Comparison of miRNA expression in patients with seasonal and perennial allergic rhinitis and non-atopic asthma

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ABSTRACT

Background. MicroRNAs (miRNA) are small non-coding molecules that play a significant regulatory role in several allergic diseases. However, their role in allergic rhinitis is still not clearly understood. The aim of this study was to identify the candidate miRNAs that can discriminate between different forms of allergic rhinitis and also differ in and out of the allergen season.

Methods. The study included 20 healthy children, 20 patients with seasonal allergic rhinitis (SAR), 20 non-atopic asthmatics (NA-A), and 12 patients with perennial allergic rhinitis (PAR). Patients with SAR were evaluated comparatively in and outside the allergen season. The changes in the expressions of selected miRNAs (miR-125b, miR-126, miR-133b, miR-181a, and miR-206) that were found related to the allergic diseases according to the literature were determined using quantitative polymerase chain reaction.

Results. In the SAR group, expression levels of miR-125b (p=0.040) and miR181a (p=0.014) were lower than in the controls outside of the allergen season. Expression levels of miR-181a were different between patients with SAR and NA-A (p=0.003), also between the SAR and PAR (p=0.001) groups in multiple comparisons. In contrast, the expression of miR-206 was found to be decreased in patients with NA-A and PAR compared with the controls (p=0.005 and p=0.024, respectively). In correlation analysis, expression levels of miR-125b and peak expiratory flow (PEF) values were found to be negatively correlated in the SAR (p=0.013) and PAR (p=0.029) groups. The expression level of miR-206 was positively correlated with total IgE levels in PAR (p=0.007). Receiver operating characteristic analysis revealed that miR-125b and miR-181a predicted the risk of SAR (p=0.040 and p=0.014, respectively), and miR-206 for NA-A and PAR (p=0.005 and p=0.024, respectively).

Conclusions. Our study showed that expression levels of miRNAs were different according to the type of allergic diseases and the presence of allergens. miR-181a and miR-125b can be candidate biomarkers for SAR, and miR-206 for NA-A and PAR.

Key words: allergic rhinitis, asthma, epigenetics, microRNA.

The prevalence of allergic diseases such as asthma and allergic rhinitis has increased in the last few decades, especially among children. Evidence from recent studies has shown that

the increase in prevalence could be explained by changing environmental conditions and some genetic factors.[1-3] Gene by environment interaction, which results in changes in the expression of genes called epigenetic changes, may contribute to this increase.[4]

Non-coding RNAs are one of the epigenetic mechanisms of the cell. MicroRNAs (miRNAs) are short, approximately 20-25 nucleotides in length, single-stranded non-coding RNA molecules, and they play a role in regulating

gene expression by targeting mRNAs.[5] miRNAs are produced by cells in different organs and are released into the blood and other body fluids, where they can have a biologic effect. This shows that miRNAs can serve as noninvasive biomarkers.[6] miRNAs play essential roles in various disease processes. miRNAs can serve as a molecular fingerprint for the characterization and diagnosis of diseases because they are detected in the blood.[7] Recent studies have demonstrated the critical role of specific miRNAs in regulating fundamental pathogenic mechanisms of allergic inflammation and the relationship between miRNAs and allergic diseases such as asthma, eosinophilic esophagitis, atopic dermatitis, and allergic rhinitis.[8-16] Let-7 microRNAs were shown to inhibit interleukin (IL)-13 expression and have positive effects on airway inflammation, airway hyperresponsiveness, and mucus secretion and subepithelial fibrosis.[8] miR-375 has a role in the modulation of (IL)-13-driven epithelial responses.[9] Lu et al.[11] showed that miR-223 was one of the most upregulated miRNAs found in eosinophilic esophagitis. miR-374a was found correlated with lung function, especially FEV1/ FVC values.[12] Panganiban et al.[5] showed that six circulating miRNAs, miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b levels were most predictive of allergic and asthmatic status. They found that the expression of miR-206 increased in plasma in patients with AR and also the expression of miR-125b increased in patients with asthma and AR.[5] In another study, 12 of the circulating miRNAs were found to be associated with exacerbations in asthma, especially miR-206.[13] miR-126 has also been reported as increased in cultured bronchial epidermal cells stimulated with IL-13.[14] A lower percentage of Treg and lower expression of IL-10 and TGF-β was shown in children with AR, and also significantly lower expressions of miR-155 and miR-181a in Treg cells in children with AR.[15]

