], Jia-Xin Bi[1], Xin-Yan Cui[1], Ruo-Xi Chen[1], Zheng Luan[1], Yun Guo[3], Mei-Ping Lu[1], Ling Li[3], Lei Cheng[1,2,4]

[1]Department of Otorhinolaryngology & Clinical Allergy Center, The First Affiliated Hospital, Nanjing Medical University, Nanjing, People's Republic of China; [2]International Centre for Allergy Research, Nanjing Medical University, Nanjing, People's Republic of China; [3]Department of Respiratory Medicine & Clinical Allergy Center, Wuxi Children's Hospital, Affiliated Children's Hospital of Jiangnan University, Wuxi, People's Republic of China; [4]Department of Allergology, The First Affiliated Hospital, Nanjing Medical University, Nanjing, People's Republic of China

Correspondence: Lei Cheng, Department of Allergology, The First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou Road, Nanjing, 210029, People's Republic of China, Email chenglei@jsph.org.cn; Ling Li, Department of Respiratory Medicine & Clinical Allergy Center, Wuxi Children's Hospital, Affiliated Children's Hospital of Jiangnan University, 299-1 Qingyang Road, Wuxi, 214023, People's Republic of China, Email liling@njmu.edu.cn

Purpose: Allergic rhinitis (AR), a chronic inflammatory disease of nasal mucosa, is considered as a classic Th2-mediated disease. We aimed to elucidate the molecular mechanisms and therapeutic potential of microRNAs (miRNAs) in AR.

Methods: Nasal mucosa was collected from patients with mite-sensitized AR and non-allergic controls for miRNA and mRNA sequencing. miRNA expression was profiled. GO and KEGG enrichment analyses were conducted. Luciferase reporter assay was implemented to identify potential targets of candidate miRNAs. An AR cell model was established through lipopolysaccharide (LPS) exposure. miR-6089 was overexpressed or downregulated to characterize its roles in the proliferation and apoptosis of human nasal epithelial cells (HNEpC). The relationship between miR-6089 and toll-like receptor 4 (TLR4) was described. PCR and ELISA were applied to quantify the expression levels of miRNAs and mRNAs.

Results: A total of 28 miRNAs and 172 mRNAs were identified to be differently expressed in the nasal mucosa of patients with AR compared to controls. The KEGG enrichment analysis showed that TLR signaling pathway, NF-κB signaling pathway, IL-17 signaling pathway and other pathways were significantly enriched in these differentially expressed RNAs. As shown by PCR results, the expression of miR-6089 decreased, and that of TLR4, IL-6, IL-8, and TSLP increased significantly in the nasal mucosa from patients with AR. Dual-luciferase reporter assay showed that miR-6089 directly bound to TLR4. miR-6089 could increase the viability, inhibit apoptosis, and relieve inflammatory response in LPS-induced HNEpC. Furthermore, miR-6089 could regulate the expression of TLR4, IL-6, IL-8, and TSLP in the LPS-induced HNEpC.

Conclusion: miR-6089 can alleviate LPS-induced inflammatory response via targeting TLR4 and may serve as a therapeutic target in the treatment of mite-sensitized AR.

Keywords: allergic rhinitis, inflammation, lipopolysaccharides, microRNAs, miR-6089, toll-like receptor 4

Introduction

Allergic rhinitis (AR) develops as allergens stimulate the interaction between Th2 cytokines and B cells, production of allergen-specific IgE, and inflammatory chain reaction.[1] AR seriously affects the quality of life of patients and brings them with a great economic burden.[2] At present, AR is commonly treated with pharmacotherapy and immunotherapy. However, these treatments are challenged by long courses, weak effects and adverse events. Therefore, new targeted therapy has become a research hotspot in the treatment of AR.

Many newly reported molecules act as drivers in AR.[3] As representative recognition receptors, toll-like receptors (TLRs) are involved in either non-specific or specific immunity in the pathogenesis of allergic airway diseases.[4] There are 10 types of

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TLRs in humans. Nuclear factor-κB (NF-κB) and interferon signals can be induced by TLR responses.[4] TLR4 recognizes lipopolysaccharides (LPS) or bacterial endotoxins to mediate innate immunity and the release of inflammatory factors. TLR4 undertakes a critical role in the development of AR.[5,6] The expression level of TLR4 increases in the nasal mucosal epithelial cells of patients with AR, but this increase is not observed in all patients.[7–9] Bergougnan et al[9] have reported that TLR4 expression decreases significantly in seasonal AR patients. We have found that the expression levels of TLR4, IL-6 and IL-8 are significantly higher in patients with persistent AR compared to control subjects, and TLR4 is mainly localized in nasal epithelium, subepithelial glands and capillary endothelial cells, and immune cells.[7] Allergens increase reactive oxygen species (ROS) levels in airway epithelial cells to cause airway inflammation. The ROS production induced by house dust mites (HDM) requires the signaling of LPS/TLR4, and overproduced ROS upregulates TLR4 surface expression and lipid raft transport to epithelial cells in the allergic airway.[10] These data support that TLR4 may regulate HDM-induced oxidative stress response to activate innate immunity and type 2 inflammation in AR.

Growing evidence implies that microRNAs (miRNAs) are powerful regulators in AR.[11,12] Therefore, we investigated the effects of certain miRNAs on TLR4-mediated inflammatory responses in AR. miRNAs, a group of short non-coding RNAs with a length of 20–24 nucleotides, can regulate the stability and/or translation of mRNAs at the post-transcriptional level. In this light, miRNAs have been widely exploited for the treatment of many diseases.[13,14] We have found a set of differentially expressed (DE) miRNAs in AR nasal mucosa.[11] Previous research has shown that exosome-encapsulated miR-6089 can target TLR4-mediated inflammatory response to regulate LPS-induced cell proliferation in rheumatoid arthritis.[15] Therefore, we infer that miR-6089 may rely on the TLR4 signal pathway to modulate type 2 inflammation.

