MicroRNA expression profile in Treg cells in the course of primary immune thrombocytopenia

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ABSTRACT
Primary immune thrombocytopenia (ITP) is an autoimmune bleeding disorder which characterizes with platelet production impairment and platelet destruction increment. CD4+CD25+Foxp3+ Treg cells (Tregs) are involved in the immune pathogenesis of ITP. MicroRNAs (miRNAs) are also involved in ITP and their loss of function is shown to facilitate immune disorders. Thus, the miRNA expression profile in Tregs from ITP was analyzed in this study. We assessed the genome-wide miRNA expression profile of three newly diagnosed adult patients with ITP and three healthy controls using microarray analysis of CD4+CD25+CD127dim−Tregs that were sorted using an immune magnetic bead kit. The miRNA microarray chip was based on miRBase 18.0 and Volcano Plot filtering software used to analyze the miRNA profile in Tregs. Distinct miRNA expression was further validated by fluorescence-based real-time quantitative PCR (qPCR). We found that 502 human miRNAs were differentially expressed (244 upregulated and 258 downregulated) in patients with ITP compared with healthy donors. We identified 37 miRNAs expressed significantly, including 26 upregulated and 11 downregulated. Among the deregulated miRNAs, three downregulated miRNAs including miR-155–5p, miR-146b–5p, and miR-142–3p were selected for qPCR verification. We confirmed that miR-155–5p, miR-146b–5p, and miR-142–3p were significantly decreased in Tregs from patients with ITP compared with healthy controls. Compared with the healthy controls, miRNAs expressed differentially in the Tregs of patients with ITP. The levels of expression of miR-155–5p, miR-146b–5p, and miR-142–3p were significantly decreased. Therefore, the deregulation of miRNAs may affect the function of Tregs in the course of ITP.

INTRODUCTION
Primary immune thrombocytopenia (ITP) is an autoimmune disease. A decreased platelet count could be observed in ITP because of autoantibody-mediated platelet destruction and insufficient platelet production.1 Massive bleeding is a serious complication in these patients. The pathogenesis of ITP, including the immune, genetic, and environmental factors remains unclear. One pathogenic factor is a regulatory Treg disorder that results in antiplatelet antibodies generated by B cells.2 Tregs are important immune-regulating cells that maintain immune tolerance and play an important role in a variety of autoimmune diseases.3 Tregs are a specialized subpopulation of T cells that act to suppress the activation of the immune system and thereby maintain homeostasis and tolerance to self-antigens. Tregs are a CD4+CD25+Foxp3+ T cell subset, characterized by CD25high expression, because immunoregulatory function is absent in CD4+CD25low/neg cells. Moreover, CD4+CD25high T cells are characterized by a high expression of the forkhead/winged-helix transcription factor 3 (Foxp3) gene. Patients with ITP have a Treg immunodeficiency, including a reduction in the number of Tregs and/or weakened immunosuppressive function.4

MicroRNAs (miRNAs) are short (about 22 nts) noncoding RNAs that control gene expression at the post-transcriptional level and have been proven to play critical roles in the development, differentiation, and function of immune cells. Recently, miRNAs have been shown to play an important role in the immunosuppressive function of Tregs.5 Tregs have their own particular miRNA expression profiles. Cobb et al reported that the miRNA expression profile in Treg is distinct from those in conventional CD4+ T cells.6 Moreover, miR-155 inhibition sensitizes CD4+ Th cells for Treg-mediated suppression.7 We therefore intended to explore
the potential correlation of miRNA during development of Treg dysfunction in the course of ITP.

MATERIALS AND METHODS

Patients and health controls

There were 21 newly diagnosed adult patients with ITP (16 females and 5 males, mean age 34±12 years) enrolled in this study. The diagnosis of ITP was based on recently reported criteria. Of the selected patients, their platelet counts ranged from 7 to 25×10⁹/L, and we excluded tumor disease, autoimmune disease, peptic ulcer, severe infection, and pregnancy.

There were 18 healthy volunteers (14 females and 4 males, mean age 35±10 years) included as the control group with platelet counts ranged from 125 to 260×10⁹/L. The differences between two groups regarding to sex and age was not statistically significant.

