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Decreased Treg-derived miR-181a and miR-155 correlated with reduced number and function of Treg cells in allergic rhinitis children

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Abstract

Background Regulatory cells (Tregs) have been proved to be deeply involved in allergic airway inflammation. This study aims to evaluate the expression of miRNA in children with AR and their association with Tregs as well as the severity of AR.

Methods Twenty-five AR children and 20 healthy children were enrolled in this study. The Treg-cell percentage and expression of IL-10 and TGF-β were detected by flow cytometry and enzyme-linked immunosorbent assay. The microRNA microarray analysis in purified Tregs was performed and differentially expressed microRNAs were confirmed by quantitative polymerase chain reaction (qPCR).

Results Children with AR had lower percentage of Tregs and expression of IL-10 and TGF-beta compared with control children. We found that significantly lower levels of miR-155 and miR-181a in Tregs from AR than healthy controls. Furthermore, intracellular miR-155 and miR-181a level were positively correlated with percentage of Tregs and expression of IL-10 and TGF-beta. Similarly, total nasal severity scores (TNSS) were found to be negatively correlated with miR-155 and miR-181a levels.

Conclusion Decreased Treg-derived miR-181a and miR-155 were correlated with reduced number and function of Tregs in AR children. The intracellular miR-155 and miR-181a levels may serve as predictors of disease severity in childhood AR.

Keywords Allergic rhinitis · Children · Regulatory T cells · miR-181a · miR-155

Introduction

Allergic rhinitis (AR) is a common chronic inflammation of the upper airway and presented as itching, rhinorrhea, sneezing, and congestion [1]. Children with AR are often affected by poor quality sleep, consequent fatigue, impair cognitive functioning, academic performance, facial abnormalities, and dental malocclusions [2, 3]. The previous paradigm of Th1/Th2 imbalance promoted AR is expanded by identification of novel T-helper families, such as regulatory T cell (Treg), Th17 and Th9 [4]. Of these cells, Tregs play central roles, since they may block the differentiation of T helper 2 (Th2), limit airway allergic inflammation, and prevent inappropriate Th2 responses to environmental allergens [5, 6]. Tregs functions by directly contacting effective immune cells or secreting anti-inflammatory cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-beta [7].

MicroRNAs (miRNAs) are 21–23-nt RNA molecules that identified in mammals and play important roles in a wide range of biological functions [8]. The miRNAs regulate target genes expression by suppressing translation or through mRNA degradation. Treg cells, which provide a critical brake on effector responses, also are regulated by miRNAs. The previous studies demonstrated that miR-182,

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miR-10a, miR-155, and miR-146 are required for proficient Treg development and suppressive capacity [9–13]. For example, miR-155 is an attractive candidate, because Foxp3 binds to the promoter region of its host gene bic, and miR-155 is highly expressed in Tregs [14].

The previous studies had showed that many miRNA expressions in nasal mucosa were altered in AR and differentially expressed miRNA may be involved in the development of AR [15]. In this study, we evaluated the serum and intracellular levels of miRNA and analyzed their correlation with the number and function of Treg cells as well as the severity of the disease.

Methods

Patients

Twenty-five children (7–12 years) with AR were enrolled at the Department of Otolaryngology, Guangzhou Women and Children's Medical Center from January 2017 to June 2017. AR was diagnosed according to symptoms, physical examination, skin prick test, and specific IgE measurement according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guideline (2010) [16]. Twenty healthy children (7–12 years) with comparable age and gender were enrolled as the control group. Children with other allergic or respiratory diseases and drug use history within the past 4 weeks were excluded. The study protocols were approved by local ethics committee boards. Informed consent was obtained from the parents of all subjects. Total Nasal Symptom Score (TNSS) was scored in our study to evaluate the symptoms, which is a daily symptom severity score that rates nasal congestion, rhinorrhea, nasal itching, and sneezing on a 0–3-point scale [17].