In this study, we profiled the expression patterns of miRNAs and mRNAs in the nasal mucosa of patients with mite-sensitized AR by high-throughput sequencing analysis, and screened aberrantly expressed miRNAs that might regulate TLR signaling. LPS causes pathological changes associated with allergic airway diseases, such as eosinophil recruitment, subepithelial fibrosis, mucus cell metaplasia, and smooth muscle hypertrophy. Given that LPS has been widely accepted in the creation of AR models[16,17] and has the ability to activate the TLR4 receptor, we stimulated nasal epithelial cells with LPS in order to construct our AR model. This study aimed to elucidate the role of miR-6089, a DEmiRNA, in TLR4-mediated inflammation in AR. The findings may provide promising therapeutic targets for the treatment of AR, a common allergic disease.

Methods

Study Subjects

Patients with AR (n = 10) and non-allergic controls (n = 10) were recruited between August 2019 and January 2020. AR was diagnosed according to the clinical guidelines.[1] The nasal mucosa tissues were collected during endoscopic septoplasty. Serum total IgE and allergen-specific IgE were measured by the Automated Immuno-Strip Analysis System (LG Life Sciences Ltd., Korea) in all the subjects. Patients with AR enrolled were sensitized mainly by HDM (Table 1).[11] The non-allergic controls had negative IgE test results. All subjects had treatment with glucocorticoids, antihistamines, antileukotrienes, and other immunomodulating drugs in the 4 weeks prior to surgery, as well as those with an immune deficiency, upper respiratory tract

Table 1 Clinical Characteristics of Patients with AR and Control Subjects

	AR (n=10)	Control (n=10)	p-value[c]
Age (years)[a]	32.50 (22.00–43.5)	26.50 (21.00–37.50)	> 0.05
Gender (male/female)	6/4	7/3	> 0.05
Total IgE (IU/mL)	> 100	≤ 100	< 0.0001
Specific IgE (kU_A/L)			
Df[b]	3.14 ± 0.76	0	0.0006
Dp[b]	6.58 ± 2.15	0	0.0066
Co-sensitization	2	0	
Asthma co-morbidity	No	No	

Notes: [a]Median (interquartile range); [b]mean ± standard deviation (SD); [c]AR vs control.
Abbreviations: AR, allergic rhinitis; IgE, immunoglobulin E; *Df*, *Dermatophagoides farinae*; *Dp*, *Dermatophagoides pteronyssinus*.

infection, chronic rhinosinusitis, nasal polyps, sinonasal neoplasms, a history of nasal and sinus surgery or allergen immunotherapy were excluded. Our study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (2019-SR-023). All participants provided written informed consent before all experiments.

RNA Sequencing

Total RNA was extracted from nasal mucosa samples, collected from three patients with AR and three controls, using TRIzol reagent (Life Technologies, California, United States). The quantity and quality of the extracted RNA were assessed with a NanoDrop ND-1000 (Delaware, United States). Raw miRNA reads were acquired through the Illumina HiSeq 2500 platform (California, United States). Following the removal of splice sequences, small RNA sequences of different lengths were obtained. Clean reads were then aligned and annotated against the Rfam, cDNA, species repeat sequence, and miRBase databases. This process aimed to eliminate as many small RNAs like tRNA, snRNA, and rRNA as possible. The remaining sequences were matched against the miRBase database to conduct statistics on known miRNAs. Additionally, the miRDeep2 software was utilized for novel miRNA prediction using the unannotated sequences. Based on the identified known miRNAs and the newly predicted miRNAs, the expression level of each miRNA was computed using the transcripts per million (TPM) metric. The TPM calculation formula is: $TPM = (N/M) \times 10^6$, where N represents the number of reads aligned to each miRNA, and M denotes the total number of reads in the sample.

The transcriptome sequencing was performed by Illumina (California, United States). The raw data underwent preprocessing with Trimmomatic software, and a statistical summary of the number of reads was maintained throughout the quality control process. Clean reads were then sequentially aligned with the designated reference genome using hisat2, revealing their location on the reference genome or gene, along with the sequenced sample's distinct sequence features. By referencing known gene sequences and annotation files, the expression abundance of each protein-coding gene in each sample was determined through sequence similarity comparison. Htseq-count software tallied the number of reads linked to protein-coding genes in each sample, while cufflinks software computed the FPKM expression of these genes. The FPKM calculation formula is: $FPKM\ (A) = N/(M \times K) \times 10^9$, where N signifies the number of fragments mapped to gene A, M denotes the total fragments mapped to all genes, and K represents the length of gene A. The high-throughput sequencing and analysis were conducted by Shanghai OE Biotech Co., Ltd (Shanghai, China).

Quantitative Real-Time PCR (qRT-PCR)

Total RNAs were isolated from nasal mucosa tissues (collected from 10 patients with AR and 10 controls) or human nasal epithelial cells (HNEpC) using TRIzol reagent (Invitrogen, California, United States) following the manufacturer's protocol. Reverse transcription was performed using miRNA specific stemloop primers and mRNA primers (Tsingke Biotechnology Co., Ltd, Beijing, China). The qRT-PCR was carried out using SYBR Green Master Mixture (Vazyme Biotech Co., Ltd, Nanjing, China) reagent. U6 and GAPDH were used as internal controls. The primer sequences were shown as follows: U6: *forward*: CGCTTCGGCAGCACATATAC, *reverse*: AAATATGGAACGCTTCACGA; GAPDH: *forward*: CACCCACTCCTCCAC CTTTG, *reverse*: CCACCACCCTGTTGCTGTAG; miR-6089: *forward*: CGCACTGGATACGACCCGCCCCG, *reverse*: TGCGCGGAGGCCGGGGTGGGGCG; TLR4: *forward*: TGAGCAGTCGTGCTGGTATC, *reverse*: CAGGGCTT TTCTGAGTCGTC; IL-6: *forward*: AGGAGACTTGCCTGGTGAAA, *reverse*: CAGGGGTGGTTATTGCATCT; IL-8: *forward*: GACATACTCCAAACCTTTCCACCCC, *reverse*: CAAAAACTTCTCCACAACCCTCTGC; TSLP: *forward*: TGGGACCAAAAGTACCGAGT, *reverse*: TGGGCACCAGATAGCTAAGG. The relative expression of miR-6089 and TLR4, IL-6, IL-8, and TSLP mRNA was shown as the fold change in relative to U6 and GAPDH.

