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*Original Article*

# The increased expression of miR-146 predicts poor prognosis of immune thrombocytopenia

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**Abstract:** Background: Previous studies showed that megakaryocytes from normal umbilical cord blood displayed some differences when cultured with or without immune thrombocytopenia (ITP) plasma. MicroRNA-146 level in megakaryocytes increased significantly after incubation with ITP plasma. Methods: The prognostic value of miR-146 in ITP was analyzed. Plasma samples were collected from 56 ITP cases and 30 healthy individuals. Megakaryocytes were isolated from bone marrow obtained from the 56 ITP patients and 15 bone marrow donors. Real-time quantitative PCR was employed to measure miR-146 expression in plasma and megakaryocytes. The co-relations between miR-146 level and clinicopathological characteristics of ITP patients were also evaluated. Results: MicroRNA-146 levels in plasma and megakaryocytes were significantly higher in samples from ITP patients compared with control values. What's more, high miR-146 expression was closely correlated with platelet count, lymphocyte count, response to steroid, infection ratio and visceral hemorrhage ratio. Conclusion: Results indicate that miR-146 upregulation may serve as a novel molecular marker to predict unfavorable prognosis in ITP patients.

**Keywords:** Immune thrombocytopenia, MicroRNA-146, prognosis

## Introduction

Adult immune thrombocytopenia (ITP) is an autoimmune disorder caused by autoantibodies raised against several platelet membrane glycoproteins. This disorder leads to inhibition of megakaryocyte proliferation, platelet maturation and destruction within the reticular endothelial system [1-3]. ITP predominantly affects young women. The overall incidence is significantly higher in women compared to men. Although severe bleeding and infection is uncommon in ITP patients, the hazard ratio for mortality among them is about 1.6 (95% CI: 1.3-1.9) compared to age- and sex-matched individuals [4]. Diagnosis of ITP is based on the exclusion of other possible underlying causes of thrombocytopenia. Only platelet count is used as a conventional monitoring marker for treatment efficacy. Bone marrow tests are carried out with platelet count < $30 \times 10^9$/L and various bleeding risk factors. The present study aimed to identify an index that can serve as a good marker for monitoring the progress of ITP.

MicroRNAs (miRNAs) constitute an abundant class of small (17-25 nucleotides), endogenous, non-coding RNAs; their primary roles being to regulate the translation of many genes, they are involved in a variety of cellular processes, including cell proliferation, differentiation, apoptosis, and immune processes [5-7]. Multiple studies have shown that dysregulated miRs are involved in various diseases, making them interesting biomarkers. However, the role of miRs in ITP remains largely unknown. Our previ-

**Table 1.** Correlation between the clinicopathologic characteristics and miR-146 expression in ITP (n = 56)

| Patients Character | | Low miR-146 group | High miR-146 group | *P* value |
|---|---|---|---|---|
| Gender | Male | 1 | 2 | 0.983 |
| | Female | 18 | 35 | |
| Age | < 14 | 4 | 10 | 0.632 |
| | ≥ 14 | 15 | 27 | |
| Platelet count | < 20 × $10^9$/L | 6 | 33 | 0.001 |
| | ≥ 20 × $10^9$/L | 13 | 4 | |
| Leucocyte count | < 2.0 × $10^9$/L | 6 | 15 | 0.651 |
| | ≥ 2.0 × $10^9$/L | 13 | 22 | |
| Absolute lymphocyte count | < 1.0 × $10^9$/L | 5 | 17 | 0.018 |
| | ≥ 1.0 × $10^9$/L | 14 | 18 | |
| Hemoglobin | < 80 × $10^9$/L | 13 | 21 | 0.652 |
| | ≥ 80 × $10^9$/L | 6 | 16 | |
| Seriuos Infection☐ | Positive | 13 | 21 | 0.031 |
| | Negative | 6 | 16 | |
| Visceral hemorrhage | Positive | 6 | 23 | 0.030 |
| | Negative | 13 | 14 | |
| Response to steroid treatment | CR☐ and R§ | 12 | 13 | 0.047 |
| | NR☐ | 7 | 24 | |

