Is There a Relationship Between CXCR4 Gene Expression and Prognosis of Immune Thrombocytopenia in Children?

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Abstract Immune thrombocytopenia (ITP) is a common autoimmune disorder characterized by decreased platelet count (thrombocytopenia) and bleeding symptoms due to production of autoantibodies against platelets. Chemokines are molecules inducing chemotaxis and play an important role in megakaryopoiesis, including CXCR4 chemokine receptor. CXCR4 is expressed on cells of megakaryocytic series, especially platelets, and triggers several mechanisms in these cells. The purpose of this study was to evaluate the pattern of CXCR4 gene changes upon diagnosis and after treatment and its comparison with laboratory findings in peripheral blood samples from newly diagnosed ITP patients. 35 newly diagnosed patients with ITP and 35 healthy controls were enrolled in this study. CXCR4 gene expression was investigated before and after treatment using real-time PCR. HPRT gene was used as the reference gene to calculate the expression rate of CXCR4 as CXCR4/HPRT ratio. CXCR4 gene expression upon diagnosis and after treatment in peripheral blood plasma of ITP patients showed a significant decrease in comparison with the control group while its expression did not change before and after treatment. No significant correlation was found between the expression of this gene and laboratory parameters. Due to unpredictable course of ITP in patients and the possibility of its progress to refractory form,

accurate choice of a biomarker is essential for evaluating prognosis and detection of resistant forms.

Keywords Immune thrombocytopenia · CXCR4 · IVIg · Methylprednisolone

Introduction

Immune thrombocytopenia (ITP) is a common autoimmune disorder, which is characterized by the production of autoantibodies against platelets, leading to thrombocytopenia and bleeding in patients [1]. Based on the presence of underlying disease, ITP is classified to primary and secondary forms. Primary ITP (formerly known as idiopathic) is identified with thrombocytopenia without any known cause or underlying disease whereas secondary ITP is associated with presence of underlying diseases, such as systemic lupus erythematosus, *Helicobacter pylori* infection, lymphoproliferative disorders and chronic infections like HIV or hepatitis C [2].

Chemokines are small proteins inducing chemotactic activity and regulating the passage of cells by binding with their receptors on various leukocyte types. Based on the conserved cysteine motif, chemokines are divided to C, CC, CXC and CX3C families, among which CC and CXC chemokines have been extensively studied [3]. As a member of CXC family, CXCL12 and its ligand (CXCR4) participate in migration, homing, proliferation and survival of hematopoietic stem cells (HSC) [4]. For the first time, Wang et al. studied the megakaryocyte lineage from CFU-Meg to platelets, and observed the expression of CXCR4 in cells of megakaryocyte lineage. In their study, evaluation of this receptor expression by flowcytometry showed that CXCR4 is increased as maturity increase, and its

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expression becomes nearly uniform in final stages in circulating platelets, showing the highest expression level in circulating platelets. These observations suggest that CXCR4 is likely to be a mediator with several cellular functions in different stages of maturity, which is due to the different biological roles of CFU-Meg and circulating platelets [5]. A few studies have examined the impact of chemokine regulation in platelet disorders. The objective of this study was to evaluate the expression pattern of chemokine receptor CXCR4 in peripheral blood plasma of newly diagnosed ITP patients upon diagnosis and after treatment compared with control group using sensitive real-time quantitative PCR method. We showed that the expression level of this receptor was decreased upon diagnosis and after treatment in ITP patients.