Cell Culture and Transfection

HNEpC cells were obtained from Shanghai Yingwan Biological Technology Co., Ltd (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (ExCell Bio., Suzhou, China) and 1% pen-strep at 37°C in a humidified atmosphere with 5% CO_2. The miR-6089 mimics, mimics negative control (mimics NC), miR-6089 inhibitors, and inhibitors NC were bought from Han Heng Biotechnology Co., Ltd (Shanghai, China). The transfection was performed by cationic liposome method in accordance with the specification of Lipofectamine 2000 (Invitrogen, California, United States). After 5h, the medium containing lipofectamine 2000 was sucked out and replaced with normal

medium and cultured at 37°C in 5% CO_2 incubator for 24h. The transfection efficiency of mimic and inhibitor labeled by FAM was observed under inverted fluorescence microscope.

MTT Assay

MTT assay (MedChemexpress, New Jersey, United States) was used to detect cell activity. After the cells were cultured for indicated time, 10 μL of MTT was added to each well, and cultured at 37°C for 4 h. Then, the medium was aspirated, and 150 μL of DMSO was added and shaken for 10 min. The absorbance at OD568 was measured with a microplate reader (BioTek Instruments, Inc., Vermont, United States).

Apoptosis Assay

HNEpC cells, stimulated by LPS and transfected with miR-6089 mimic or inhibitor, stained with Annexin V-fluorescein isothio-cyanate/propidium iodide (KeyGen Biotech Co., Ltd, Nanjing, China) for 15 min at room temperature in the dark (negative control was also set). Cell apoptosis was then detected using a flow cytometry at 488 nm.

Detection of Oxidative Stress Indexes

The supernatant was obtained from LPS-induced HNEpC transfected with miR-6089 mimics or inhibitors. The levels of malondialdehyde (MDA), ROS and glutathione (GSH) in each supernatant sample were determined using detection kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Dual-Luciferase Reporter Assay

The targeting binding of miR-6089 and TLR4 were predicted by TargetScan V7.2. The predicted and mutated sequences targeting the 3'UTR of TLR4 were amplified and cloned into the luciferase vector pmirGLO (YouBio Co., Ltd, Changsha, China). pmirGLO-TLR4-3'UTR wild-type (Wt) and pmirGLO-TLR4-3'UTR mutated (Mut) were synthesized for the luciferase reporter assay. HNEpC cells were co-transfected with the luciferase reporter vectors and miR-6089 mimics or corresponding negative control, using Lipofectamine 2000 reagent. After 24 h of incubation, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega E1910, Madison, Wisconsin, United States) according to the manufacturer's protocol. In TLR4 3'UTR mutation analysis, the focus was on the 6-nucleotide (6-nt) target site, which was the sequence CCGGAG, and its complementary strand GGCCTC.

Enzyme-Linked Immunosorbent Assay

ELISA kits for the detection of IL-6, IL-8, and TSLP protein levels were obtained from Elabscience Biotechnology Co., Ltd (Wuhan, China). The levels of IL-6, IL-8, and TSLP in the culture supernatant of cells were analyzed with ELISA kits according to the manufacturer's instructions. Each experiment was independently triplicated.

Statistical Analysis

Data were presented as mean ± SEM. Statistical analysis was performed on SPSS 21.0 software (IBM SPSS Inc., Chicago, Illinois, United States). Between-group differences were analyzed using Student's *t*-tests. Significance levels were denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

miRNA and mRNA Expression Profiles in Nasal Mucosa from AR Patients

The expression patterns of miRNAs and mRNAs in the nasal mucosa of patients with AR were profiled by RNA sequencing analysis. A total of 1,784 miRNAs and 2,030 mRNAs were screened in nasal mucosa from three patients with AR and compared with those from three non-allergic controls. Box-whisker plots were generated to visualize whether the symmetry of miRNA data, as well as the degree of dispersion in the distribution (Figure 1A). The density distribution of TPM reflected the miRNA expression pattern in each sample (Figure 1B). The overall distribution of DE miRNAs was