☐Seriuos Infection is defined as antibacterial more than 10 days, or deep-seated fungal infection; ☐CR means complete response defined as any platelet count of at least 100 × $10^9$/L; §R means response defined as any platelet count between 30 and 100 × $10^9$/L and at least doubling of the baseline count. ☐NR means no response defined as any platelet count lower than 30 × $10^9$/L or less than doubling of the baseline count [9].

ous study indicated that the expression levels of certain viral miRNAs in normal megakaryocytes are increased after treatment with plasma from ITP patients [8]. Thus, we analyzed the correlation between miR-146 levels and ITP prognosis.

## Material and methods

*Patients and tissue samples*

This study was approved by the Research Ethics Committee of the Zhujiang Hospital and Sun Yat-sen Memorial Hospital. Written informed consent was obtained from all patients. All specimens were handled in a blinded manner according to ethical and legal standards. Plasma and bone marrow tissues were obtained from patients who underwent treatment or health examination in Hematology Department of Zhujiang Hospital or Sun Yat-sen Memorial Hospital, from 2008 to 2013. Plasma was isolated by centrifugation and stored at -80°C until use. Megakaryocytes were isolated from bone marrow using CD41 immuno-magnetic beads. The patients were staged according to the American Society of Hematology Criteria, and received corticosteroids as a first-line treatment. In most cases, this consisted of a standard dose of methylprednisolone (1-2 mg/kg/day), while some cases with severe thrombocytopenia received high-dose steroids (dexamethasone 40 mg/day for 4 days or methylprednisolone 15 mg/kg/day for 4 days). Besides standard steroid therapy, intravenous immunoglobulin (1 g/kg for 2 days), azathioprine, and danazol were used as first-line treatment in combination with steroids or as second-line management. Cyclosporine, cyclophosphamide, vincristine, and rituximab were used as salvage therapy. The clinicopathological information of patients is shown in **Table 1**.

*Evaluation of miR-146 expression by quantitative RT-PCR*

Total RNA was extracted from plasma and megakaryocytes by TRIzol Reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Primers for miR-146 and the endogenous control U6 were purchased from Jima Com (Shanghai City, China). RNA concentration and purity were determined spectrophotometrically using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Delaware, USA). cDNA was generated using the PrimeScript RT reagent kit (Takara Co. Ltd, Dalian, China) in a 20 μl final reaction volume containing 0.5 μg of RNA, 0.5 μl Prime-Script RT enzyme mix, 4 μl 5 × PrimeScript buffer, and 1 μl RT primer, at 42°C for 60 min and 85°C for 5 min. Quantitative real-time PCR was performed to evaluate miR-146 expression using SYBR Premix Ex Taq (Takara Co. Ltd) on

