Biomedicine & Pharmacotherapy 101 (2018) 195–200

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# MiR-133a/133b inhibits Treg differentiation in IgA nephropathy through targeting FOXP3

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ARTICLE INFO

*Keywords:*
IgA nephropathy
Treg differentiation
miR-133a/133b
FOXP3

ABSTRACT

*Objective:* The aim of this study was to investigate the effect of miR-133a and miR-133b on regulatory T cell (Treg) differentiation in IgA nephropathy (IgAN) through targeting forkhead box P3 (FOXP3).

*Methods:* Peripheral blood mononuclear cells (PBMCs) were isolated from IgAN patients (n = 20) and healthy controls (n = 20). Percentage of Tregs defined as CD4 + CD25 + FOXP3 + T cells were determined by flow cytometry. The mRNA expression levels of miR-133a, miR-133b and FOXP3 were measured by real-time PCR. FOXP3 protein level was analyzed by western blotting.

*Results:* Tregs percentage in PBMCs of IgAN patients was significantly lower than that of healthy controls, whereas the expression levels of miR-133a and miR-133b in IgAN patients were dramatically higher than that in the control group. Treg percentage was negatively correlated with miR-133a and miR-133b expressions. Meanwhile, miR-133a and miR-133b modulated FOXP3 expression by detecting of its gene 3′-untranslated region. MiR-133a or miR-133b overexpression significantly decreased the % Tregs (CD4 + CD25 + FOXP3 + ) of the total CD4 + T cells while miR-133a or miR-133b knockdown led to an opposite effect. Moreover, FOXP3 levels in IgAN patients was significantly lower than that in the control group and was negatively correlated with miR-133a and miR-133b expression.

*Conclusion:* MiR-133a and miR-133b inhibited Treg differentiation in IgA nephropathy through targeting FOXP3.

## 1. Introduction

Primary immunoglobulin A nephropathy (IgAN) is one of the most common forms of primary glomerulonephritis [1]; it is the main cause of end-stage renal disease (ESRD) in patients with primary glomerular disease in China [2]. About 30% patients became permanently work disabled during the first 2–3 years of the disease if insufficiently treated [3]. IgAN is the most common primary glomerulonephritis worldwide. Approximately 20–40% of patients with biopsy-proven IgAN progress to end-stage renal disease (ESRD) within 10–20 years [4].

CD4 + T cells expressing both interleukin-2 receptor α chain (CD25) and the transcription factor forkhead box P3 (FOXP3) are called regulatory T cells (Tregs). FOXP3, together with a high expression of a high affinity IL-2 receptor α chain (CD25), has been described as the best and most specific markers of Tregs development and function [5–7]. Except for soft tissue and bone lesions, rheumatoid arthritis (RA) synovial fibroblasts can spontaneously secrete numerous proinflammatory cytokines such as interleukin-6 (IL-6) and IL-8 and matrix metalloproteinases (MMPs) including MMP-1 and MMP-3, which result in infiltration of inflammatory cells and play critical roles in progressive destruction of articular cartilage and bone [8–10]. Previous studies showed that abnormalities of regulatory T-lymphocytes including Tregs were considered important in patients with IgAN. Numerical and functional Treg deficit was observed in patients who have autoimmune diseases [11,12]. Compared with environmental influences, genetics and immunology mechanisms were more important in the development of IgAN, which have been suggested to play a central role in the pathogenesis of IgAN [13,14].

MicroRNAs function to directly bind to the potential target site in the 3′-untranslated region (3′ UTR) of specific target mature mRNA, leading to the repression of protein expression and the production of target mRNA degradation [15]. In previous studies, miRNAs were derived from precursors with a characteristic hairpin secondary structure and functioned as critical gene regulators [16]. Accumulating evidence suggested that miRNAs were involved in the pathogenesis and histological changes of kidney diseases by targeting various types of mRNAs [17]. However, the detailed roles of miRNAs in kidney disease, especially in IgAN, still need to be further investigated. Potential biological

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https://doi.org/10.1016/j.biopha.2018.02.022
Received 4 December 2017; Received in revised form 8 February 2018; Accepted 9 February 2018

**Table 1**
Characteristics of immunoglobulin A nephropathy patients and controls.

| Characteristic | Patients (n = 20) | Controls (n = 20) |
|---|---|---|
| Male/female | 10/10 | 10/10 |
| Age (year) | 38.34 ± 8.27 | 40.23 ± 7.52 |
| Serum creatinine (μmol/L) | 88.36 ± 6.21 | 74.13 ± 8.39 |
| Estimate GFR (mL/min/1.73 $m^2$) | 73.52 ± 16.92 | 72.65 ± 17.21 |
| Proteinuria (g/24 h) | 1.48 ± 0.81 | ND |
| Hypertension | 121 ± 12 | 138 ± 13 |
| With | 8 | 0 |
| Without | 12 | 20 |

Note: Values are expressed as the mean ± SD. GFR, glomerular filtration rate; ND, not determined.

roles and molecular mechanisms of miR-133a and miR-133b in Treg differentiation in the IgAN pathogenesis have been poorly investigated.