Percentage of Treg in CD4 + T cells

Peripheral blood mononuclear cells (PBMCs) from AR and control children were isolated by Lymphoprep density-gradient centrifugation from heparinized leukocyte-enriched buffy coats. CD4 + T cells were purified by magnetic activated cell sorting (Miltenyi Biotec) with purity > 95%. Anti-human CD4-FITC, CD3-PE, CD25a-APC, and Foxp3-PE (Becton Dickinson Biosciences, San Diego, USA) were used for staining. Stained CD4 + T cells were detected on a FACSCalibur cytometer (BD Bioscience, USA) and analyzed using FACS Diva software (BD Bioscience, USA).

Treg-cell purification and culture

Treg cells were separated from PBMCs using the CD4 + CD25 + Regulatory T-Cell Isolation Kit (Miltenyi Biotec, Germany). For stimulation, cells were co-cultured with anti-CD3/CD28-coated beads at a 1:1 cell-to-bead ratio (Dynabeads, Life Technologies, Carlsbad, California, USA) in the presence of 100 U/mL IL-2 (Chiron, Emeryville, California, USA) in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 50 U/mL penicillin G, 50 µg/mL streptomycin, 2 mM L-glutamine (all from Life Technologies), and 10% normal human serum.

RNA extraction labeling and microRNA microarray in Tregs

The microRNA microarray of Tregs was performed separately in five AR children and five healthy controls. MicroRNA was labeled by a miRCURY™ Hy3™/Hy5™ Power Labeling Kit (Exiqon, Denmark). Then, the samples were hybridized onto miRCURYTM LNA Array (version 16.0) (Exiqon, Denmark) slides in a 12-Bay Hybridization System (Nimblegen Systems, USA) and scanned using an Axon GenePix 4000B microarray scanner (Axon, USA). GenePix Pro 6.0 software (Axon, USA) was used for data analysis.

Determination of serum and Treg-derived miRNA level by quantitative real-time PCR (qRT-PCR)

For miR-181a and miR-155, total RNA from serum sample or Treg cells were reverse transcribed into cDNA using the Taqman microRNA reverse transcription kit and Taqman microRNA assays (Invitrogen), as per manufacturer's instructions. The results were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to RNA U6 controls.

Enzyme-linked immunosorbent assay (ELISA) for protein expression

ELISA kits (R&D systems, USA) were performed for detecting levels of IL-10 and TGF-β from serum according to the protocol provided by manufacturer. The detection limits of the assays were as follows: IL-10, 3.9 pg/mL, TGF-β, 15.4 pg/mL. The eosinophil cationic protein (ECP) was measured by Unicap system (Phadia AB, Uppsala, Sweden). Total serum IgE level was detected by an ELX-800 system.

Statistical analysis

The data are presented as mean ± SD. The differences between groups were determined by Student *t* test. Correlations between the various parameters were assessed by the Spearman rank correlation analysis. A *P* value of less than 0.05 was defined as statistically significant.

Results

Demographic and laboratory characteristics of the study population

We recruited 25 children with AR and 20 normal controls to the study. Demographic parameters of the AR children and controls are summarized in Table 1. The mean age and gender distribution were comparable between AR and healthy children. The eosinophil cationic protein (ECP) and IgE level in AR group were significantly compared with control group ($P < 0.05$).

Number and function of Treg

Flow cytometric analysis demonstrated that the percentages of peripheral blood CD4+ CD25+ Foxp3+ Tregs were significantly lower in AR patients than in normal controls (0.46 ± 0.16 vs 1.91 ± 0.42, $P < 0.001$). Meanwhile, serum protein level of IL-10 (40.42 ± 6.01 vs 70.36 ± 14.32, $P < 0.001$) and TGF-β (14.83 ± 5.49 vs 37.97 ± 10.29, $P < 0.001$) were significantly lower in AR patients than in normal controls (Fig. 1).

Table 1 Demographic characteristic of AR children and normal controls

Groups	AR group	Control
Number	25	20
Sex (male:female)	12:13	12:8
Age (months)	102.3 ± 22.6	99.5 ± 18.2
ECP[a] (ng/ml)	38.5 (12.0–139.0)*	7.6 (2.8–41.0)
IgE[a] (IU/ml)	155.2 (34.1–1125.0)*	36.7 (3.1–46.0)

ECP eosinophil cationic protein

*Compared with control group, $p < 0.05$

[a]Data presented as median values (minimum–maximum)

Treg miRNA assay

A total of 986 microRNAs were successfully measured in the microarray analysis. Among them, 517 were up-regulated and 469 were down-regulated compared to healthy controls. Several microRNAs related to T-cell differentiation and regulation are listed in Table 2. We found that the miR-155 and miR-181a levels in the Tregs of AR children were dramatically decreased compared to healthy controls.