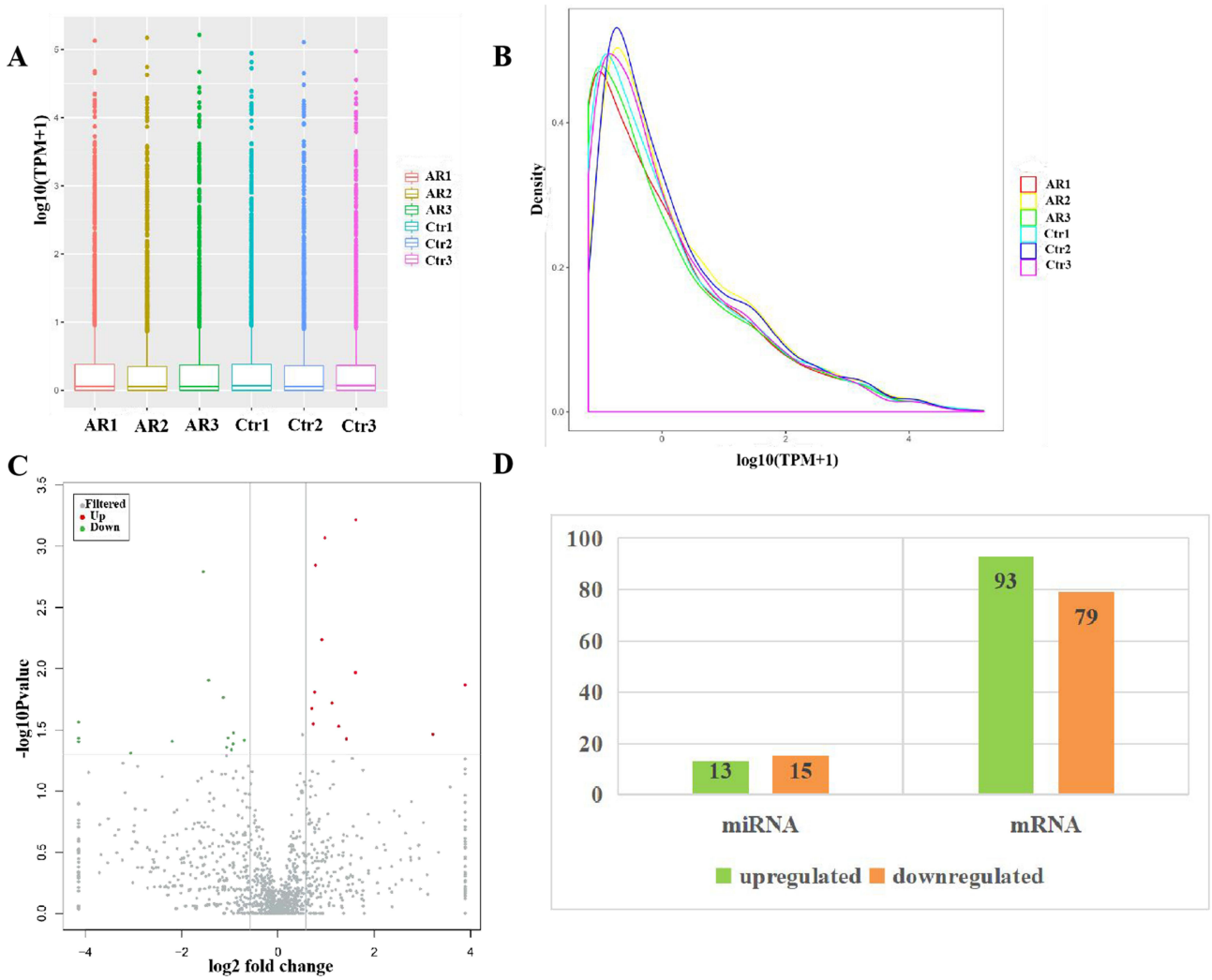

Figure 1 miRNA expression profiling in the nasal mucosa from patients with AR. (**A**) Box whisker plot for the degree of dispersion in the distribution. (**B**) The density distribution of TPM reflected the miRNA expression patterns in each sample. (**C**) The volcano map for the overall distribution of DEmiRNAs. (**D**) The distribution of DEmiRNAs and DEmRNAs by RNA sequencing (> 1.5-fold change, $p < 0.05$). n = 3 each group.
Abbreviations: AR, allergic rhinitis; DE, differentially expressed; miRNAs, microRNAs; TPM, transcripts per million.

shown by a volcano map (Figure 1C). A total of 13 DEmiRNAs were upregulated, 15 DEmiRNAs downregulated, 93 DEmRNAs upregulated, and 79 DEmRNAs downregulated (Figure 1D).

Enrichment Analysis

To identify the functions of DEmiRNAs and mRNAs, 172 DEmRNAs were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The top 30 GO terms related to Biological Processes (BPs), Molecular Functions (MFs), and Cellular Components (CCs) were screened at $p < 0.05$ (Figure 2A). BP terms were enriched in immune response and immune cell activity; MF terms in molecular binding, cytokine activity; CC terms in secretory granule and multiple extracellular components. KEGG pathway enrichment analysis showed the top 20 pathways enriched in toll-like receptor signaling pathway, NF-κB signaling pathway, IL-17 signaling pathway and other pathways (Figure 2B).

Expression of miR-6089, TLR4 and Its Downstream Pathway in AR

Our previous studies have found that TLR4 acts on the pathogenesis of AR through the NF-κB pathway. Our sequencing data indicated that the content of miR-6089 was reduced in AR (Figure 3A), and bioinformatics analysis showed that

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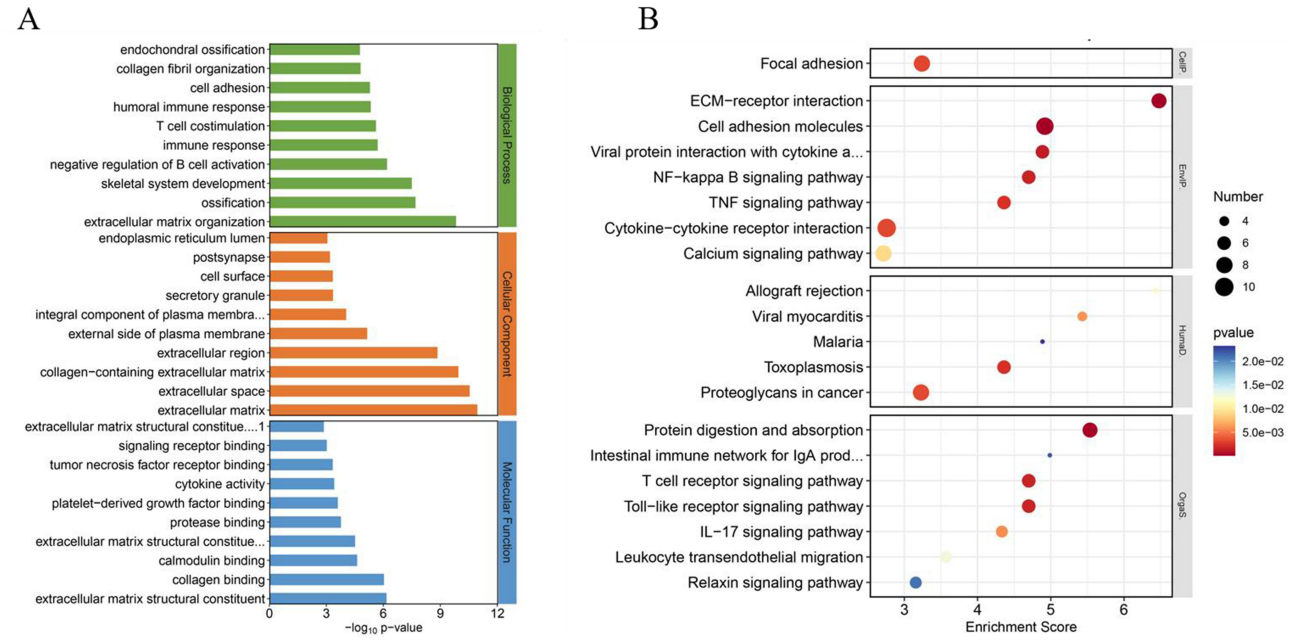

