

Microarray analysis of differentially expressed microRNAs in allergic rhinitis

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ABSTRACT

Background: Allergic rhinitis (AR) is a common disease characterized by chronic inflammation of the nasal mucosa, but we have not fully understood the mechanism responsible for the development of AR. MicroRNAs (miRNAs) are short endogenous noncoding RNAs regulating protein translation through a mechanism known as RNA interference. To understand the molecular mechanisms of miRNA involved in the pathogenesis of AR, expressed miRNAs in AR were investigated through genomewide microarray analysis.

Methods: Mammalian miRNA microarrays containing whole human mature and precursor miRNA sequences were used for analyzing eight samples of nasal mucosa of AR and eight samples of nonallergic patients. Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) of some different expressed miRNAs was used to confirm the array results.

Results: The miRNA microarray chip analysis identified 421 miRNAs differentially expressed in the nasal mucosa of AR, and a total of 9 miRNAs were identified in the AR group with twofold change compared with control samples ($p < 0.05$). These included up-regulated miRNAs, hsa-hsa-miR-7, and hsa-miRPlus-E1194, and down-regulated miRNAs, hsa-miR-498, hsa-miR-187, hsa-miR-874, hsa-miR-143, hsa-miR-886-3p, hsa-miR-224, and hsa-miR-767-5p. RT-PCR results also confirmed that part of differentially expressed miRNAs as hsa-miR-224, hsa-miR-187, and hsa-miR-143 were down-regulated in AR.

Conclusion: The report indicated that many miRNA expressions were altered in AR and differentially expressed miRNAs appear to be involved in the development of AR. The study of miRNAs may lead to a better understanding about the roles of identified miRNAs in the pathogenesis of AR; this would be considered in future therapeutic strategies.

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Allergic rhinitis (AR) is a chronic inflammatory condition of the nasal mucosa affecting both children and adults. Along with the burden of the disease and decrease in quality of life in individuals who suffer from AR symptoms, there are high costs related to the disorder.^{1,2} To date, the factors that influence nasal conditioning in subjects with AR still have not been thoroughly investigated. Recently, it has been found that microRNAs (miRNAs) play an important role in regulating gene expression by inhibiting protein translation. The miRNAs are a family of recently discovered, small RNAs, produced by cells via a unique process, involving RNA polymerase II, microprocessor protein complex, RNase III/Dicer endonuclease complex, and, subsequently, an miRNA ribonucleoprotein complex.³ The biological functions of miRNAs depend on their ability to silence gene expression, primarily through degradation of the target mRNA and/or translational suppression mediated by the RNA-induced silencing complex.³

In mammals, >1000 miRNAs have been identified performing diverse functions, including the regulation of cellular differentiation, proliferation, and apoptosis.⁴ Allergic diseases are also likely to be regulated by miRNAs. Li *et al.* found that T-cell receptor sensitivity and signaling strength can be modulated at the posttranscriptional level by miR-181a and that the increase in miR-181a expression in mature T cells augments their sensitivity to peptide antigens.⁵ The miR-150 has been found to express in the lymph nodes and spleen and is highly up-regulated during the development of mature T and

B cells,⁶ and miRNA-155 has been associated with the generation of immunoglobulin class-switched plasma cells.⁷ Overexpression of miR-17-92 was observed in pre-B cells, indicating that miR-17-92 takes part in the pro-B to pre-B-cell transition.⁸ However, at present, research focusing on the relationship between miRNAs and the immune system is still in the nascent stage; so, what influence miRNAs have over human immune-related diseases such as AR is not fully understood to date.

Only scarce data are available on research about miRNAs in AR. To classify the genes regulation of miRNAs in AR, it is necessary to conduct the studies profiling miRNA expression. Various methods have been used to profile miRNA expression, including Northern blotting with radiolabeled probes,⁹ oligonucleotide microarrays,¹⁰ quantitative polymerase chain reaction (PCR)-based amplification of precursor or mature miRNAs,¹¹ bead-based profiling methods, and microarrays spotted onto glass surfaces. In contrast to mRNA profiling technologies, miRNA microarrays offer a new tool that can facilitate the studies focused on understanding the biological roles of miRNAs.¹² miRNA profiling must address the short nature (~22 nucleotide [nt]) of miRNAs and should be able to distinguish miRNAs differing from each other by just a single nt. Microarray-based miRNA profiling assay is an efficient solution to screen the expression of a large number of miRNAs in a parallel fashion. Here, this study attempts to reveal a difference between the miRNA profiles of nasal mucosa biopsy specimens from patients with AR compared with those from healthy volunteers; this helps in further understanding the molecular mechanisms involved in the pathogenesis of AR.