Decreased Treg-derived miR-181a and miR-155 correlated with the number and function Tregs in children with AR

Lower Treg-derived miR-155 (0.013 ± 0.004 vs 0.0283 ± 0.005, $P < 0.001$) and miR-181a (0.054 ± 0.01 vs 0.08 ± 0.006, $P < 0.001$) levels in AR group were found compared with the control group (Fig. 2). The correlation analysis revealed that Treg-derived miR-155 ($r = 0.58$, $P < 0.001$) was positively associated with the number Treg in AR group and Treg-derived miR-181a was positively associated with expression of serum IL-10 ($r = 0.71$, $P < 0.001$) and TGF-β ($r = 0.78$, $P < 0.001$) in AR group (Fig. 2). However, the correlation analysis showed no relationship between serum

Table 2 Expression profile of several microRNAs in Tregs of AR patients (shown as fold changes or percent decrease)

Up-regulated			Down-regulated		
microRNA	Fold	*P*	microRNA	%Decrease	*P*
miR-18	12.23	0.23	miR-143	23	0.11
miR-19	3.25	0.07	miR-146	33	0.28
miR-26	5.61	0.31	**miR-155**	**59**	**0.01**
miR-126	2.77	0.28	**miR-181a**	**68**	**0.02**
miR-203	3.47	0.11	miR-191	41	0.07
miR-233	8.12	0.46	miR-224	29	0.09
miR-326	9.65	0.18	miR-498	18	0.33
miR-7	10.29	0.09	miR-let7e	27	0.26

$P < 0.05$ was defined as statistically significant (bold)

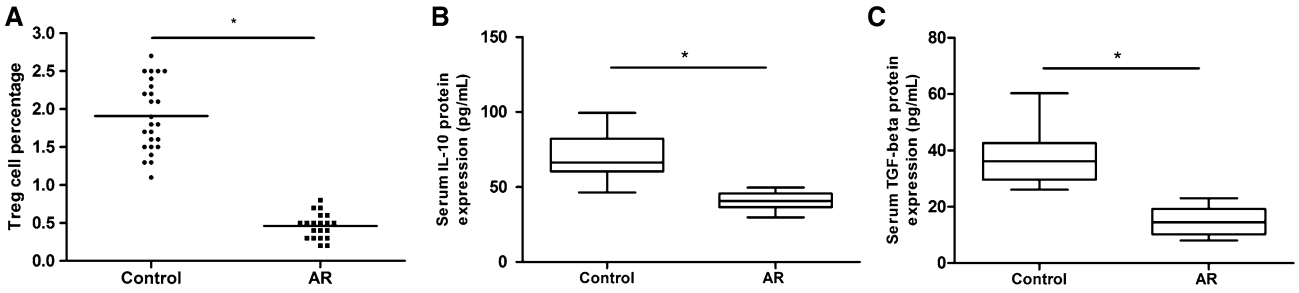

Fig. 1 Treg-cell percentage (**a**), serum IL-10 (**b**) and TGF-beta (**c**) levels in the AR group was significantly higher than that in the control group. *$P < 0.001$