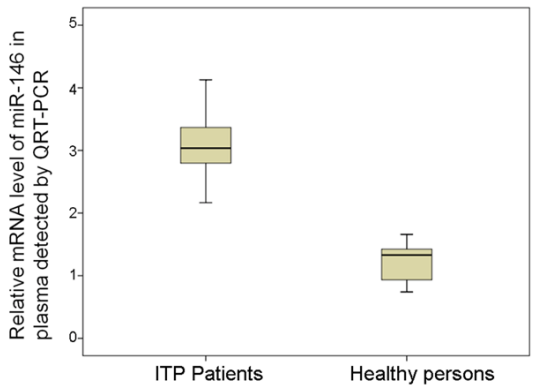


**Figure 1.** Comparison of miR-146 expression levels in plasma between ITP patients and healthy individuals.

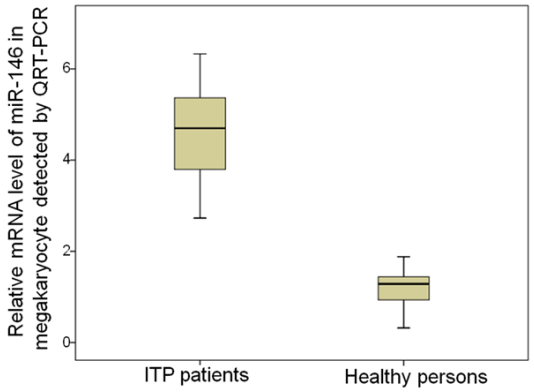


**Figure 2.** Comparison of miR-146 expression levels in megakaryocyte between ITP patients and healthy donor.

LightCycler 480 System (Roche, Basel, Switzerland). Amplification was carried out at 95°C for 10 min (initial denaturation), followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Relative quantification of miRNA expression was performed using the $2^{-\Delta\Delta CT}$ method. Raw data were presented as relative target miRNA levels, normalized to U6 and relative to a calibrator sample.

*Statistical analysis*

All computations were carried out using the software of SPSS version 18.0 for Windows (SPSS, Inc, Chicago, IL). Data were expressed as mean ± SD. We analyzed the difference of the miR-146 level in both plasma and megakaryocyte between ITP patients and control using independent-samples T test. By means of the multivariable correlation analysis were used to evaluate the statistical differences of all the clinicopathological characteristics between the high miR-146 group and low miR-146 group. Differences were considered statistically significant when *P* was less than 0.05.

## Results

*The expression of miR-146 in plasma from ITP patients*

MiR-146 expression levels were detected in plasma samples from 56 ITP patients and 30 healthy individuals. As shown in **Figure 1**, miR-146 levels in plasma specimens from ITP patients were significantly higher compared with those from healthy individuals (1.19 ± 0.27 vs. 3.01 ± 0.66, P < 0.0001) after normalization to U6 expression.

*The expression of miR-146 in megakaryocyte from ITP patients*

MicroRNA-146 expression was detected in megakaryocytes from 56 ITP patients and 15 bone marrow donors. As shown in **Figure 2**, miR-146 expression levels in megakaryocytes from ITP patients were significantly higher compared with those from donors (1.25 ± 0.27 vs 4.63 ± 0.92, P < 0.0001) after normalization to U6 expression. In ITP patients, miR-146 levels in megakaryocytes were about 5-10% higher than those in plasma samples. However, the same trend was observed for various patients: the higher the miR-146 level in megakaryocyte, the higher the amount in plasma.

*Association of miR-146 expression with clinicopathological characteristics of ITP patients*

Routine blood examination is a common monitoring tool for ITP. To evaluate the mean miR-146 level for ITP, plasma was selected as classification index system. Accordingly, the median fold change of miR-146 expression was used as a cutoff value to divide the 56 patients into two groups: the low (n = 37) and high (n = 19) expression groups included patients with levels below and equal or above median fold change, respectively. **Table 1** summarizes the association between miR-146 expression and the clinicopathological characteristics of ITP patients. Interestingly, high miR-146 expression level was closely correlated with platelet count (P =

0.018), lymphocyte count (P = 0.019), infection ratio (P = 0.031) visceral hemorrhage (P = 0.030), and response to steroid treatment (P = 0.047). In contrast, no statistically significant association was found between miR-146 expression and other clinical factors, such as gender, age, leucocyte count, lymphocyte count and hemoglobin level (all P > 0.05). Finally, miR-146 upregulation was associated with poor prognosis in ITP patients.

