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Aberrant expression of RUNX3 in patients with immune thrombocytopenia

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ABSTRACT

Immune thrombocytopenia (ITP) is an autoimmune disease, characterized by dysregulation of cellular immunity. Previous studies demonstrated that immune imbalance between Th1 and Th2 was associated with the pathogenesis of ITP. Runt-related transcription factor 3 (RUNX3) is a member of the runt domain-containing family of transcription factors and plays an important role in the regulation of T cell differentiation into Th1 cells. Whether RUNX3 was involved in the pathogenesis of ITP remains unclear. In this study, 47 active ITP patients, 18 ITP with remission and 26 age and gender matched healthy control were included. Peripheral blood mononuclear cells (PBMCs) were isolated from ITP and control for isolation of RNA and plasma which were used to measure mRNA level of RUNX3 and T-box transcription factor (T-bet) by quantitative real-time PCR and interferon γ (IFN-γ) plasma level by ELISA. Meanwhile, protein was also extracted from PBMCs for Western blot analysis of RUNX3 expression. Our results showed a significantly higher expression of RUNX3, T-bet and plasma level of IFN-γ in active ITP patients compared to control. No differences were observed between ITP with remission and control. Furthermore, a positive correlation of RUNX3 with T-bet was found in active ITP patients. In conclusion, aberrant expression of RUNX3 was associated with the pathogenesis of ITP and therapeutically targeting it might be a novel approach in ITP treatment.

1. Introduction

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disease, which is characterized by accelerated platelet destruction and impaired platelet production, leading to thrombocytopenia (low platelet counts) which may have a propensity to bleeding [1,2]. The pathophysiology of ITP is becoming more and more complicated, with lots of factors involved. Several studies demonstrate that ITP is mainly due to immunoglobulin G (IgG) autoantibodies opsonizing individual's platelets, leading to markedly enhanced Fc receptor (FcR)-mediated phagocytosis and destruction by macrophages in the reticuloendothelial system in the spleen [3].

T cells are known to play a critical role in regulating immune response towards disease as demonstrated by the closely association of dysregulation of T cell activity and cytokine abnormalities with several autoimmune diseases, including ITP [4,5]. Platelet autoreactive T cells in ITP are demonstrated to be less apoptotic and more clonal expansion, leading to an imbalance of cytokines and subsequent reduced expression of Tregs with abnormal function, which are important in controlling appropriate immune response and autoreactivity [6–8]. Among T cell subtypes, T helper cells (Th cells) are shown to play a critical role in regulation of immune system, particularly in adaptive immune system, possibly through secretion of T cell-related cytokines [9]. Meanwhile, Th cells are also essential in B cell antibody class switching [10]. Th1 and Th2 are two major subtypes of Th cells, with the former characterized by secreting IFN-γ cytokine (pro-inflammatory) and latter by secreting IL-4 cytokine (anti-inflammatory) [9,11]. Imbalance of Th1/Th2 has been reported to be associated with the pathogenesis of ITP, with Th1-polarized immune response [12,13,3].

Runt-related transcription factor 3 (RUNX3) is a member of the runt domain-containing family of transcription factors [14]. RUNX proteins are a family of evolutionarily conserved heterodimeric transcription factors that have critical roles during the development of immune system [14] and many tissues [15,16]. The role of RUNX proteins in

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the regulation of T-cell development was first described as sequential interaction of RUNX3 with the Cd4 silencer. The interaction region is a cis-acting element in Cd4 locus which restricts CD4 expression to appropriate thymocyte populations independent of its position and orientation [17,18]. Beyond its role in the regulation of stage-specific CD4 expression, RUNX3 have also been shown to be involved in Th cells differentiation [19,20].

Considering Th1 polarization in the pathogenesis of ITP and RUNX3 in Th1 cells differentiation, whether RUNX3 was associated with the development of ITP remains unclear. In this study, we aimed to evaluate the expression profile of RUNX3, T-bet as well as plasma level of IFN-γ in 47 patients with active ITP, 18 ITP patients with remission and 26 healthy individuals.