Materials and Methods

Patients and Controls

Among patients with suspected platelet disorders referred to Shafa Hospital of Ahvaz Jundishapur University of Medical Sciences in Ahvaz, Iran, we chose 35 newly diagnosed ITP patients (14 male and 21 female). Peripheral EDTA blood samples (5 mL) were obtained from all of them in two stages, upon diagnosis and after treatment (3–5 days after the initial diagnosis). Diagnosis was based on elimination of other causes of thrombocytopenia (diseases such as HIV infection, lymphoproliferative disorders, *Helicobacter pylori* infection and Systemic Lupus Erythematosus) as well as morphological examination of peripheral blood (PB) and bone marrow (BM) films. ITP was confirmed by the presence of thrombocytopenia and the clinical criteria adopted by the physician. 35 individuals (with no hematopoietic disease) were also selected as control. This study was approved by the local ethics committee of Ahvaz Jundishapur University of Medical Sciences, and written informed consent was obtained from all the patients and normal controls.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from peripheral blood plasma using Quick-RNATM MiniPrep kit (Zymo Research, Germany) based on manufacturer's instructions. RNA was spectrophotometrically quantified at 260 nm. cDNA was synthesized from 2 µg of total RNA in 20 mL reaction mixture using AccuPowerCycleScript RT PreMix (dN12) (Bioneer, Germany) instrument according to the manufacturer's instructions. The mixture was incubated at 20 °C

for 30 s, 42 °C for 4 min and 55 °C for 30 s, followed by 95 °C for 5 min performed at eight cycles.

Quantitative Real-Time Polymerase Chain Reaction (Q-RT-PCR)

HPRT gene was used as a reference gene because it was validated according to our experimental conditions. In addition, its expression was in variant and stable by experimental factors. The use of reference gene as an internal control is essential for normalizing real-time data (mRNA concentration of target gene/reference genes), and it should be experimentally validated for specific cell types or tissues [6]. The expression level of genes was determined by Step one Plus Real-Time RT-PCR system (ABI, USA) using SYBR green master mix (Takara, Japan) according to the manufacturer's instructions. The following specific primers were used: CXCR4 forward (F): 5'-CGC CACCAACAGTCAGAG-3'; CXCR4 reverse (R): 5'-AAC ACAACCACCCACAAGTC-3' and HPRT1 F: 5'-CCTG GCGTCGTGATTAGTG-3'; HPRT1 R: 5'-TCAGTCCTG TCCATAATTAGTCC-3'. 20 µL PCR reaction mixture was prepared containing 4 µL SYBR green, 0.5 µL each primer, 2 µL cDNA and 13 µL DEPC water. CXCR4 RT-PCR was performed in 45 cycles, and each cycle consisted of 95 °C for 15 min, 95 °C for 10 s, 60° for 20 s and 72° for 20 s. RT-PCR data were analyzed by $2^{-\Delta\Delta CT}$ method and were presented as fold change in gene expression. The results were presented as relative index of CXCR4/HPRT1 ratio. All samples were tested in duplicate.

Statistical Analysis

Data were analyzed by SPSS software for windows v.13.0. Spearman's rho method was used to examine the correlation between quantitative data. Kruskal–Wallis test was used to compare quantitative data between three groups of ITP patients. Analysis of RT-PCR data was performed using REST Software (2009, QIAGEN, Valenica, USA). $p < 0.05$ was considered statistically significant.

Results

Patient Data upon Diagnosis

Among 35 patients evaluated in this study with an average age of 5.5 years (age range 1–15.5 years), 18 patients were from consanguineous marriages, of which two had a history of ITP in their family. BM smear of patients showed normal cellularity with increased megakaryocytes in 26 patients, while other nine patients had normal BM with no increase in megakaryocytes.

Patient Data After Treatment

Due to ITP heterogeneity, a range of therapies are available that target different aspects of its pathogenesis. These treatments include splenectomy, corticosteroids, immunoglobulins (IVIG and anti-D), cytotoxic drugs, anti CD-20 (Rituximab) and thrombopoietin (TPO) receptor agonist. The first-line therapy is commonly corticosteroid (prednisone, prednisolone, dexamethasone or methylprednisolone) or immunoglobulin (IVIG or anti-D) [7]. Given that all patients in our study were new cases, they were subject to first-line treatment and treatment period was 5–7 days. The response rate to treatment was assessed as follows, based on an increase in platelet count:

1. Patients with platelet counts less than $30 \times 10^3/\text{mm}^3$ or with two times increase in platelet count after therapy relative to original count with bleeding were considered as not response (NR).
2. Patients with platelet counts equal to or higher than $30 \times 10^3/\text{mm}^3$ or with at least two times increase in platelet count after therapy relative to original count without bleeding were considered as response (R).
3. Patients with platelet counts equal to or higher than $100 \times 10^3/\text{mm}^3$ without bleeding after therapy were considered as completely response (CR) [8].