miR-21 and miR-146 were found to have a role in the polarization of adaptive immune responses and activation of T cells.[16] However, studies on this subject are quite limited.

In this study, we aimed to identify candidate miRNAs that could discriminate between different forms of allergic rhinitis, also in and out of the allergen season in children with perennial (PAR) and seasonal allergic rhinitis (SAR). Also, we tried to establish whether different miRNAs were effective in patients with non-atopic asthma and allergic rhinitis. To achieve this aim, we selected five miRNAs (miR-126, miR-125b, miR-181a, miR-133b, and miR-206) as candidate miRNAs according to literature that might play a role in the pathogenesis of the allergic disease.

Material and Methods

This study was approved by the Ethics Committee of Hacettepe University (GO 18/433) and informed consent was obtained from all individual participants before being included in the study.

Study population

Twenty children with non-atopic asthma (NA-A), 20 children with SAR, and 12 children with PAR who presented to the Pediatric Allergy and Asthma Unit, and 20 children with no history of allergy and asthmatic disease (control group) who presented to the Department of Child Health and Diseases, of Hacettepe University Faculty of Medicine between November 2018 and May 2019 were included in the study. Patients with SAR were evaluated comparatively in and out of the allergen season. In the SAR group, off-season samples were collected in November (2018), while in-season samples were collected in April-May (2019).

All the patients in SAR group had grass pollen allergy and all the patients in PAR group were positive for house dust mites, both Der p and

Table I. The demographics and clinical characteristics of the study population.

	SAR(n=20)	NA-A(n=20)	PAR (n=12)	Controls (n=20)
Age (year)	12.55 ± 2.44	10.85 ± 2.85	10.42 ± 2.15	10.98 ± 3.02
Gender (F (%))	8 (40)	7 (35)	4 (33.3)	8 (40)
FEV1	100 (93.50-106.75)	96 (84.25-102)	98.50 (95.5-100.5)	-
FVC	100 (98.20-101.50)	97.50 (87-100)	99.50 (97.75-100)	-
MEF/25-75	112 (101.50-131.70)	93 (84.25-107.50)	113 (97.25-143.50)	-
PEF	91.0 (84.8-100.1)	85.7 (74.0-97.5)	92.6 (92.3-97.3)	-
Total IgE	85.50 (33.05-333.25)	25.65 (10.84-93)	171 (94.75-391)	-
Eos number	150 (100-200)	100 (100-200)	250 (100-925)	-
Eos %	2.3 (1.6-2.9)	1.7 (1.2-2.8)	4.50 (1.83-12.78)	-

SAR: seasonal allergic rhinitis, NA-A: non-atopic asthmatics, PAR: perennial allergic rhinitis, F: Female, FEV1: Forced expiratory volume in 1 second, FVC: Forced vital capacity, MEF: maximal (mid-)expiratory flow, PEF: Peak expiratory flow, IgE: Immunoglobulin E, Eos: Eosinophils

Der f, except 2 out of 12 patients. Two patients in PAR group had only Der p allergy. The diagnosis of AR was made based on history, clinical examination, skin prick test, and specific immunoglobulin (Ig)-E measurement according to the Allergic Rhinitis and Its Impact on Asthma (ARIA) guideline.[17] The diagnosis of asthma was made based on history and lung function tests according to the Global Initiative for Asthma (GINA).[18] Eight patients in SAR group also had asthma besides AR. The characteristics of the study population are summarized in Table I.