Figure 2 GO functional annotation and KEGG pathway enrichment analyses for DEmRNAs. (**A**) Top 30 GO items enriched in all DEmRNAs. (**B**) Top 20 KEGG pathways enriched in DEmRNAs screened by mRNA sequencing (> 1.5-fold change, $p < 0.05$).
Abbreviations: DE, differentially expressed; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

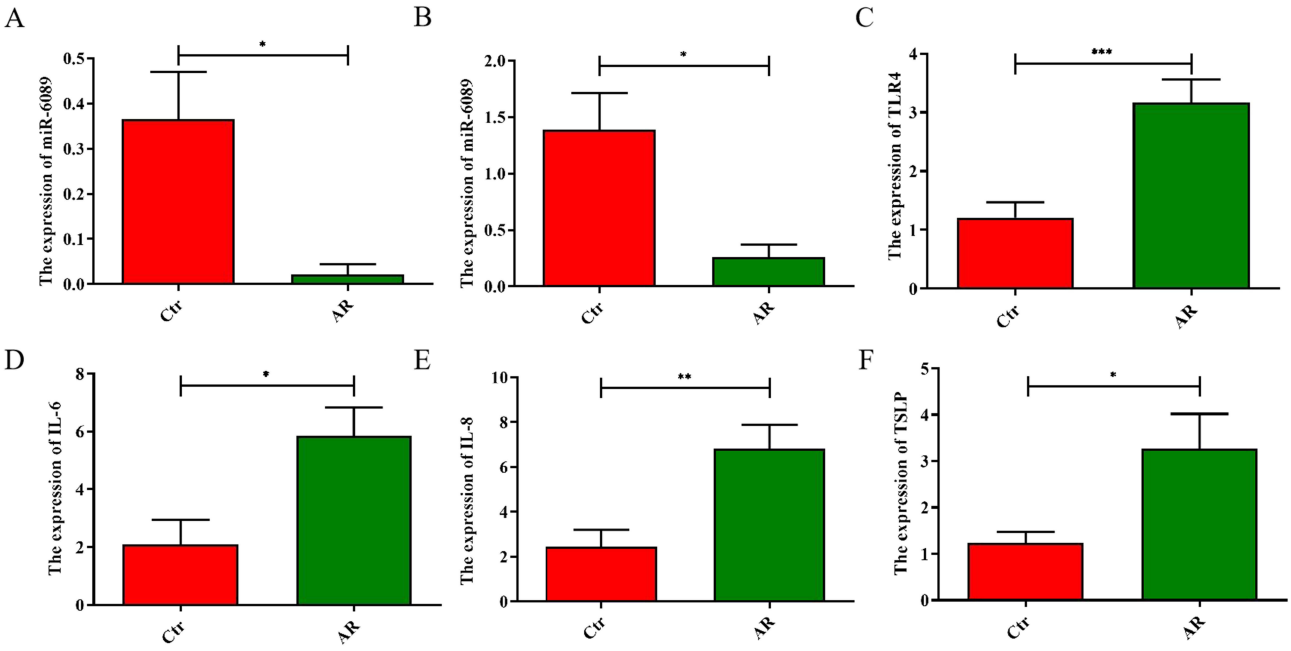

Figure 3 Validation of miRNAs and mRNAs in patients with AR (n=10) versus controls (n=10). (**A**) Sequencing data revealed that the expression of miR-6089 was downregulated. (**B**) The expression level of miR-6089 was decreased by qRT-PCR. (**C–F**) The expression levels of TLR4 (**C**), IL-6 (**D**), IL-8 (**E**), and TSLP (**F**) were increased by qRT-PCR. n = 10 each group. *p < 0.05, **p < 0.01, ***p < 0.001.
Abbreviations: AR, allergic rhinitis; Ctr, control; IL, interleukin; miRNAs, microRNAs; qRT-PCR, quantitative real-time polymerase chain reaction; TLR, toll-like receptor; TSLP, thymic stromal lymphopoietin.

miR-6089 could target TLR4. qRT-PCR revealed that the expression of miR-6089 was downregulated (Figure 3B), and TLR4 and its downstream effectors (IL-6, IL-8, and TSLP) were upregulated in AR (Figure 3C–F).

Inhibition of miR-6089 on LPS-Induced Apoptosis and Inflammation

To further investigate the role of miR-6089 in AR, HNEpC cells were treated with 1 μg/mL LPS and transfected with miR-6089 mimics/inhibitors or miR-6089-NC. Under an inverted fluorescence microscope, the transfection efficiencies of FAM-miR-6089 mimic-NC, miR-6089 mimic, miR-6089 inhibitor-NC, and miR-6089 inhibitor were observed to be remarkably high, with rates of 82.84%, 85.19%, 86.46%, and 83.84%, respectively (Figure 4A–D). Compared with the control group, the HNEpC in the LPS group showed evident inflammatory changes. The results showed that transfection with miR-6089 mimics inhibited the apoptosis and increased the activity of HNEpC after LPS treatment, and miR-6089 inhibitors weaken this effect (Figure 4E–L). miR-6089 mimics downregulated the expression of ROS and MDA, upregulated that of GSH, whereas miR-6089 inhibitors reversed these effects (Figure 5A–C).