The miRNA array study was carried out in 3 patients and 3 age-matched and sex-matched healthy donors and then used fluorescence-based real-time quantitative PCR (qPCR) to measure selected miRNAs from the remaining 18 patients and 15 healthy controls. This study was approved by our hospital-based ethics committee and informed consent was obtained from the patients as legal guardians.

Isolation of peripheral blood mononuclear cells

Peripheral blood was collected into EDTA-anticoagulated vacuum tubes. Peripheral blood mononuclear cells were isolated using lymph preparation density gradient centrifugation (Reagent Factory, Shanghai, China). A microscope was used to count cells.

Isolation of Tregs using immune magnetic beads

Purification of CD4⁺CD25⁺high/CD127dim⁻ regulatory cells was achieved in a two-step procedure, following the protocols of two Miltenyi Biotec kits for isolation of Tregs. Non-CD4⁺ MACSxpress depletion cocktail and CD25 MicroBeads were used for labeling. In the first step, we used immunomagnetic depletion with MACSxpress Beads to remove the MACSxpress Separator non-CD4⁺ cells and the majority of CD127hi cells after erythrocytes were aggregated and sedimented. The CD25 MicroBeads may not be influenced by the magnetic field of the MACSxpress Separator (Miltenyi Biotec, Germany) because of its small nature. In the second step, anti-CD25-coated microbeads were directly labeled to the CD4⁺CD127dim⁻ T cells. Then, CD4⁺CD127dim⁻ T cells in the pre-enriched CD4⁺ T cell fraction were isolated according to positive selection. After eluting, the eluted cells were regarded as CD4⁺CD25⁺high/CD127dim⁻ regulatory cells for further analysis.

Assessment of Treg purity

CD4 FITC/CD25 APC (BD Company, USA) and eBioscience Flow Cytometry Staining buffer (eBioscience, USA) were used in two groups (experimental and negative groups). We followed the eBioscience Treg kit procedure in which cells were suspended by vortex shock and an eBioscience Fixation and Permeabilization set added. After a depleting wash and repeated removal of supernatant, cells were reacted with Foxp3 antibody. Multiparametric FACS analysis for the detection of the various markers was performed using a FACS Calibur system (BD, USA).

RNA extraction

Total RNA was extracted from Tregs using Trizol reagent (Invitrogen, Carlsbad, California, USA) and following the protocol for an miRNeasy mini kit (Qiagen, USA). The quantity of the RNA samples was assessed using an ultraviolet spectrophotometer (NanoDrop-1000 Technologies).

miRNA array analyses

The miRCURY LNA Array (miRBase 18.0) system was performed in three patients with ITP and three healthy donors as previously described. The RNA samples were then labeled with a miRCURY Array Power labeling kit (Exiqon, Denmark) and hybridized on a miRCURY LNA Array (miRBase 18.0) station and scanned with an Axon GenePix 4000B microarray scanner (Applied Biosoftware Systems, USA). The significance of upregulation or downregulation of miRNAs was determined with a fold change of >1.3 and a padj<0.05 calculated by Student’s t-test.

TaqMan qPCR for quantification of miRNAs

Total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer’s protocol. Primers (Invitrogen, Shanghai) were designed using Primer 5.0 software (tables 1 and 2). A reverse transcriptional kit (Epicentre, USA) were used in two groups (experimental and negative groups). We followed the manufacturer’s instructions. The manufacturer’s instructions were strictly followed.

Table 1

| Primer name | RT primer |
|-------------|-----------|
| U6          | 5'CCTTCGCAATTTGCCAGCAT3' |
| mir-155-5p  | 5'GTCGTATACAGGGCCAGTGGGAGTCCGCAATTTGCAGATCAGACAACCCT3' |
| mir-146b-5p | 5'GTCGTATACAGGGCCAGTGGGAGTCCGCAATTTGCAGATCAGACCGCGGCTAAG3' |
| mir-142-3p  | 5'GTCGTATACAGGGCCAGTGGGAGTCCGCAATTTGCAGATCAGACCGCGGCTAAG3' |

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U6 small nuclear RNA was quantified as a control to normalize differences in total RNA levels.

PCRs were amplified on an ABI Prism-7900 Sequence Detection System (Applied Biosystems, USA). An initial denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 10 s, and extension at 60°C for 1 min. A dissolution curve was drawn to confirm that the PCR specific product was as specified.