The aim of present study was to investigate the effect of miR-133a and miR-133b on Treg differentiation in IgA nephropathy through targeting FOXP3. We found that miR-133a and miR-133b were related to Treg percentage in PBMCs from IgAN patients and healthy controls. Thus, we speculated that miR-133a and miR-133b might regulate Treg differentiation in IgAN through targeting FOXP3.

## 2. Materials and methods

### 2.1. Patients and controls

The present study was approved by the Medical Ethical Committee of Department of Nephrology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (Wenzhou, China). Twenty kidney biopsy-proven IgAN patients and twenty healthy subjects in Huashan Sub-Hospital of Fudan University were recruited and enrolled in the study. We recorded related clinical data at the time of kidney biopsy (Table 1). Signed informed consent was obtained from all patients and control subjects and no patients underwent treatment with steroids, immunosuppressive agents, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

### 2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was obtained from IgAN patients and healthy individuals. PBMCs were isolated from venous blood collected from the basilic vein by density gradient centrifugation using Gradisol L (Polfa, Kutno, Poland). Separation of monocytes (95% purity) was performed with the acceleration of 600 × *g* for 20 min at RT. Finally, the PBMCs layer was collected and washed twice by PBS (250 × for 10 min).

### 2.3. T cell isolation and transfection

$CD4^+$ T cells (95%purity) separated from PBMCs of healthy subjects were isolated and purified by negative selection using the untouched $CD4^+$ T cell isolation kit purchased from Miltenyi Biotec (Shanghai, China) according to the manufacturer's protocols. After isolation of $CD4^+$ T cells, the naïve $CD4^+$ T cells were further purified by EasySep enrichment kits (StemCell Technologies, Vancouver, Canada) as recommended by the manufacturer's guidelines. A portion of the $CD4^+$ T cells was cryopreserved for later use.

### 2.4. Transient miRNA and siRNA transfection

PBMCs were isolated and cultured for miR-133a and miR-133b transfection. PBMCs were grown overnight and then transfected with above miRNAs. According to the manufacturer's instructions, complexes of Lipofectamine 2000 (Invitrogen, CA, USA) and miRNA mimics and inhibitors were directly mixed with cells in 6-well cell culture plates at a density of $2 \times 10^5$ cells per well. Cells were harvested for real-time PCR and western blotting, respectively, 24 h and 48 h post transfection.

### 2.5. Real-time PCR (RT-PCR)

Real-time PCR was performed to detect miR-133a, miR-133b, FOXP3expression. RNA was isolated from cells using the TRIzol reagent (Invitrogen) as recommended by the manufacturer's guidelines. For reverse transcription, cDNA was synthesized using the iScript kit (Bio-Rad, PA, USA). Real-time PCR analysis was performed using Power SYBR Green RT-PCR Reagents on an ABI thermal cycler Step-One Plus (Life Technologies, Shanghai, China). PCR reactions were performed using a TaqMan Master Mix (Applied Biosystems, CA, USA). Data were collected and quantitatively analyzed on the ABI 7500 Fast Sequence Detection System and software (Applied Biosystems) using 2-delta delta CT method relative to 18S.

### 2.6. Western Blotting for FOXP3

Western blot analyses were performed for the determination of protein expressions. Primary hepatocytes were washed with ice-cold PBS once and lysed with lysis buffer supplemented with protease inhibitors to prepare cell lysates. Protein extracted from the cultured PBMCs (containing approx. $4 \times 10^6$ cells) lysed in cell lysis buffer containing 1mm phenylmethanesulfonyl fluoride (PMSF). Protein lysates from each sample were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Bio-Rad). Subsequently, membranes were blocked in 5% milk/1 × TBST for 1 h and then probed with the corresponding primary antibodies, anti-FOXP3 (Abcam, CA, USA) and anti-GAPDH (Tebu-Bio, Columbia, USA). Following thorough wash, membranes were incubated with appropriate horseradish peroxidase-coupled secondary antibodies (GE Healthcare, Michigan, USA) for 1 h. Finally, blots were detected by ECL western blotting detection reagent (GE Healthcare). Total protein levels were normalized to Tubulin and bands were quantified with Image Guage 4.0 (Fujifilm). Each sample was replicated triple.

### 2.7. Statistical analysis

Differences in the Treg levels and miRNA expression between the two groups were analyzed by Student's *T* test. A $p$ value of $< 0.05$ was considered to be statistically significant. When multiple groups were compared, One Way ANOVA and Kruskal Wallis test were used for data fulfilled normal distribution and for those did not, respectively. Spearman correlation was used to examine the relationship between the variables. All experiments were performed at least three times and data are expressed as mean ± D. For all tests, two-sided p values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Relationship between Tregs population and the expression levels of miR-133a as well as miR-133b

Tregs in PBMCs obtained from 20 IgAN patients and 20 healthy controls were defined based on their expression of CD4, CD25 and FOXP3 and quantified by flow cytometer analysis. Statistical analysis showed that the percentage of Tregs in peripheral blood of IgAN patients was significantly lower than that of the healthy control (Fig. 1A). Some researchers have shown that miR-133a and miR-133b were significantly upregulated and correlated with disease severity and progression in patients with IgAN [18]. In this study, the mRNA expression levels of miR-133a and miR-133b were dramaticly higher in IgAN patients than that in the control group (Fig. 1B, C). We further explored relationship between the percentage of Tregs and the expression levels of miR-133a as well as