METHODS

Tissue Specimens

Human inferior turbinate nasal mucosa was obtained from eight patients with perennial AR (five women and three men; mean age, 35 years; range, 22–50 years) and eight nonallergic patients (four women and four men; mean age, 42 years; range, 21–45 years). All underwent surgery for nasal obstruction. All patients in the group with AR had a history of sneezing, secretion, and nasal obstruction during natural

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Table 1 Oligonucleotides used in this study

Primer Set Name	Reverse Transcriptase Reaction Primer (5' to 3')	Real-Time Quantitative PCR Primer (5' to 3')
U6	5'CGCTTCACGAATTTCGCTGTCAT3'	F: 5'GCTTCGGCAGCACATATACTAAAAT3' R: 5'CGCTTCACGAATTTCGCTGTCAT3'
hsa-miR-224	5'GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACAACGGAAC3'	F: 5'GGGGGCAAGTCACTAGT3' R: 5'TGCGTGTCTGGAGTC3'
hsa-miR-187	5'GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCGGCT3'	F: 5'GAGTCGTGTCTTGTGTTGC3' R: 5'CAGTGCGTGTCTGGA3'
hsa-miR-143	5'GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAGCTA3'	F: 5'GGGATGAGATGAAGCACT3' R: 5'TGCGTGTCTGGAGTC3'

F = forward; R = reverse; PCR = polymerase chain reaction.

exposure to allergen; a positive skin-prick test to mixed grass pollen, tree pollen, and/or weeds; and a positive screening for specific immunoglobulin E (IgE). Subjects in the nonallergic group with no history of allergy had negative skin-prick tests to a panel of common aeroallergens (mixed grass pollen, tree pollen, weeds, house-dust mites, cat hair, dog hair, and molds) and a negative screening for specific IgE. The specimens were obtained after the patients refrained from the use of topical or systemic corticosteroid therapy for 2 weeks.

RNA Microarray and Hybridization

RNA Extraction. Total RNA was prepared using Trizol (Invitrogen Inc, Carlsbad, CA) in accordance with the manufacturer's protocol. RNA quality was measured by denaturing agarose gel electrophoresis. The total RNA (10 µg) was ligated to a RNA-linker (p-rUrUrU-dACy3-dye, Dharmacon Inc, Lafayette, CO) labeled at 3'-end with 3 fluorescent T4 RNA ligase overnight at 37°C using miRCURYTM Array Labeling kit (Exiqon Inc, Vedbaek, Denmark).

Microarray Preparation and Hybridization

The miRNAs expression profiles were performed by applying the miRCURY locked nucleic acid (LNA) microarray platform (Exiqon).¹³ All procedures were conducted according to manufacturer's protocol of miRCURY Array Power Labeling kit (cat. no. 208032-A; Exiqon). Briefly, 1 µg of total RNA was labeled with the Hy3Fluorescent, using miRCURY Array Power Labeling kit. After stopping the labeling procedure, the reaction mixture was briefly spun and left it at 4°C. The samples were hybridized on a hybridization station using miRCURY LNA miRNA Array (14.0)—it contains >1700 capture probes, covering all miRNAs annotated in miRBase 14.0.¹⁴ Microarrays with labeled samples were hybridized at 56°C for overnight using a heat-shrunk hybridization bag, then were washed by miRCURY array wash buffer (cat. no. 208021; Exiqon). After hybridization, the chip slides were washed, dried, and scanned immediately. Each miRNA spot was replicated four times on the same slide.

Image Acquisition and Quantification

The slides were scanned using the Genepix 4000B (Axon Instruments Inc, Foster City, CA). The data were analyzed in Genepix Pro 6.0 (Axon Instruments), where average values of the triplicate spots of each miRNA were background subtracted, normalized, and further analyzed. GeneSpring 7.2 (Silicon Genetics, Redwood City, CA) was used for further data analysis. The intensity of green signal is calculated after background subtraction and four replicated spots of each probe on the same slide have been averaged. We used the median normalization method to obtain "normalized data" (normalized data = (foreground - background)/median. The median is 50%

quantile of miRNA intensity, which is larger than 50 in all samples after background correction. After normalization, the statistical significance of differentially expressed miRNA was analyzed by *t*-test.