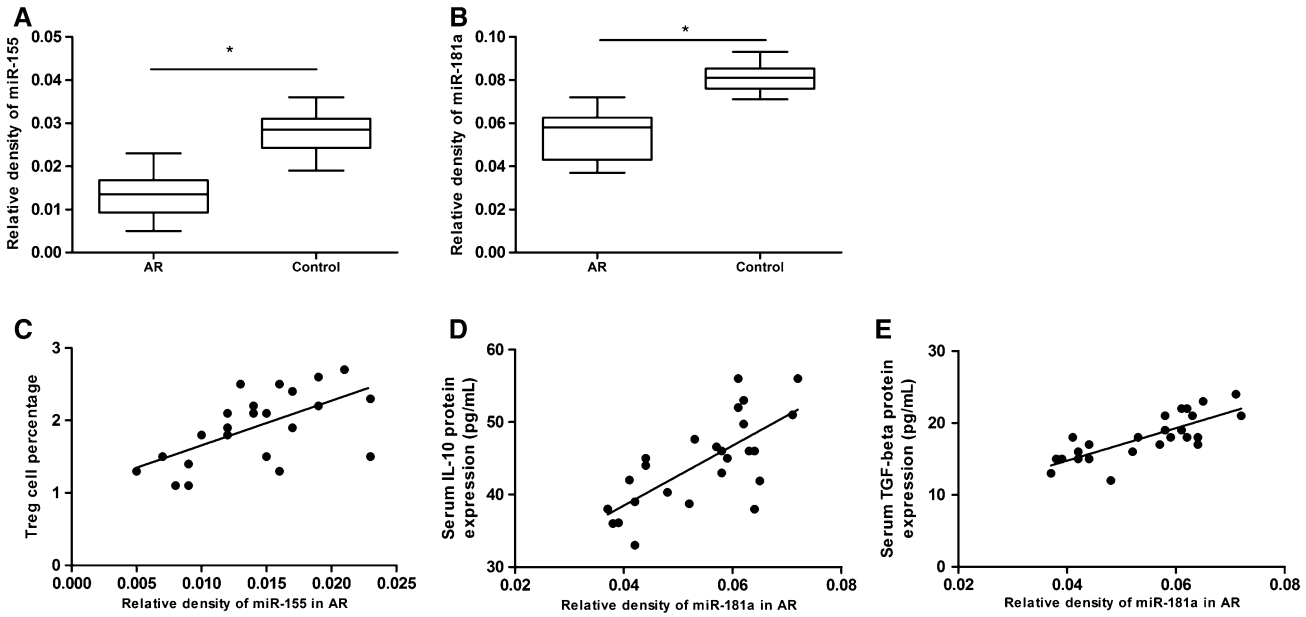

Fig. 2 Treg-derived miR-155 and miR-181a in the AR group was significantly higher than that in the control group (**a, b**). The Treg-derived miR-155 was significantly associated with Treg-cell percentage AR (**c**). The Treg-derived miR-181a was significantly associated with serum IL-10 (**d**) and TGF-beta (**e**) levels. $^{*}P < 0.001$

miR-155 or miR-181a with the number and function of Tregs.

Decreased expression of IL-10 and TGF-β from Tregs in AR children and controls

Our results showed that both the mRNA of IL-10 (28.37 ± 9.33 vs 55.24 ± 21.79, $P < 0.001$) and TGF-β (18.77 ± 8.51 vs 45.26 ± 21.18, $P < 0.001$) from Tregs stimulated with PHA were significantly lower in AR children than controls.

Correlation of Treg-derived miR-181a and miR-155 with clinical severity in children with AR

To identify if miR-181a and miR-155 have relationship with the severity of pediatric AR patients, the TNSS of the children was assessed. As shown in Fig. 3, there was a significant correlation between Treg-derived miR-181a and miR-155 and TNSS ($r = -0.57$, $P < 0.05$; $r = -0.58$, $P < 0.05$). However, no correlation was found between serum miR-181a and miR-155 expression and TNSS (data not shown).

Discussion

There is limited literature regarding the roles of miRNAs and Tregs in airway allergic inflammation and most relates to asthma and adult population. In this study, we demonstrated that lower Treg-derived miR-155 and miR-181a in AR group were associated with the decreased number and function of Treg in AR compared with controls.