Selection of miRNAs

We selected five miRNAs (miR-126, miR-125b, miR-181a, miR-133b, and miR-206) as candidate miRNAs that might play a role in the pathogenesis of the allergic disease according to the literature.

Sample Collection and miRNA Isolation

Blood samples were collected from the study subjects, and serum was separated and aliquoted within one hour after collection. Isolation of circulating miRNAs was performed using the miRNeasy Serum/Plasma Kit (QIAGEN, Germany) according to the manufacturer's instructions. miRNA purity and quantification were determined using NanoDrop Nucleic Acid

Quantification (Thermo Scientific, Waltham, MA). miRNAs were stored at -80°C until they were translated into complementary DNA or required for further use.

Determination of miRNA levels by qRT-PCR

The obtained miRNA samples were reverse transcribed into cDNA using a miScript II RT Kit (QIAGEN, Germany). miScript Primer Assays (QIAGEN, USA) were purchased from a supplier for selected miRNAs and the control miRNA (miR-16).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using miScript SYBR® Green PCR Kit (QIAGEN, Germany) on the Mx3005P Real-Time PCR system (Agilent Stratagene, United States). The reactions were set up according to the manufacturer's instructions. The mean value of the cycle threshold of duplicates for each sample was calculated, and the expression level of each miRNA was calculated using the 2–ΔΔCt method and normalized to miR-16.

Statistics

The SPSS 22 for Windows program was used for statistical analysis. The comparison of numerical variables was performed using parametric or

non-parametric tests depending on whether they were normally distributed. Clinical data were compared using an independent t-test. Categorical variables were evaluated using the Chi-square test or Fisher's exact test. Expressions of target miRNAs were normalized according to the expression of control miRNA. The results were analyzed using the 2−ΔΔCt method. The Mann-Whitney U test was used to analyze miRNA expression levels. In the SAR group, the comparison of miRNA expression levels in and outside the allergen season was performed using the Wilcoxon test with two related samples. Spearman's correlation test was also used to determine correlations between miRNA expression and clinical characteristics. Receiver operating characteristic (ROC) curves were generated for miR-125b, miR-181a, and miR-206. The sensitivity, specificity, cut-off value, and 95% confidence intervals (CI) were calculated based on the ROC curves. P-values <0.05 were considered to be statistically significant.