**RESULTS**

**Assessment of Treg purity**

Purified Tregs were analyzed for their surface expression of CD4, CD25, Foxp3 antigens, and the cell counts were about 1–2×10^5. FACS analysis showed that CD4^+ Foxp3^+ Tregs were more than 80% and CD4^+CD25^+ Foxp3^+ Tregs were more than 70% (figure 1).

**Differential expression of miRNAs in Tregs of patients with ITP compared with healthy controls**

We chose qualified RNA for microarray and found no signal in negative controls. Experiment quality and sample properties were assessed by the correlation of gene hybridization signal (figure 2). The figure shows an increased, but not strong hybridization signal. One reason for this outcome may be the quality of miRNAs; other reasons might include the weak expression of some miRNAs in the sample or the presence of as yet unknown genes.

We assessed the levels of expression of human miRNAs in Tregs from three patients with ITP and three healthy donors using an miRCURY LNA Array (figures 3 and 4). We found that 502 genes were expressed differentially; 244 had increased expression and 258 decreased expression. Because too few transcripts showed a fold-change >1.3, genes that reached the significance level of p<0.05 and with a fold-change >1.3 were analyzed further. We identified 37 miRNAs differentially expressed in Tregs from patients with ITP compared with healthy controls (tables 3 and 4). Among them, 26 miRNAs were statistically upregulated and 11 were statistically downregulated.

Decreased expression level of miR-155–5p, miR-146b-5p, and miR-142–3p in Tregs of patients with ITP compared with healthy controls (figures 3 and 4). We found that 502 genes were expressed differentially; 244 had increased expression and 258 decreased expression. Because too few transcripts showed a fold-change >1.3, genes that reached the significance level of p<0.05 and with a fold-change >1.3 were analyzed further. We identified 37 miRNAs differentially expressed in Tregs from patients with ITP compared with healthy controls (tables 3 and 4). Among them, 26 miRNAs were statistically upregulated and 11 were statistically downregulated.
microarray analysis revealed that 37 aberrant miRNAs were identified. We validated the results by TaqMan qPCR in 18 patients with ITP and 15 healthy controls and found three abnormally expressed miRNAs in Tregs from patients with ITP (table 5, figure 5). Then, we compared the level of expression of miRNA in Tregs by correlation analysis and found none were significantly different (table 6).

**DISCUSSION**

The expression of CD25 and the Treg-specific Foxp3 were the characteristics of Treg which is essential for basic function. Severe inflammation and autoimmunity could be observed in mice and humans with dysfunctional Tregs, indicating that Tregs may help prevent harmful autoimmune responses. Dysregulation of Tregs could also
be considered to associate with ITP pathophysiology. A decreased frequency of Tregs in peripheral blood is found in patients with ITP, and their immunosuppressive function was inferior in patients with ITP compared with healthy controls. In the present study, we sorted Tregs from patients with ITP and healthy controls using immunoreactive magnetic beads to study the pathogenesis of ITP and dysfunction in Tregs.

It has become more clear that miRNAs form a complex signaling pathway and aberrant expression of miRNAs is implicated in the pathogenesis of autoimmune diseases. miRNAs also play a part in growth development, maintaining immune function and inhibiting the inflammatory response in Tregs.

### Table 3

| miRNA            | Fold change | P value |
|------------------|-------------|---------|
| hsa-miR-106b-3p  | 4.134943    | 0.037759|
| hsa-miR-200b-3p  | 3.214671    | 0.025018|
| hsa-miR-668      | 2.985206    | 0.001874|
| hsa-miR-548j     | 2.359644    | 0.023893|
| hsa-miR-3138     | 2.743772    | 0.04483 |
| euv-miR-BART19-3p| 3.184072    | 0.04792 |
| hsa-miR-3677-5p  | 2.444652    | 0.036854|
| hsa-miR-3632-5p  | 4.319884    | 0.001374|
| hsa-miR-337-3p   | 1.49291     | 0.00463 |
| hsa-miR-4540     | 2.454396    | 0.039986|
| hsa-miR-4667-5p  | 1.752593    | 0.02491 |
| hsa-miR-548h-3p/hsa-miR-548z | 1.660791 | 0.030347|
| hsa-miR-183-3p   | 2.171599    | 0.020913|
| hsa-miR-4522     | 2.317606    | 0.047429|
| hsa-miR-548t-5p  | 1.306963    | 0.040787|
| hsa-miR-3922-3p  | 1.762495    | 0.047063|
| hsa-miR-4441     | 1.823415    | 0.032323|
| hsa-miR-337-3p   | 1.962377    | 0.008488|
| hsa-miR-5197-5p  | 2.819095    | 0.025433|
| hsa-miR-4540     | 2.454396    | 0.039986|
| hsa-miR-4667-5p  | 1.752593    | 0.02491 |