Quantitative Reverse Transcriptase-PCR

miRNA levels were determined by real-time quantitative reverse transcriptase-PCR (qRT-PCR). Briefly, RNAs of nasal mucosa from AR and control group were isolated with the mirVana miRNA isolation kit (Ambion Inc, Austin, TX). qRT-PCR was performed on cDNA generated from 300 ng of total RNA using the protocol of the mirVana qRT-PCR miRNA detection kit (Ambion). A total of 30 ng of cDNA, 200 nmol/L of both sense and antisense primers, and SYBR Green PCR Master Mix (PE Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) in a final volume of 25 µL were used for PCR. Amplification and detection of specific products were performed with the Roter-Gene 3000 real-time PCR (Corbett Research, Pty Ltd, Sydney, Australia). The F primer (forward primer) is miRNA specific and is the same as the mature miRNA sequence. The R primer (reverse primer) corresponds to the stem-loop RT primer sequence (for probe sequences for qRT-PCR, see Table 1). As an internal control, U6 primers were used for RNA template normalization. Fluorescent signals were normalized to the internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. Relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting it from the U6 Ct value, which was given as the ΔCt value. The relative expression level between treatments was then calculated using the following equation: relative gene expression = $2^{-(\Delta Ct \text{ sample} - \Delta Ct \text{ control})}$. The experiment was conducted in triplicate.

miRNA Target Prediction

To identify potential binding genomic sites for candidate miRNA, genes predicted by TargetScan (www.targetscan.org) were considered as potential targets of a certain miRNA. The miRanda uses dynamic programming alignment to identify highly complementary sites, which are predicted miRNA targets in human genomes. To investigate predicted targets of candidate miRNAs, gene ontology (GO) categories were examined with DAVID bioinformatics resources to provide laboratory and bioinformatic support to the National Institute of Allergy and Infectious Diseases.

Statistical Analysis

Data are expressed as means ± SE and the data were analyzed using SPSS Version 11.5 (SPSS, Inc., Chicago, IL). Independent-samples *t*-test was used for comparing the data between two groups. A value of *p* < 0.05 was considered significantly different.

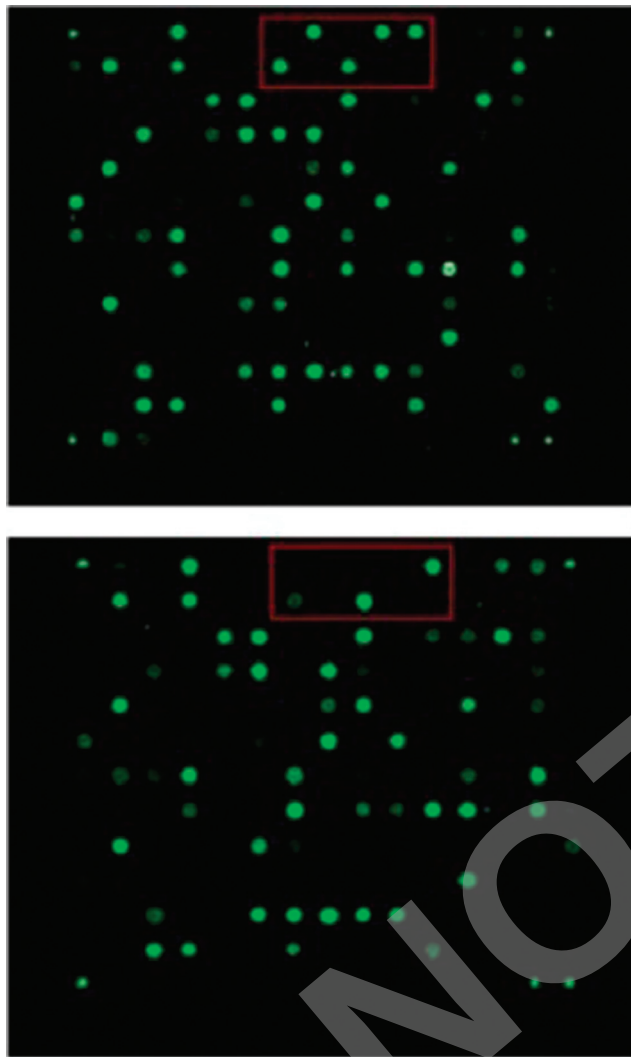


Figure 1. Representative microRNA (miRNA) microarray of this study. More than 1700 miRNAs were detected at each microarray. One small area is shown indicating the difference between allergic rhinitis (AR; up) and control (down). The chip profile contains >1700 capture probes, total (2.5–10 mg) RNA was ligated to an RNA-linker (p-rUrUrUdA- Cy3-dye) labeled at the 3' end. The intensity of each spot represents the amount of Cy3-dye. The same location in the profile from AR and control with different amounts of miRNA expression is seen.