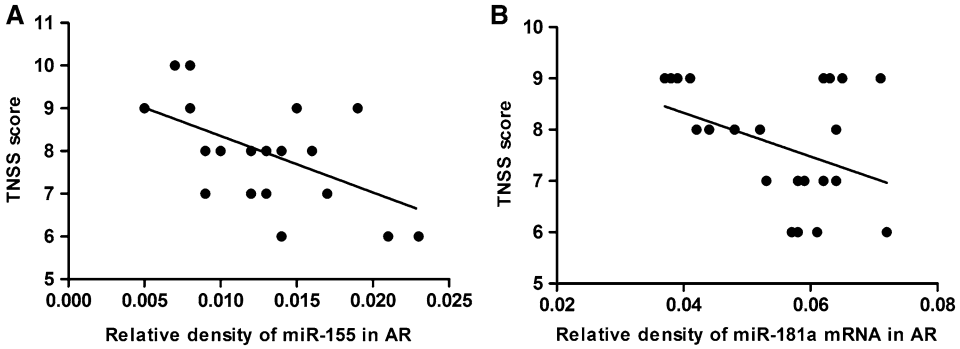

Fig. 3 Negative correlation between Treg-derived miR-155 and miR-181a and total nasal symptom score (TNSS)

Treg maintains self-tolerance and prevents the development of various inflammatory diseases by directly contacting effective immune cells and secreting anti-inflammatory cytokines, such as IL-10 and TGF-β [7]. TGF-β is essential for both Treg differentiation through induction of Foxp3 expression and promoting Treg-cell function by activating SMAD5-signaling pathway [18]. Consistent with the previous reports, our results suggest the number and function of Treg in AR were inhibited compared with controls, manifested as decreased both serum and Treg-derived IL-10 and TGF-β levels. Allergic patients were believed to be have an allergen-specific functional defect of Treg cells and decreased production of TGF-β and IL-10 [19]. The higher level of TGF-βand IL-10 expression in control groups suggested that non-allergic patients showed as immune tolerance even without allergen exposure. However, the mechanism was not fully understood.

Altered miRNAs' profiles in nasal mucosa and bronchoalveolar lavage fluid exosomes in allergic airway inflammation have been reported in several studies [20–22]. Therefore, we supposed that miRNA may be involved in the regulation of Tregs. We screened differentiated miRNA in Tregs and found that lower Treg-derived miR-155 and miR-181a were correlated with the number and function of Treg in AR. However, we did not found correlation between serum miR-155 or miR-181a with the number and function of Tregs, suggesting that miR-155 or miR-181a may have multiple regulatory targets rather than only on Tregs.

MiR-155 was up-regulated in several activated immune cells, such as T lymphocytes, B lymphocytes, macrophages, and dendritic cells [23]. Moreover, miR-155-deficient mice presented with decreased numbers of Tregs in the thymus and periphery, but these Tregs express normal levels of surface markers such as CD25, CTLA-4 [13]. In agreement with these findings, miR-155-deficient mice also have reduced numbers of FOXP3 + cells among CD4 + lymphocytes in lymph nodes [24]. Consistently, our data also showed that Treg-derived miR-155 rather than serum miR-155 was correlated with Treg frequency rather than function of Treg in AR.

The previous studies demonstrated that the inhibition of miR-181a significantly impaired both positive and negative selections of double positive cells [25]. Its expression is high in immature T cells and low in more differentiated Th1 and Th2 cells. MiR-181a repressed expression of a set of genes involved in T-cell maturation (TCRα, CD69, BCL-2) and as such regulated T-cell signaling and migration [26]. However, the role of miR-181a in regulation in Tregs was not clear. Our study preliminarily suggested that miR-181a may be involved in the function of Tregs, presented as negative correlation with IL-10 and TGF-βlevels. However, the direct effect of miR-181a on Tregs should be proved by in vitro experiments in the future.

We also found that Treg-derived miR-155 and miR-181a had a negative correlation with TNSS in pediatric AR patients. These findings suggest that miR-155 and miR-181a play a crucial role in driving the progression of childhood AR and miR-181a is more likely to have opposing effect. Nonetheless, the question of how miR-155/miR-181a regulates the proliferation and function of Treg needs further investigation.

In summary, children with AR showed a marked lower Treg-derived miR-155 and miR-181a, which were involved in the regulation of Tregs. The intracellular miR-155 and miR-181a levels may serve as predictors of disease severity in childhood AR and appear to be promising targets for modulating AR.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Research involving human participants All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all guardians of children included in the study.

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