Results

Circulating miRNAs showed altered expression levels between study subgroups

We compared the miRNA expression levels between healthy subjects, patients with SAR (out of season), PAR, and NA-A (Fig. 1). The expressions of miR-125b and miR-181a were lower in the SAR group than in the control group (p=0.039 and p=0.014, respectively) (Figs 1B and 1C). A comparison of expression levels of selected miRNAs between the NA-A and control groups revealed that miR-206 expression was lower in asthmatics than in healthy subjects (p=0.002), and the same result was found with PAR (p=0.024) (Fig. 1E). Expression levels of miR-181a were different between patients with SAR and NA-A (p=0.003), and also between the SAR and PAR (p=0.001) groups in multiple comparisons.
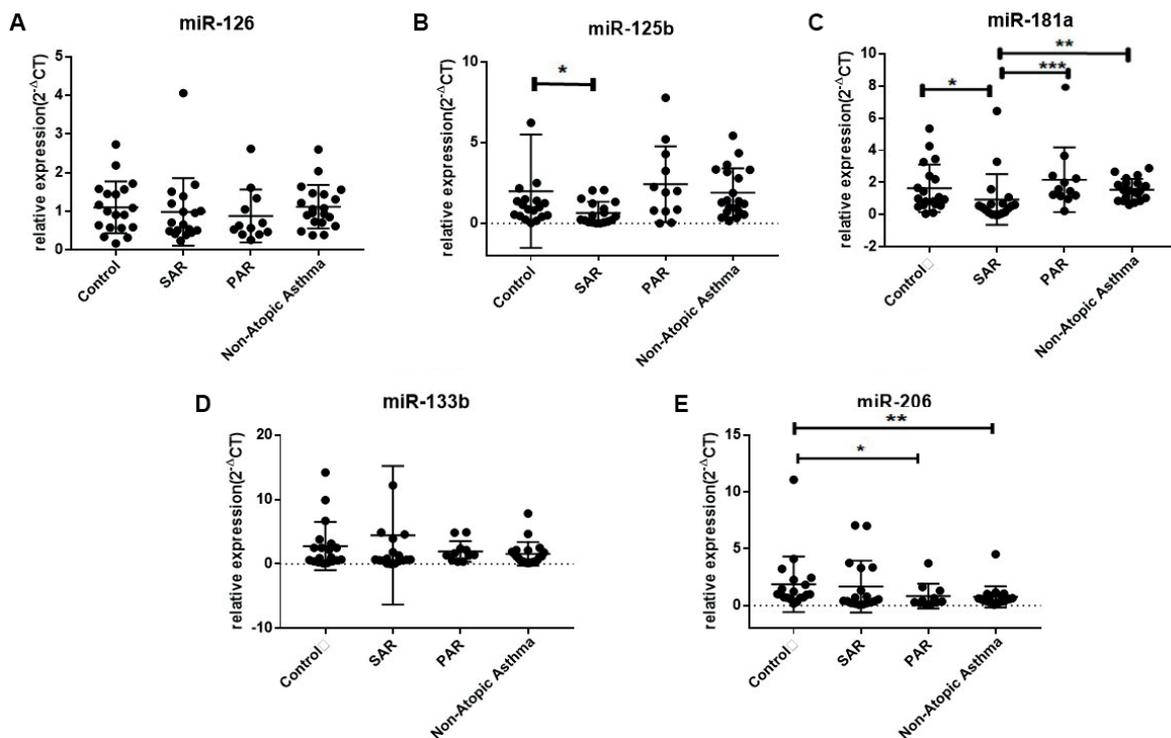

Fig. 1. Comparison of relative expression levels of serum miRNAs miR-126 (A) miR-125b (B) miR- 181a (C) miR-133b (D) and miR-206 (E) in healthy controls, SAR, PAR and asthma patients. *P<0.05 **P<0.01 ***P<0.001
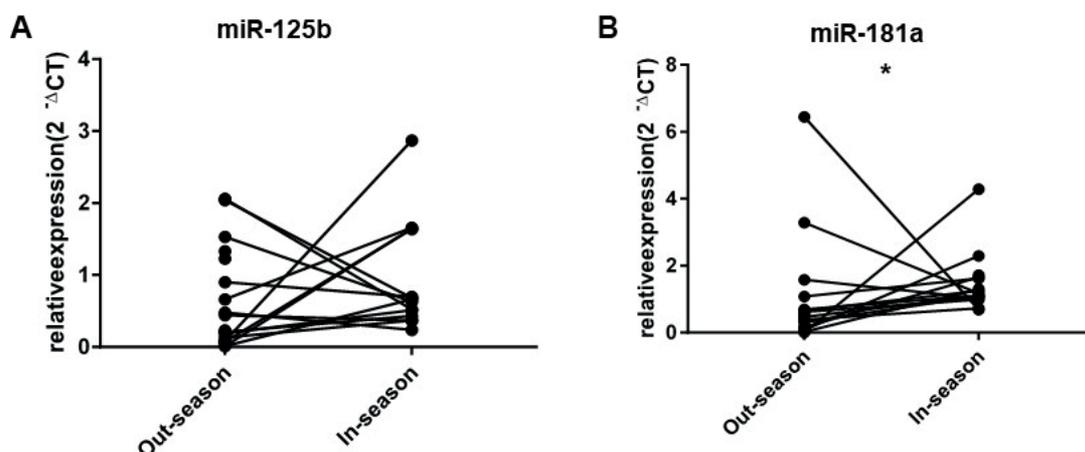
Fig. 2. Comparison of relative expression levels miR-125b (A) and miR-181a (B) between out of and in allergy seasons among SAR patients. *P<0.05

There was no difference in the expression of miR-126 and miR-133b between all study subgroups.