CXCR4 Gene Expression Before Treatment Relative to Control Group

The expression level of CXCR4 gene was assessed in peripheral blood plasma samples from 35 newly diagnosed ITP patients. CXCR4/HPRT ratio was calculated for all patients compared to normal controls. Accordingly, CXCR4 gene expression before treatment was decreased in 15 patients and increased in one patient ($p < 0.05$), while the expression of this gene was not different from normal controls in 19 patients. Generally, the expression of CXCR4 in ITP patients was reduced upon diagnosis compared to control ($p < 0.05$) (Fig. 1).

CXCR4 Gene Expression After Treatment Compared with the Control Group

CXCR4 gene expression after treatment was evaluated in ITP patients relative to normal subjects, which was decreased in 22 patients and increased in 2 patients ($p < 0.05$). However, the expression of this gene was not different from control group in 11 patients. In general, the CXCR4 gene expression was reduced after treatment compared with the control group ($p < 0.05$).

Fig. 1 The overall levels of CXCR4 genes expression. 1 The overall decrease in CXCR4 gene expression before treatment, 2 the overall decrease in CXCR4 gene expression after treatment, 3 the overall levels of CXCR4 genes expression after treatment than before treatment

Comparison of CXCR4 Gene Expression Before and After Treatment

CXCR4 gene expression was not different in ITP patients before and after treatment in 29 patients, while decreased and increased expression was observed in 4 and 2 patients, respectively ($p < 0.05$). Finally, CXCR4 gene expression was not different before and after treatment. No significant correlation was found between CXCR4 gene expression and clinical and laboratory parameters before and after treatment (Table 1).

Discussion

ITP is a heterogeneous disorder with reduced platelet count due to accelerated immune destruction of platelets as well as defective platelet production by megakaryocytes [9]. The cause of ITP is not clear but involvement of multiple defects in immune system has been widely accepted in the development of the disease [10]. Survival, proliferation, differentiation and function of normal hematopoietic cells is negatively or positively regulated by various cytokines. Megakaryopoiesis and thrombopoiesis are complicated processes among the hematopoietic cell lineages [11]. While substantial progress has been made in understanding the mechanisms of thrombopoiesis regulation, signaling pathways initiating and regulating this process have not been well established [12]. Cytokines and chemokines play an important role in megakaryopoiesis, and exert their regulatory mechanisms in proliferation, differentiation and release of platelets [13]. Chemokines are a family of proinflammatory molecules that can be used as activators of platelet function [14, 15]. Several chemokines (CCL5, CCL17, CXCL4 and CXCL8) stored in high levels in platelet alpha granules, are released during platelet

Table 1 The correlation between the CXCR4 gene expression and the number of platelets at before and after treatment in ITP patients

	Platelets before treatment	Platelets after treatment
<i>CXCR4 genes expression before treatment</i>		
Correlation coefficient	−0.065	−0.172
<i>p</i> value	0.713	0.324