### Table 4

| miRNA            | Fold-change | P value |
|------------------|-------------|---------|
| hsa-miR-409-3p   | 0.281322    | 0.003373|
| hsa-miR-125a-5p  | 0.6731      | 0.044941|
| hsa-miR-548e     | 0.560181    | 0.029698|
| hsa-miR-5694     | 0.408008    | 0.021335|
| hsa-miR-559      | 0.611151    | 0.003017|
| hsa-miR-654-3p   | 0.198366    | 0.021035|
| hsa-miR-4780     | 0.126252    | 0.00205 |
| hsa-miR-4286     | 0.46205     | 0.029317|
| hsa-miR-337-5p   | 0.470205    | 0.019236|
| euv-miR-BART18-3p| 0.686011    | 0.00396 |
| hsa-miR-4722-5p  | 0.585287    | 0.019703|

### Table 5

| miRNA            | Healthy control | Patient with ITP | P value |
|------------------|-----------------|-----------------|---------|
| miR-155-5p       | 6.633 (4.03–9.53)| 3.390 (1.68–5.44)| 0.002866|
| miR-146b-5p      | 0.08 (0.07–0.11)| 0.015 (0.01–0.05)| 0.001374|
| miR-142-3p       | 0.36 (0.10–0.42)| 0.08 (0.05–0.22)| 0.000374|

Our previous studies confirmed that Treg cells were involved in the pathogenesis of ITP. We found that the number of Treg cells in peripheral blood of patients with ITP was significantly lower than that of normal control group, and the immunosuppressive function of Treg cells was significantly weakened. In this experiment, although 37 kinds of miRNAs were abnormally expressed, combined with previous literature, it was reported that miR-155-5p, miR-146b-5p, and miR-142-3p may be involved in regulating the development and function of Treg cells. On the other hand, as the limited sample size of patients, we initially selected miR-155-5p, miR-146b-5p, and miR-142-3p in patients with ITP for qPCR verification. We confirmed by qPCR that miR-155-5p, miR-146b-5p, and miR-142-3p were downregulated in Tregs of patients with ITP compared with healthy controls. miR-155 is located in exon 3 of chromosome 21 and is regulated by cytokines expressed by T cells, including IL-10 and TGF-β.

A suppressor of cytokine signaling (SOCS1) and signal transduction activated factor are targets of miR-155. Kohlhass reported that miR-153-deficient mice have reduced numbers of Tregs, both in the thymus and periphery, because of impaired development and increasing miR-155 expression.
level that contributes to enhancing the immunosuppressive function of Treg. These findings indicate that miR-155 contributes to the function and development of Treg. We found that miR-155 was significantly downregulated in Treg, which may lead to Treg dysfunction and contribute to ITP pathogenesis. miR-146 is composed of miR-146a and miR-146b, which locate on chromosomes 5 and 10, and have only two bases different between them. Therefore, it is not surprising that their mechanism of action and target gene are similar. miR-146 inhibits tumor necrosis factor receptor-related factor 6 and interleukin-1 receptor-associated kinase 1 at a post-transcriptional level through NF-κB signaling pathways and negatively regulates immune signal transduction. miR-146a contributes to the development of Treg and to maintaining their immunosuppression function. We found that miR-146a is significantly decreased in peripheral blood cells from patients with ITP. We know that in patients with ITP, T helper Type 1 (Th1) cells are in a polarization mode, and damaging Treg function results in that in patients with ITP, T helper Type 1 (Th1) cells are in a polarization mode, and damaging Treg function results in...
In silico mapping of polymorphic miRNA-mRNA interactions in autoimmune thyroid diseases. *Autoimmunity* 2014;47:327–33.

Yao R, Ma YL, Liang W, et al. Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents. *Blood* 2010;116:4639–45.