## Discussion

Immune thrombocytopenia (ITP) is a common autoimmune bleeding disorder, in which platelet glycoproteins are targeted by autoantigens. The guidelines (GL) endorsed by the American Society of Hematology (ASH) defined primary vs secondary ITP, with a clear distinction between the different phases of disease newly diagnosed, persistent, and chronic (after 12 months from diagnosis) [10]. Primary ITP identifies an autoimmune disorder characterized by an isolated platelet count < $100 \times 10^9$/L, with or without bleeding manifestations; secondary ITP includes all other forms of immune thrombocytopenia. Severe ITP indicates cases with bleeding symptoms at onset, requiring therapeutic intervention, or those with development of new bleeding symptoms demanding additional therapies with different drugs or a higher dose. For refractory ITP, two criteria have to be fulfilled simultaneously: (1) the lack of response or relapse after splenectomy with severe ITP or a bleeding risk requiring treatment according to the glycoprotein (GP); (2) temporary response to corticosteroids or to intravenous immunoglobulins does not exclude a refractory form. ITP is a diagnosis of exclusion; at present there are gold standards. The current guidelines suggest that bone marrow examination "is not necessary" for children and adolescents in cases showing typical ITP features, even in those not responding to intravenous immunoglobulins (IVIg); in addition, they propose bone marrow evaluation is not necessary before starting treatment with corticosteroids or before splenectomy. Bone marrow examination is only carried out for patients older than 60, before splenectomy in adults, before corticosteroid administration in children, and for subjects unresponsive to IVIg. MicroRNAs are abundantly present in plasma in a remarkably stable form. Circulating miRs are increasingly reported as new biomarkers in multiple diseases [11-13]. In a previous research, we added plasma samples from adult patients with acute ITP and healthy volunteers to umbilical cord blood mononuclear cell cultures. By microarray and qRT-PCR, we found not only 14 upregulated viral miRs, but also some induced hsa-miRs, e.g. miR-146 that was increased by 2.99 fold. Therefore, we hypothesized that miR-146, an endogenous miR, would be a marker for ITP. MicroRNA-146 is involved in cell differentiation and development. Xiao et al. reported that overexpression of miR-146 induces the differentiation efficiency of glial cells from neural stem cells (NSCs) [14]. In addition, miR-146 family members are also involved in DC cell differentiation and maturation [15, 16]. As monocytes differentiate into imDCs and mDCs, the expression levels of miR-146a and miR-146b increase significantly. Furthermore, miR-146 modulates DC apoptosis and cytokine production through the miR-146a/b-TRAF6/IRAK1-NF-κB axis. Consequently, miR-146a and/or miR-146b silencing in imDCs and mDCs significantly prevents DC apoptosis, whereas miR-146a and/or miR-146b overexpression induces DC apoptosis. A previous study proposed miR-146 to be a biomarker for inflammation and cancer [17]. Here, we detected miR-146 levels in plasma samples from 30 healthy individuals, megakaryocytes from 15 bone marrow donors, and plasma specimens and megakaryocytes from 56 ITP cases, by qRT-PCR. We found that miR-146 levels in both plasma and megakaryocytes from ITP patients were significantly higher compared with those from healthy individuals. Additionally, high miR-146 expression was closely correlated with gender, platelet count, and bleeding symptoms. In the high miR-146 group, 89.2% (33/37) of patients had platelet count below $20*10^9$/L, with 62.2% (23/37) suffered visceral hemorrhage. All of indicators are significant higher than those of the low miR-146 group. Most noteworthy is the incidence of infection. Not only the bacterial infection ratio, but also the fungal infection ratio is increasing year by year. In our study, one 44 year female of high miR-146 group died for serious pulmonary fungal infection, who just reveived 3 month of standard dose of methylprednisolone. She was nonlocal patient, after treated with methylprednisolone for 15 d, the platelet up from $1.7*10^9$/L to $4.3*10^9$/L, and go home. She detected the blood routine examination twice a week. At the

end of the second month, she had petechiae and ecchymoses all over the body and the platelet count decreased gradually. As her rehospitalization, we found although she had no sputum-coughing without fever, her was increasing shortness of breath. The CT scan of the chest shew bilateral lungs ground-glass and tree-in-bud opacities. Although Micafungin was started in the hospital the next day, her condition appeared to deteriorate rapidly. The patient's antibody of CMV is also positive. Only after 15 days of comprehensive therapy, the patient died due to breathing failure. ITP has previously been shown to be associated with an increased risk of infection relative to normal populations. Maybe the miR-146 is a biomarker to predicted the risk of infection in ITP patients.

In this retrospective study, our observations suggest that circulating miR-146 may be useful to serve as sensitive biomarkers for predicting the progress of ITP.

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## Disclosure of conflict of interest

None.

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