In the allergen season, expression levels of miR-125b and miR-181a were higher than outside of the allergen season (Fig. 2), but this increase was significant only for miR-181a (p=0.041).

Correlations between circulating miRNAs and clinical parameters

There was a negative correlation between miR-125b expression levels and PEF measurements in the SAR (r=-0.572, p=0.013) and PAR (r=-0.685, p=0.029) groups. Total IgE levels were positively correlated with miR-206 levels (r=0.817, p=0.007) in the PAR group. Although there was no differential expression level of miR-126 in the study populations, there was a positive correlation between miR-126 levels and MEF/25-75 values in patients with NA-A

(r=0.491, p=0.028). Correlation analysis results are summarized in Table II.

miRNAs as a diagnostic marker

To evaluate whether miRNAs that were found differentially expressed in SAR, PAR, and NA-A could discriminate AR and NA-A, we performed a ROC curve analysis. As shown in Fig. 3, the area under the curve (AUC) of individual miR-125b and miR-181a were 0.697 (95% CI: 0.526-0.869, p=0.040) and 0.735 (95% CI: 0.567-0.903, p=0.014), respectively. The sensitivity and specificity values at the best cut-off points that yielded the maximum value of sensitivity plus specificity for miR-125b were 61.1% [positive predictive value (PPV) 68.8%] and 73.7% [negative predictive value (NPV) 66.7%], respectively. The sensitivity and specificity values for miR-181a were 61.1% (PPV=78.6%) and 84.2% (NPV=69.6%), respectively.

Table II. Correlations between clinical findings and miRNA levels.

Disease Group	miRNA	Clinical Finding	Correlation (R)	P value
SAR	125b	PEF	-0.572	0,013
PAR	125b	PEF	-0,685	0,029
PAR	miR-206	Total IgE	0.817	0.007
Non-atopic Asthmatic	miR-126	MEF/25-75	0.491	0.028

SAR: seasonal allergic rhinitis, PAR: perennial allergic rhinitis

ROC curve analysis performed to evaluate the diagnostic value of miR-206 for NA-A revealed that the AUC was 0.763 (95% CI: 0.607-0.919, p=0.005) with good sensitivity and specificity, 70% (PPV=82.4%) and 84.2% (NPV=72.7%) respectively (Fig. 4A).

The diagnostic value of miR-206 in diagnosing PAR showed an AUC value of 0.751 (95%

CI: 0.548-0.954, p=0.024) and sensitivity and specificity at the best cut-off points were obtained as 72.7% and 84.2%, respectively (Fig. 4B).