RESULTS

Differential Expression of miRNAs between Normal and AR Nasal Mucosa

By using a highly sensitive, high-throughput and specific miRCURY LNA microarray platform, miRNA expression profiles for the two groups were determined. Microarray hybridized with total RNAs of nasal mucosa was shown (Fig. 1). After normalization, average values obtained for each miRNA spot were used for statistics. When compared with the healthy group, different changes were found in 421 miRNAs of the AR group; moreover, 2 overexpressed and 7 down-regulated miRNAs with twofold change were identified in AR group with statistical significance ($p < 0.05$). These included up-regulated miRNAs (hsa-miR-7 and hsa-miRPlus-E1194), and down-regulated miRNAs (hsa-miR-498, hsa-miR-187, hsa-miR-874, hsa-miR-143, hsa-miR-886-3p, hsa-miR-224, and hsa-miR-767-5p; Table 2), implying

these miRNAs have a pivotal role in the mechanism of the allergic disease.

Validation of Microarray Results Using qRT-PCR

To validate our microarray results, qRT-PCR analysis of nasal mucosa of AR and normal was performed for part of altered miRNAs as hsa-miR-224, hsa-miR-187, and hsa-miR-143. The RT-PCR analysis is consistent with microarray data: expression of hsa-miR-224, hsa-miR-187, and hsa-miR-143 was down-regulated with statistical significance ($p < 0.05$) in nasal mucosa of AR (Fig. 2).

Genomic Location of Differentially Expressed miRNAs

Chromosomal locations of miRNA genes have frequently provided important insight in understanding the role of miRNAs in specific diseases. Chromosomal distribution of some differentially expressed miRNAs in the human genome was also determined using University of California Santa Cruz Genome Browser (www.genome.ucsc.edu). By mapping miRNA genes onto the chromosomes, it has been found that chromosomes 5, 18, 19, and X appear to contain the most differentially expressed miRNA genes (Table 2).

Predicted Targets of Candidate miRNAs

Computational analysis of miRNA targets in TargetScan shows that >1500 target genes of 9 differentially expressed miRNAs in this study (Table 2). To evaluate the potential functions of these miRNA target genes, GO categories were assigned to the putative targets of the nine differentially expressed miRNA genes. GO is organized into three partially overlapping categories that consider three different aspects of each gene: biological processes, molecular functions, and cellular component. Based on "molecular function," the genes were finally classified into 91 categories, the 3 most overrepresented GO terms are binding (77.7%), protein binding (57.5%), and cation binding (27.3%). Categories based on "biological processes" revealed that the miRNA target genes were related to 235 biological processes; the 3 most frequent terms are cellular process (64.6%), biological regulation (47.9%), and regulation of biological process (46%). Based on "cellular component," it was found that the miRNA target genes were related to 93 biological processes; the 3 most frequent terms are cell (83.9%), cell part (83.9%), and intracellular (68.3%).

DISCUSSION

miRNAs are short (20–24 nt) noncoding RNAs that are involved in posttranscriptional regulation of gene expression in multicellular organisms as they affect both the stability and the translation of mRNAs. miRNAs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein coding or noncoding. The primary transcript is cleaved by the Drosha ribonuclease III enzyme to produce an ~70-nt stem-loop precursor miRNA (pre-miRNA), which is further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature miRNA and antisense miRNA star (miRNA*) products. The mature miRNA is incorporated into an RNA-induced silencing complex, which recognizes target mRNAs through imperfect base pairing with the miRNA usually resulting in translational inhibition or destabilization of the target mRNA.