De Santis S, Ferracin M, Biondani A, et al. Altered miRNA expression in T regulatory cells in course of multiple sclerosis. *J Neuroimmunol* 2010;226(1-2):165–71.

Cui Y. In silico mapping of polymorphic miRNA-mRNA interactions in autoimmune thyroid diseases. *Autoimmunity* 2014;47:327–33.

Yao R, Ma YL, Liang W, et al. MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. *PLoS One* 2012;7:e46082.

Singh Y, Garden OA, Lang F, et al. MicroRNA-15b/16 Enhances the Induction of Regulatory T Cells by Regulating the Expression of Rictor and mTOR. *J Immunol* 2015;195:5667–77.

Li S, Fan Q, He S, et al. MicroRNA-21 negatively regulates Treg cells through a TGF-β1/Smad-independent pathway in patients with coronary heart disease. *Cell Physiol Biochem* 2015;37:866–78.

Borjigin M, Hou W, Gong C, et al. Profiling of miRNA expression in immune thrombocytopenia patients before and after Qishunbaolier (QSBLE) treatment. *Biomed Pharmacother* 2015;75:196–204.

Bay A, Cossu E, Gotuzzo S, et al. Plasma microRNA profiling of pediatric patients with immune thrombocytopenic purpura. *Blood Coagul Fibrinolysis* 2014;25:379–83.

Li H, Zhao H, Xue F, et al. Reduced expression of MiR409-3p in primary immune thrombocytopenia. *Br J Haematol* 2013;161:128–35.

Cheung ST, So EY, Chang D, et al. Interleukin-10 inhibits lipopolysaccharide induced miR-155 precursor stability and maturation. *PLoS One* 2013;8:e71336.

Das LM, Torres-Castillo MD, Gill T, et al. TGF-β conditions intestinal T cells to express increased levels of miR-155, associated with down-regulation of IL-2 and rtk mRNA. *Mucosal Immunol* 2013;6:167–76.

Lu C, Huang X, Zhang X, et al. miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27Kip1, KIF1C, and SOCS-1. *Blood* 2011;117:4293–303.

Kohanbash G, MicroRNAs OH, and STAT Interplay. *Seminars in cancer biology* 2012;22:70–5.

Kohlihas S, Garden OA, Scudamore C, et al. Cutting edge: the FOXP3 target miR-155 contributes to the development of regulatory T cells. *J Immunol* 2009;182:2578–82.

Stanczyk J, Pedrioli DM, Brentano F, et al. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum* 2008;58:1001–9.

Liu R, Liu C, Chen D, et al. FOXP3 Controls an miR-146/NF-kB Negative Feedback Loop That Inhibits Apoptosis in Breast Cancer Cells. *Cancer Res* 2015;75:1703–13.

Lu LF, Boldin MP, Chaudhry A, et al. Function of miR-146a in controlling T cell-mediated regulation of Th1 responses. *Cell* 2010;142:914–29.

Hurst DR, Edmonds MD, Scott G, et al. Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. *Cancer Res* 2009;69:1279–83.

Riesco-Eizaguirre G, Wert-Lamas L, Perales-Patón J, et al. The miR-146b-3p/PAX8/NIS Regulatory Circuit Modulates the Differentiation Phenotype and Function of Thyroid Cells during Carcinogenesis. *Cancer Res* 2015;75:4119–30.

Walker SR, Xiang M, Frank DA. STAT3 Activity and Function in Cancer: Regulation by STAT5 and miR-146b. *Cancers* 2014;6:958–68.

Bissels U, Wild S, Tomiuk S, et al. Combined characterization of microRNA and mRNA profiles delinates early differentiation pathways of CD133+ and CD134+ hematopoietic stem and progenitor cells. *Stem Cells* 2011;29:847–57.

Flamant S, Ritchie W, Guilhot J, et al. Micro-RNA response to imatinib mesylate in patients with chronic myeloid leukemia. *Haematologica* 2010;95:1325–33.

Huang B, Zhao J, Lei Z, et al. miR-143-2p restricts CAMP production in CD4+CD25- T cells and CD4+CD25+ TREG cells by targeting AC9 mRNA. *EMBO Rep* 2009;10:180–5.

Buckner JH. Mechanisms of impaired regulation by CD4+CD25+FOXP3+ regulatory T cells in human autoimmune diseases. *Nat Rev Immunol* 2010;10:849–59.