Comparisons in ROC curve analyzes were made between the control group and the other groups.
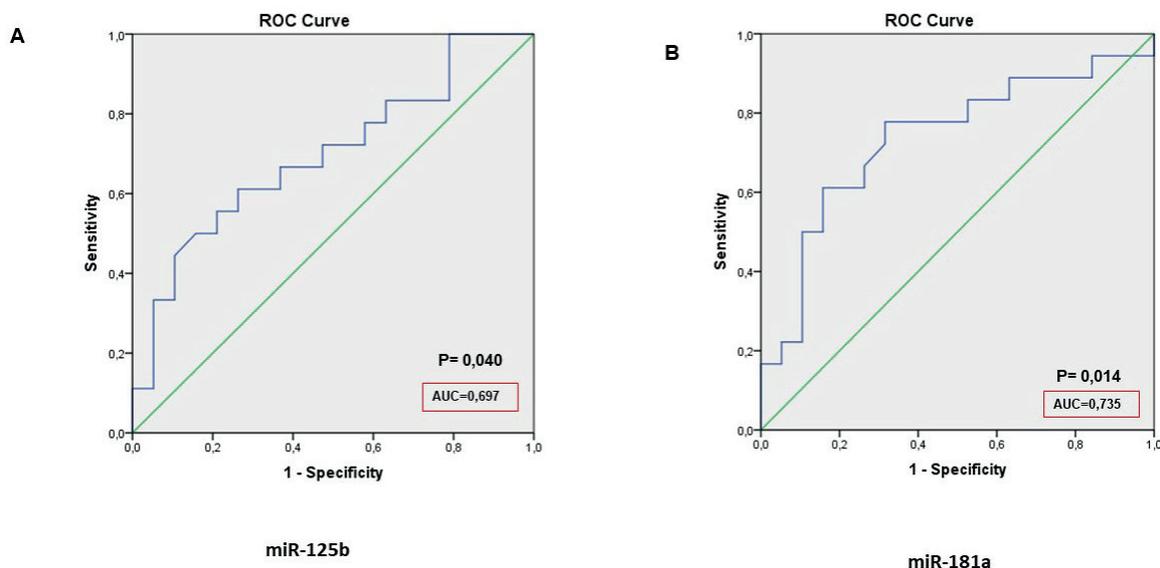

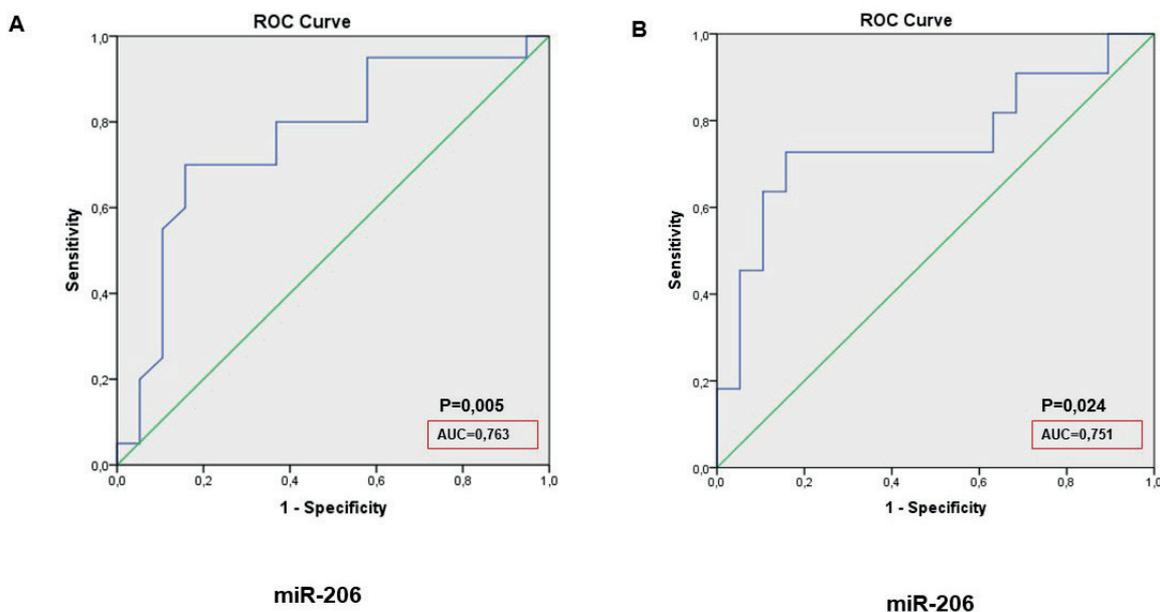**Fig. 3.** ROC curve analyses of serum miR-125b (A) and 181a (B) in SAR patients.

Fig. 4. ROC curve analyses of serum miR-206 in (A) NA-A and (B) PAR patients.