The regulatory potential of miRNAs is vast in the sense that most miRNAs are predicted to target many hundreds of mRNAs.¹⁵ Although >900 miRNAs have been identified in the human genome, the biological function of the majority of these is unknown. Furthermore, there was no information on the research about miRNA in AR, and it is unclear as to how miRNAs contribute to AR. To initially explore the role of miRNA in AR, microarray profile was selected in the present experiments, and qRT-PCR method, based on highly sensitive Taq-

Table 2 Differentially expressed miRNAs in study

miRNA	Fold	p Value	Chromosomal Location
hsa-miR-874	0.46	0.01	5q31.2
hsa-miR-143	0.17	0.001	5q32
hsa-miR-498	0.36	0.02	19q13.42
hsa-miR-187	0.35	0.04	18q12.2
hsa-miR-767-5p	0.18	0.04	*
hsa-miR-224	0.35	0.02	Xq28
hsa-miR-886-3p	0.47	0.004	*
hsa-miR-7	2.67	0.01	9q21.32, 15q26.1, 19p13.3
hsa-miRPlus-E1194	5.13	0.02	*

Each miRNA spot was replicated four times on the same slide. After normalization, obtained average values for each miRNA spot were used for statistics. The p values for these miRNAs were <0.05 in nasal mucosa of AR tissue compared with normal tissue.

*No detail about location.

AR = allergic rhinitis; miRNA = microRNA.

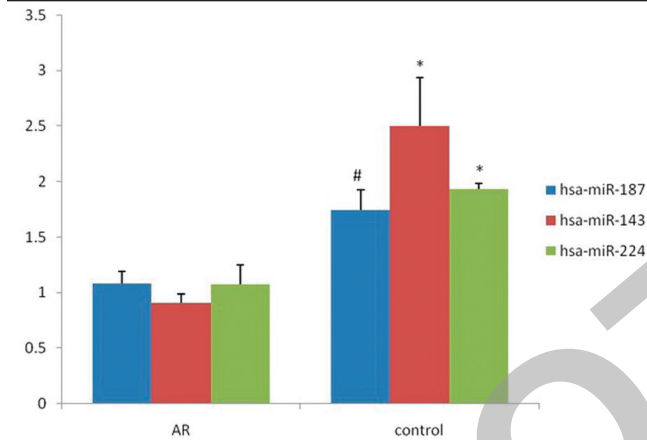


Figure 2. Down-regulation of microRNA (miRNA) 187, miRNA-143, and miRNA-224 in allergic rhinitis (AR). miRNA was extracted from nasal tissue from AR ($n = 8$) and non-AR ($n = 8$) and relative expression of miRNAs was analyzed using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The experiment was conducted in triplicate ($\#p < 0.05$ versus control; $*p < 0.01$ versus control).

Man technology, was also used to verify the results of the miRNA expression profile.

miRNAs constitute a recently identified class of small endogenous noncoding RNAs that act as negative regulators of the protein-coding gene expression and may impact cell differentiation, proliferation, and survival. It has reported that all fundamental signals transgress the pathway implicated in allergic disease. To study the molecular biology of miRNAs in AR, we used a microarray to profile and identified 421 miRNAs showing altered expression in nasal mucosa of the AR group when compared with the control group. All of these miRNAs are likely to represent those that have a significant influence on gene expression in the airways of AR. Moreover, the expression levels of nine miRNAs were significantly altered in the AR group. To verify the results of microarray, three differentially expressed miRNAs as hsa-miR-224, hsa-miR-187, and hsa-miR-143 were determined by qRT-PCR, and the results also confirmed these changes, suggesting that they may play important roles in the course of allergic reaction.

It has been reported that has-miR-224 is involved in cell proliferation and apoptosis regulation and has important pathophysiological functions in carcinoma.¹⁶ The has-miR-224 also has an important role in the inflammation process, which is regulated by transforming growth factor (TGF), and inhibition of endogenous miR-224 partially suppressed granulosa cell proliferation induced by TGF- β 1. The miR-224 was also involved in promoting TGF- β 1-induced granulosa cell

proliferation and ovarian estrogen release.¹⁷ Such miR-224-mediated effects could be potentially used for regulation of TGF- β 1-induced disorder processes of AR.

We observed that hsa-miR-143 was the most significant down-regulated miRNA in nasal mucosa of AR when compared with normal tissue. It has been reported that miR-143 plays an important role in regulating smooth muscle cell (SMC) fate and plasticity. Thomas Boettger's study revealed that the expression of miR-143, which forms a small cluster on mouse chromosome 18, strongly correlated with the number of SMCs; vascular SMCs from miR-143-deficient mice were locked in the synthetic state, which incapacitated their contractile abilities and favored neointimal lesion development, thereby revealing an unanticipated role of miR-143 in the regulation of vascular SMC phenotype.¹⁸ SMC is an important organizational structure of airway; pleiotropic cytokines interferon β and interferon γ could stimulate miR143 miRNA expression of airway SMC and contribute to airway allergic disease such as asthma.¹⁹ The miR-143 may also be responsible in regulating the upper airway SMC of AR.