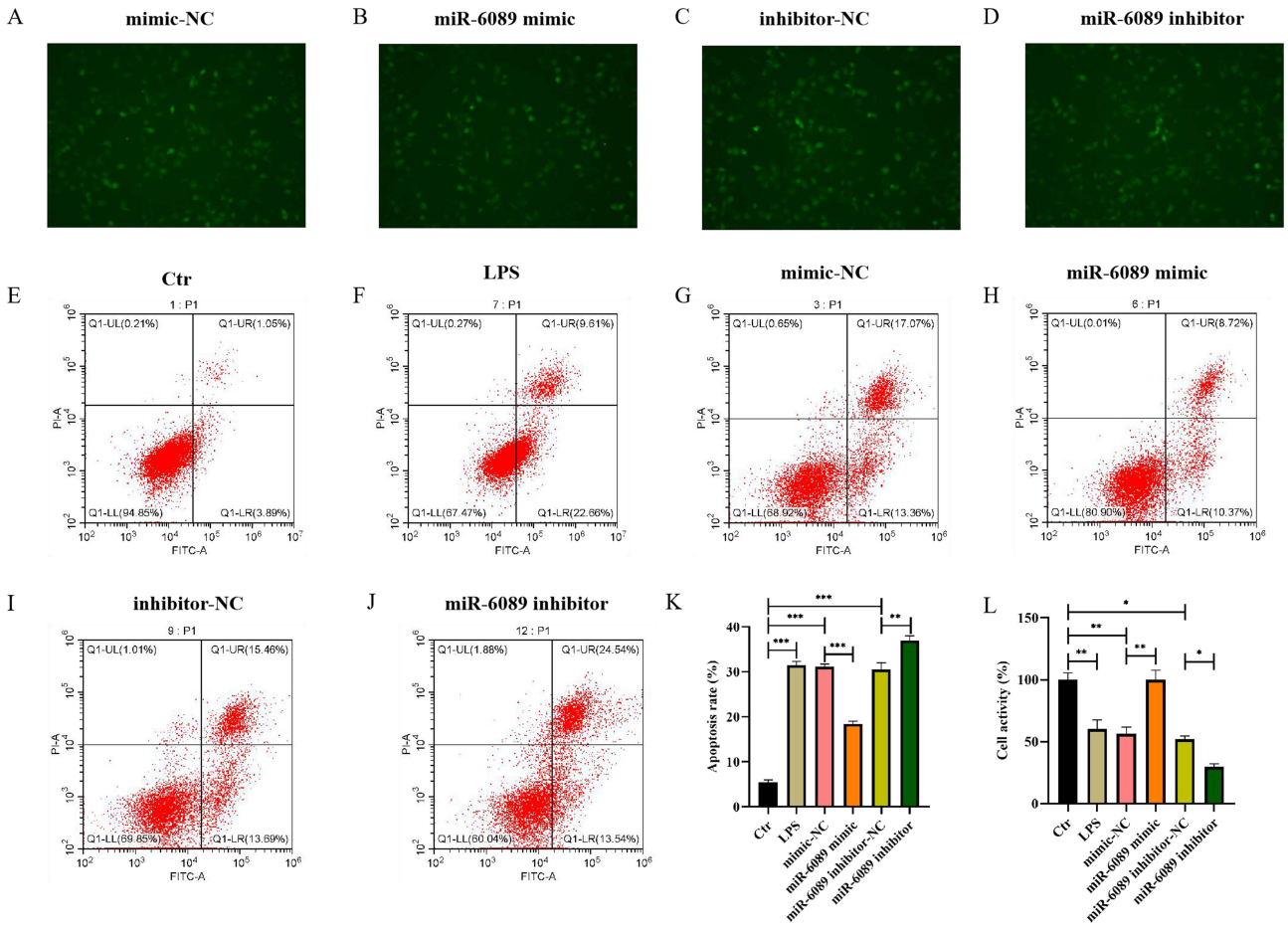

Figure 4 miR-6089 suppressed the apoptosis and promoted the growth of LPS-treated HNEpC. HNEpC cells were treated with LPS (1 μg/mL) for 24 h, and then transfected with miR-6089 mimics/inhibitors or miR-6089-NC for 24 h. The cells and cell supernatants were used for further analysis. The transfection efficiencies of FAM-miR-6089 mimic-NC, miR-6089 mimic, miR-6089 inhibitor-NC, and miR-6089 inhibitor were observed to be remarkably high, with rates of 82.84%, 85.19%, 86.46%, and 83.84%, respectively (**A–D**). Compared to Ctr (**E**), stimulation with LPS (**F**) and LPS + mimics-NC (**G**) and inhibitors-NC (**I**) promoted cell apoptosis significantly (**K**). Transfection with miR-6089 mimics inhibited cell apoptosis significantly (**H, K**). Transfection with miR-6089 inhibitors promoted cell apoptosis significantly (**J, K**). MTT assay demonstrated that the proliferation of cells was promoted following transfection with miR-6089 mimics, and inhibited following transfection with miR-6089 inhibitors (**L**). n = 3 each group. *p < 0.05, **p < 0.01, ***p < 0.001.

Abbreviations: Ctr, control; HNEpC, human nasal epithelial cells; LPS, lipopolysaccharide; miR, microRNA; NC, negative control.