Discussion

To identify the candidate miRNAs that can discriminate between different forms of allergic rhinitis, that also differ in and out of the allergen season and to establish whether various miRNAs were effective in patients with non-atopic asthma and allergic rhinitis we investigated the expression levels of miR-125b, miR-126, miR-133b, miR-181a, and miR-206 that were found related to allergic diseases according to the literature. As a result of our study, we found that miR-181a and miR-125b are differentially expressed in patients with seasonal AR in and out of allergen season also compared to the patients with perennial AR and non-atopic asthmatics. Results also revealed that the expression of miR-206 is decreased in patients with NA-A and PAR compared with the controls. We also found the expression levels of miRNAs correlated to clinical parameters such as lung function and total IgE levels.

Recent studies identified that specific miRNAs had critical roles in the pathogenesis of several allergic diseases including asthma, eosinophilic esophagitis, atopic dermatitis, and AR. These studies also showed that expression levels of miRNAs are different according to the type of allergic disease and miRNAs might serve as noninvasive biomarkers for diseases. In accordance with the literature, our study showed that the expression levels of miR181a and miR-125b are different according to the type of allergic disease and the presence of allergens.

Many studies investigating the role of miR-125b in immune response and inflammation have been conducted in recent years.[19-23] Several studies have shown that miR-125b expression decreases under inflammatory conditions[19,21-23], whereas other studies found it was related to increased inflammation.[5,20] Liu et al.[16] demonstrated that the expression level of miR-125b was decreased in the sputum of asthmatics compared with controls and miR-125b could inhibit goblet cell differentiation. Tili et al.[21] reported that the expression of miR-125b decreased in macrophages of LPS-stimulated mice. In another study, miR-125b was upregulated in naïve T cell and inhibits T cell differentiation.[23] In contrast to these studies, in another study, miR-125 was shown to be effective in signaling pathways such as STAT3, MAPK, and PI3K-Akt. Quantitative RT-PCR analysis showed that the expression of miR-125b increased in patients with asthma and AR.[5] In our study, we found the expression of miR125b was significantly lower in patients with SAR than in the controls and also increased in the allergen season. Since miR-125b has been shown to inhibit T cell polarization, we can say that its lower expression in SAR compared with the controls allows for more T cell polarization, in favor of Th2-type inflammation. It is also acceptable to consider that under allergen attack during the allergen season the expression of miR125b increases to suppress polarization of naive T cell to Th2 T cells, as an immune response to prevent the development of excessive Th2 response.

Another miRNA that we found with lower expression in patients with SAR compared with controls in this study was miR-181a. However, the expression of miR-181a was found to be increased during the allergen season compared with outside the allergen season. Neilson et al. reported that miR-181a expression was elevated in immature T cells and decreased in Th1 and Th2 cells. miR-181a has been reported to suppress the expression of several genes (TCRa, CD69, BCL-2) that are effective in T cell maturation.[24] Blevins et al.[25] reported that miR-181a was a negative regulator of TCR signaling and limited T cell activation. A lower percentage of Treg and lower expression of IL-10 and TGF-β was shown in children with AR, and also significantly lower expressions of miR-155 and miR-181a in Treg cells in children with AR.[15] In another study, miR-181a was found to be inversely correlated with serum osteopontin levels, IL-4/IL-5, and total nasal symptom scores, but positively correlated with IFN-γ/IL-12 in patients with pediatric AR.[26] The increase of inflammation during the allergen season results in an increased number of Tregs and positive

regulation of the expression of cytokines IL-10, transforming growth factor (TGF)-β, and interferon (IFN)-γ, which is known as the anti-inflammatory response.[15,27,28] In our study, the increase in miR-181a levels during the allergen season is thought to be due to the anti-inflammatory response of the cell to suppress Th2 inflammation, since that miR-181a limits T cell activation and its level positively correlated with IFN-γ/IL-12 according to the literature.