Hsa-miR187 was found differentially expressed in ischemia kidneys, alveolar macrophages of bovine lung, thyroid tumors, and osteoblast exposed to titanium. Its expression level could be affected by TGF- β .²⁰ Because all researches about miR187 were focused on tumors, the information about how it was affected by allergy is scarce. Here, miR-187 expression has shown decreased levels in the nasal mucosa of AR allergic disease, implying that the lack of miR-187 may impair the immune condition through an allergic disease.

Other differentially expressed miRNA as has-miR-7 is perfectly conserved from annelids to humans indicating that miR-7 is essential for stable gene expression and cell fate determination under conditions of environmental flux.²¹ The has-miR-7 was found to play important roles in multiple fields such as the pancreas, nasopharyngeal carcinoma, islets, and intestinal epithelial cell. miR-7 has been proven to enter into novel genetic relationships to buffer developmental programs against variation and impart robustness to diverse regulatory networks. miR-7 was found differentially expressed in neuronal differentiation of embryonic stem (ES) cells, implying that it regulates both the development and the function of the nervous system, and miR-7 potently and directly regulates epidermal growth factor receptor.²² It has been found to be differentially expressed in AR in our study showing that the function for miR-7 may involve the networks of allergic inflammation.

There were not many reports about other differentially expressed miRNAs, including hsa-miR-498, hsa-miR-886-3p, hsa-miRPlus-E1194, and hsa-miR-874. The miR-498 expression was significantly down-regulated in CD4⁺ T cells of patients with rheumatoid arthritis; so it is possible that it plays an important role in the function of CD4⁺ T cells.²³ Its down-regulation in nasal mucosa of AR may be associated with the process of CD4⁺ T cell-mediated inflammation in AR.

Hsa-miR-886-3p was observed to be down-regulated greater than twofold in the squamous cell lung carcinoma tissues; no more research information about it is available in the allergic field.²⁴ Although this study supports that hsa-miRPlus expression was altered in AR, additional investigations are required to understand the relationship between them and their roles in mechanisms of AR.

Chromosomal locations of miRNA genes have frequently provided important insight into the roles of miRNAs in specific diseases.²⁵ Of all the chromosomes, chromosomes 5, 18, 19, and X appear to contain the most differentially expressed miRNA genes. Genes encoding down-regulated miRNAs—hsa-miR-874 and hsa-miR-143—were found on chromosomes 5q31.2 and 5q32. Genetic studies have indicated chromosome 5q31-33 as a major susceptibility locus for high IgE values and airway allergic disease. This region includes a cluster of cytokine genes encoding IL-3, IL-4, IL-5, IL-9, IL-13, granulocyte macrophage colony stimulating factor, and the β -chain of IL-12.²⁶ Our results indicated that hsa-miR-874 and hsa-miR-143 may be involved in regulating the cytokine genes and causing the allergic inflammation.

At present, the lack of knowledge about lavish miRNA gene targets hampers our understanding completely the biological effects caused by different miRNA expression. Computational analysis of miRNA targets in TargetScan were used to predict gene targets, and >1500 genes were shown as 9 miRNA target genes. Clustering of overrepresented GO classes in predicted targets of altered miRNAs by molecular function classifications were binding, protein binding, ion binding, metal ion binding, and cation binding. The GO categorization of the cellular component suggests enrichment in genes related to cell, cell part, intracellular, intracellular part, *etc.* Based on biological process, the most overrepresented GO terms showed that most target genes were involved in cellular process, biological regulation, regulation of biological process, metabolic process, regulation of cellular process, and primary metabolic process, suggesting that a set of significantly dysregulated miRNAs might promote the progression of biological regulation, metabolic process, and regulation of cellular process in allergic inflammation.

The presented differential miRNA pattern provides a solid basis for identification of an increasing number of other potential miRNAs in AR. The results in this study verified that miRNAs are specifically involved in AR and differentially expressed miRNAs appear to be involved in the development of AR. Furthermore, more work needs to be done to investigate the potential role of an individual miRNA in allergic reaction and inflammation.

Taken together, we identified nine miRNAs as differentially expressed in nasal mucosa of AR; the expression profiling of these nine miRNAs may provide a useful clue for pathophysiology research of allergic disease. Our work indicates that miRNAs are potentially important members involved in the pathogenesis of AR. The study of miRNAs may lead to a better understanding about the roles of identified miRNAs in the pathogenesis of AR; this would be considered in future therapeutic strategies.

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