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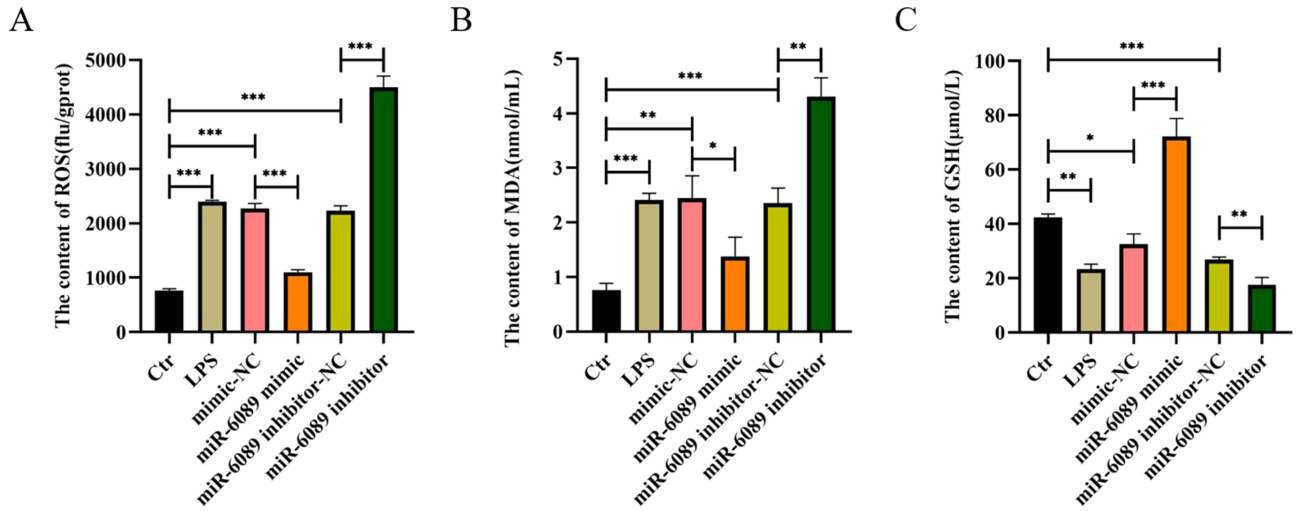

Figure 5 miR-6089 inhibited oxidative stress in LPS-treated HNEpC. ROS, MAD and GSH levels in HNEpC were detected using the kit. (**A**) The levels of ROS in LPS-treated HNEpC cells treated with miR-6089 mimics/inhibitors. (**B** and **C**) The expression levels of MDA and GSH in LPS-treated HNEpC cells treated with miR-6089 mimics/inhibitors. n = 3 each group. *p < 0.05, **p < 0.01, ***p< 0.001.

Abbreviations: Ctr, control; GSH, glutathione; HNEpC, human nasal epithelial cells; LPS, lipopolysaccharide; MDA, malondialdehyde; miR, microRNA; NC, negative control; ROS, reactive oxygen species.

Effect of miR-6089 on AR Inflammation by Targeting TLR4 Signal Pathway

The binding site of miR-6089 with TLR4 in human predicted by TargetScan is shown in Figure 6A. To verify whether miR-6089 directly binds to TLR4, a dual luciferase reporter assay was performed. It was observed that miR-6089 overexpression decreased the relative luciferase activity in the presence of the Wt 3'UTR. Similarly, no significant change in luciferase activity was observed when the targeted sequence of TLR4 was mutated in the miR-6089-binding

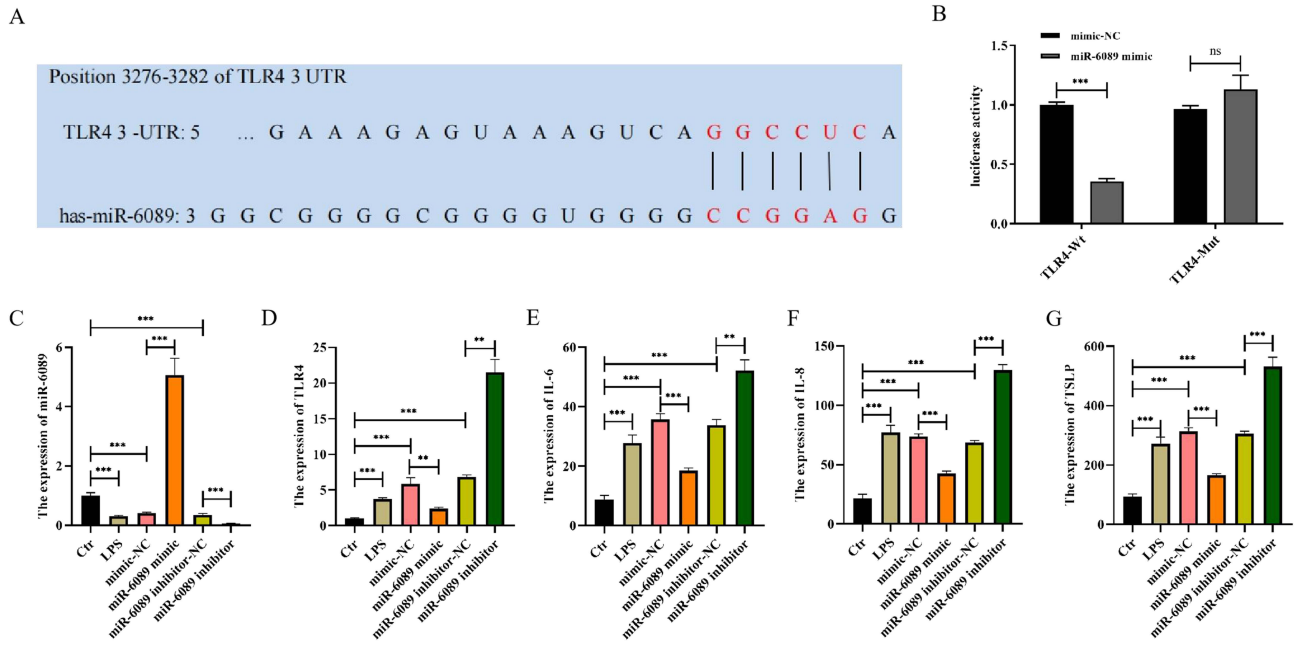

Figure 6 miR-6089 inhibited AR inflammation by targeting the TLR4 signal pathway. (**A**) The binding site of miR-6089 with TLR4 in human predicted by TargetScan. (**B**) Luciferase assay of HNEpC co-transfected with firefly luciferase constructs containing TLR4 wild-type (Wt) or mutated (Mut) 3'UTRs, miR-6089 mimics, and mimics NC. qRT-PCR to detect the expression of miR-6089 (**C**) and mRNA expression of TLR4 (**D**). ELISA to detect the protein expression of IL-6 (**E**), IL-8 (**F**), and TSLP (**G**). n = 3 each group. **p < 0.01, ***p < 0.001.