2. Materials and methods

2.1. Patients

From October 2012 to October 2014, 47 primary ITP patients (13 males and 34 females with a median age of 42, ranged from 8 to 78 years old) (Table 1) from Department of Hematology, the Affiliated Hospital of Xuzhou Medical College, Xuzhou China were enrolled in this study. The diagnosis of ITP was based on the criteria from international working group [21]. These 47 diagnosed ITP patients with a platelet count of $<50 \times 10^9$/L were defined as active ITP, who had a median platelet count of 15×10^9/L, ranged from 1 to 49×10^9/L (Table 1). Patients with hypertension, cardiovascular diseases, diabetes, active or chronic infection were excluded. Eighteen ITP patients (4 males and 14 females with a median age of 44 ranged from 14 to 81 years old) (Table 1) achieved remission after treated with glucocorticoid, prednisolone or intravenous immunoglobulin, including complete response and response after treatment. According to the consensus report of international working group [21], complete response is defined as any platelet count of at least 100×10^9/L and response is defined as any platelet count between 30 and 100×10^9/L and at least doubling of the baseline count. These 18 ITP patients in remission had a median platelet count of 103×10^9/L ranged from 50 to 187×10^9/L. Meanwhile, 26 age and gender matched healthy individuals, consisted of 9 males and 17 females with a median age of 44 ranged from 6 to 84 years old, were served as a control (Table 1). The platelet counts in healthy individuals ranged from 129 to 300×10^9/L with a median count of 233×10^9/L. Ethical approval for this study was obtained from the Medical Ethics Committee of the Affiliated Hospital of Xuzhou Medical College, Xuzhou China. Informed consent was obtained from all participants before enrollment in the study.

2.2. Plasma isolation

Venous whole blood was drawn from patients or control into tubes with EDTA as anti-coagulant. Plasma was obtained by centrifuging anti-coagulant blood at 2000 rpm for 20 min and stored at −80 °C until analysis.

2.3. Peripheral blood mononuclear cells isolation and RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from collected venous blood by gradient centrifugation on Ficoll-Paque Plus (Sinopharm Chemical Reagent Ltd., China) at 2000 rpm for 20 min. RNA was extracted from isolated PBMCs by using TRIZOL reagent (Life Technology, Carlsbad, CA, USA) according to manufacturer's instruction. RNA quantitation and quality was assessed by measuring the absorbance of RNA at 260 nm on a spectrophotometer and agarose gels, respectively.

2.4. Quantitative real-time PCR

Extracted RNA was reverse-transcripted to cDNA which was used for quantification of mRNA expression of RUNX3 and T-bet by real-time PCR with GADPH as internal control. Amplification was performed in triplicate on LightCycler® R480 II (Roche Life Science) in a total volume of 20 μl, including 10 μl SYBR Green qPCR Super Mix, 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 5 μl cDNA and 4 μl sterile water. The primers for RUNX3, T-bet and GADPH were listed in Table 2. The PCR reaction conditions were as follows: initial denaturation (95 °C for 5 min), 40 cycles of denaturation (95 °C for 20 s); annealing (60 °C for 15 s) and extension (72 °C for 15 s). The melting curve analysis of the amplification products was performed at the end of each PCR reaction. The relative mRNA expression of target genes was calculated by comparative Ct method which is performed by using the following formula: relative expression $= 2^{-\Delta\Delta}$Ct.

2.5. Western blot

Whole proteins were extracted from PBMCs of patients or control and were separated on 10% SDS-PAGE, followed by transferring to an NC membrane. The membranes were then blotted with mouse anti-human RUNX3 antibody (Cat No. 653601, BioLegend, San Diego, CA, USA). Bound antibody was visualized using HRP-conjugated anti-mouse secondary antibody and enhanced chemiluminescence.

2.6. Statistical analysis

Data was presented as mean ± SE. For comparison of relevant parameters among different groups, data were assessed by one-way ANOVA using GraphPad Prism software. For correlation, liner regression analysis was applied. P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. RUNX3 expression in ITP patients and control

Peripheral mononuclear cells, isolated from active ITP patients or ITP patients with remission were used for measuring expression level of RUNX3 by qRT-PCR and Western blot. As seen in Fig. 1A, a significantly higher expression level of RUNX3 was observed in active ITP patients

Table 1
Clinical characteristics of ITP patients and control.

	Active ITP	ITP in remission	Control
N	47	18	26
Male/female	13/34	4/14	9/17
Age (range)	42 (8–78)	44 (14–81)	44 (6–84)
Platelet count (range) ($\times 10^9$/L)	15 (1–49)	103 (50–187)	233 (129–300)

Table 2
Primers for quantitative real-time PCR.

Gene	Primer Sequence (5′–3′)	Length (bp)
RUNX3	F: TCTGTAAGGCCCAAAGTGGGTA R: ACCTCAGCATGACAATATGTCACAA	197
T-bet	F: CCCTTGGTGTGGACTGAGAT R: GTCGGTGTCCTCCAACCTAA	245
GADPH	F: TGAAGGTCGGAGTCAACGGATT R: CCTGGAAGATGGTGATGGGATT	225