miR-206 expression was found to increased in human macrophages and related to an increase in the secretion of inflammatory cytokines and MMP-9, and reduced tissue inhibitor of matrix metalloproteinase (TIMP-3) expression.[29] In a study of breast cancer, it was shown that increased expression of miR-206 significantly impaired migration and the invasive ability of cancer cells, and suppressed epithelial-mesenchymal transition (EMT) via TGF-β.[30] It is known that TGF-β acts on eosinophils, macrophages, monocytes, and neutrophils, and also reduces IgE secretion.[28] The effect of miR-206 on TGF-β and EMT is thought to be related to remodeling in asthma. In another study, 12 of the circulating miRNAs were found to be associated with exacerbations in asthma, and particularly, miR-206 had an important role in exacerbation.[13] Panganiban et al.[5] showed that six circulating miRNAs, including miR-206, were effective in allergic states, and expression of miR-206 increased in plasma in patients with AR. The expression level of miR-206 seems related to the atopic status of patients. Briefly, the role of miR-206 in the pathogenesis of diseases can change depending on whether it is suppressive or inflammatory. In our study, it was found that miR-206 expression was lower in the circulation of patients with NA-A and patients with PAR compared with healthy controls, but did not differ between SAR and controls. In addition, the expression level of miR-206 was positively correlated with the total IgE values of patients with PAR. These data are partly inconsistent with the role of miR-206 in allergic inflammation in the literature, due to

lower or similar expression status of miR-206 in PAR and SAR patients compared to healthy controls.

In parallel with the expression results, the evaluation of the predictive value of miRNAs, which were found differentially expressed between the groups, as a diagnostic biomarker by using ROC curve analysis, showed that both miR125b and miR-181a for SAR, and miR-206 both for NA-A and PAR, could be used as biomarkers.

In the literature, expression of miR-126 has been reported as increased in patients with asthma and AR compared with controls, and also in cultured bronchial epidermal cells stimulated with IL-13.[5,14,31] In our study, we found no change in miR-126 expression in the study groups. However, there was a positive correlation between the expression levels of miR-126 and the MEF/25-75 values of patients with NA-A (p=0.028). We interpreted this result as follows: because the higher MEF/25-75 values are associated with better lung function, lower miR-126 levels in asthmatics are associated with worse respiratory function. Suojalehto et al.[10] reported low miR-126 expression in patients with chronic asthma, and their findings were consistent with the low MEF/25-75 values and baseline levels of miR-126 observed in our patients.

Although miR-133b has been found related to AR in both mouse models and studies with human subjects[5,32], in our study miR-133b expression was not significantly different in the study groups and was also not correlated with any clinical parameters.

Our study has some limitations. One is the low number of patients in the study groups. Secondly, the correlation between miRNA levels and inflammation markers such as cytokine levels is the missing part of the story in our study. Showing a correlation between inflammation markers and levels of circulating miRNAs, which was found significant in this study, may have supported our hypothesis.

We demonstrated that the expression of several circulating miRNAs was different between healthy individuals and allergic subjects, and between subjects with different allergic diseases. We also found the expression levels of miRNAs correlated to clinical parameters such as lung function and total IgE levels. According to our findings, we suggest that circulating miR-181a and miR-125b could be candidate biomarkers for SAR, and miR-206 for NA-A and PAR. Studies with larger numbers of patients are needed to more clearly assess the role of these circulating miRNAs as a biomarker in allergic diseases.

Ethical approval

This study was approved by the Ethics Committee of Hacettepe University (GO18/433). All procedures performed in this study were in accordance with the ethical standards of the Hacettepe University Ethical Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EB, FT, ÜMŞ; data collection: FT, MO, HÜ, ÖUS, BES; analysis and interpretation of results: FT, EB, ÜMŞ; draft manuscript preparation: EB, FT, ÜMŞ. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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