Abbreviations: AR, allergic rhinitis; Ctr, control; HNEpC, human nasal epithelial cells; IL, interleukin; LPS, lipopolysaccharide; miR, microRNA; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction; TLR, toll-like receptor; TSLP, thymic stromal lymphopoietin; UTR, untranslated region.

site (Figure 6B). We further confirmed that TLR4 was negatively regulated by miR-6089, using an AR cell model. LPS treatment decreased the level of miR-6089 and increased the levels of TLR4 and its downstream signaling effectors (IL-6, IL-8, and TSLP); however, this effect was reversed by transfection with miR-6089 mimics. In contrast, miR-6089 inhibitors enhanced the effect of LPS in decreasing the levels of miR-6089 and increasing the levels TLR4 and its downstream effectors, suggesting that miR-6089 also negatively regulated the expression of TLR4 in LPS-induced HNEpC (Figure 6C–G). Given that TLR4 signaling is associated with inflammation in AR, miR-6089 might protect against LPS-induced inflammation by targeting TLR4.

Discussion

Over the past decades, the efficacy of many natural or synthetic TLR4 inhibitors for inflammatory diseases has been evaluated in clinical trials.[18–21] For example, TAK-242, a TLR4 antagonist, ameliorates combined AR and asthma syndrome by reducing inflammatory monocyte infiltration in a mouse model.[22] Dong et al[23] have found that luteolin ameliorates inflammation and reconstructs Th1/Th2 balance via regulating the TLR4/NF-κB pathway in AR rats. LPS, a key ligand for TLR4, initiates a signaling cascade upon binding to TLR4. This interaction activates the MyD88/NF-κB pathway, which in turn triggers the transcription of genes crucial for immune-inflammatory responses. Once activated, this pathway sets off a cascade of pro-inflammatory cytokine production, specifically including IL-6, IL-8, and TSLP.[24,25] Furthermore, the generation of ROS is contingent upon the presence of a functional TLR4, highlighting the intricate interplay between TLR4 signaling and ROS in modulating inflammatory pathways. Therefore, new specific and targeted treatments are still needed for the treatment of AR.

Increasing evidence has indicated the implication of miRNAs in the pathogenesis of AR.[11,26,27] Jia et al[28] have uncovered that miR-126 drives AR by regulating the expression of regulatory T cells (Treg), Th1 and Th2 cytokines. Chen et al[29] have found that miR-466a-3p negatively regulates GATA-3 expression, and that intra-articular injection of lentiviral miR-466a-3p can turn the condition of AR mice similar to that of normal mice. In this study, we uncovered that miR-6089 suppressed the inflammatory response and apoptosis through an inhibition of TLR4 activation, highlighting the possibility of using miR-6089 to counter TLR4 synthesis and AR. Previous studies have shown that miR-6089 can inhibit the proliferation and induce the apoptosis of fibroblast-like synoviocytes by targeting CCR4,[30] and regulate the generation of IL-6, IL-29, and TNF-α by targeting the TLR4/NF-κB pathway in rheumatoid arthritis.[15]

In the present study, we first found that miR-6089 was significantly downregulated in the nasal mucosa tissues from patients with AR induced by HDM, suggesting that miR-6089 may be involved in the pathogenesis of AR. Second, miR-6089 overexpression protected against AR through increasing cell activity and suppressing oxidative stress response in LPS-induced HNEpC. Finally, miR-6089 could negatively regulate the TLR4 pathway in the LPS-induced HNEpC. miR-6089 could regulate the expression of TLR4, providing preliminary evidence that miR-6089 influences the occurrence and development of AR through modulating the TLR4-ROS pathway. TLR4 plays an active role in the arousal of inflammation and the release of inflammatory cytokine (such as TSLP, IL-6 and IL-8).[31,32] Some studies have demonstrated that miR-182-5p, miR-224-5p and miR-345-5p alleviate nasal symptoms and type 2 inflammation in AR mice by regulating the TLR4/NF-κB pathway.[33–35] Xu et al[15] have reported that the miR-6089 poses a protective effect by directly targeting TLR4 in an LPS-induced rheumatoid arthritis model. To the best of our knowledge, no other data have been released to characterize the roles of miR-6089 in the inflammatory responses associated with AR. Unprecedentedly, our results demonstrated that miR-6089 counteracts AR-related inflammation via targeting the TLR4 pathway, suggesting that the miR-6089/TLR4 axis may represent a novel and promising target for the treatment of AR.

There are some limitations in the present study. First, we did not conduct an animal model experiment to validate the therapeutic efficacy of miR-6089 on AR. The second aspect is that we did not investigate the downstream regulatory pathway of TLR4. The ongoing implementation of these studies will be necessary in the future.

Conclusions

miR-6089 plays an important role in AR pathogenesis. It can evoke the anti-inflammatory cascade by damping the expression of TLR4 and proinflammatory cytokines, as well as LPS-mediated HNEpC cell apoptosis and oxidative stress response. However, more studies, particularly in vivo studies on TLR4 and its downstream effectors, are required to

warrant the underlying molecular mechanisms of miR-6089 in pathogenesis and progression of mite-sensitized AR. These molecules may serve as novel therapeutic biomarkers for AR.

Abbreviations

AR, allergic rhinitis; BPs, biological processes; CCs, cellular components; DE, differentially expressed; ELISA, enzyme-linked immunosorbent assay; GO, gene ontology; GSH, glutathione; HDM, house dust mite; HNEpC, human nasal epithelial cells; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; MDA, malondialdehyde; MFs, molecular functions; miRNAs, microRNAs; NF-κB, nuclear factor-κB; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; Th, helper T cell; TLR, toll-like receptor; TPM, transcripts per million; TSLP, thymic stromal lymphopoietin; UTR, untranslated region.

Data Sharing Statement

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Informed Consent

This study was conducted in strict adherence to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (Approval No. 2019-SR-023). Written informed consent was obtained from each participant.

Consent for Publication

Institutional consent forms, obtained from the patients, were available for our study.

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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.

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Disclosure

The authors have no relevant financial or non-financial interests to disclose.

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