activation and act as autocrine factors, which represents the important role of chemokines in homeostasis and inflammation [16]. Chemokines and their receptors contribute to pathogenesis of these diseases by forming a complex network [3]. Moreover, there are several reports of expression of chemokine receptors on platelets, including CCR5, CXCR1, CXCR2 and CXCR4 receptors [17]. CXCR4 receptor and its ligand, Stromal cell-derived factor-1 (SDF1), are expressed on all cells of megakaryocytic lineage, showing increased expression with maturation [18]. Studies have shown that CXCR4 inhibition blocks normal megakaryopoiesis and thrombopoiesis, indicating the critical role of CXCR4 in these processes [19]. Several studies have examined the role of this chemokine receptor in various diseases, including systemic lupus erythematosus, HIV and hematologic malignancies such as acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), essential thrombocythemia (ET) and aplastic anemia. In all these studies, the importance of this chemokine in disease prognosis has been emphasized [20–22]. In the study of Ahn et al. [23], it was found that CXCR4 expression in AML patients is associated with poor prognosis. Despite many studies on the role of CXCR4 in various diseases, the effect of platelet disorders on regulation of chemokines has been rarely studied. Reduced expression of CXCR4 on platelets has been described in essential thrombocythemia patients [24].

Although CXCR4 is expressed on platelets and binds SDF1 with high affinity, no platelet activation or aggregation response is observed due to this binding [25]. Therefore, there are few evidences of biological CXCR4 expression on platelets. Several inflammatory factors have been studied in ITP but chemokines have been rarely considered in this disease. Given the important role of chemokines in megakaryopoiesis, more attention should be paid to the role and contribution of these molecules to ITP pathogenesis. In our study, reduced CXCR4 gene expression was observed in samples of newly diagnosed ITP patients before and after treatment compared to the control group, which was contrary to the results of the two previous studies. In the study of Jiaan et al., it was found that CXCR4 level on platelets of children with chronic ITP is increased compared with the control group, which leads to activation of AKT in platelets of patients with chronic ITP as well as resistance to apoptosis. In this study, an inverse relationship was found between the expression of

CXCR4 and platelet count, such that the patients with lower platelet counts showed a higher expression level of CXCR4 [26]. In addition, Olsson et al. [27] showed that the expression of CXCR4 and CX3CR1 chemokine receptors is increased on T-cells in BM of patients with chronic ITP but is not different with control group in peripheral blood T-cells of these patients. Disease phase may be a reason for the difference between our findings with previous studies. All our patients were newly diagnosed while all patients had chronic ITP in the two previous studies. Therefore, it can be concluded that CXCR4 level is likely different in acute and chronic ITP and also in different stages of disease progress. Genetic factors can also account for difference in our results because 18 patients in our study were from consanguineous marriages, including two patients with a history of ITP in their family. These two patients showed decreased expression of CXCR4 upon diagnosis and after treatment, which can indicate the impact of genetic factors on the difference between our results with previous studies, and additional studies are necessary to prove this hypothesis.

Overexpression of CXCR4 chemokine receptor is associated with maturation of megakaryocytes. Binding of this receptor to its ligand in megakaryocytes increases the migration of these cells from BM niche to vascular niche. Therefore, increased megakaryocytic maturation and platelet production [28]. SDF1-CXCR4 reaction leads to a large number of intracellular signals, including increased concentration of calcium ions, extracellular signal-regulated kinase (ERK1/2) phosphorylation, activation of phosphoinositide 3-kinase (PI3K) and increased activity of nuclear factor KB (NF-KB), which all indicate the physiological role of this receptor in megakaryocytic maturation and production of platelets (Fig. 2) [26]. In this study, the effect of current treatments on the expression of CXCR4 gene was studied in an attempt to find a correlation between the expression levels of this gene in plasma of patients with disease prognosis. After giving the standard dose of IVIg and corticosteroids to treat our patients, there was no significant relationship between the expression level of gene and prognosis of disease, while these treatments were effective in increasing the platelet count and improvement of clinical symptoms. In 11 patients, no difference was observed in CXCR4 expression levels upon diagnosis and after treatment while only one patient did not respond to treatment, and the other patients showed

Fig. 2 CXCR4 launched intracellular signaling pathways by its activated in Megakaryoblast. This receptor binds to its ligand, SDF-1, that trigger MAPK and PI3K/AKT ways activation and finally induced proliferation, resistance to apoptosis and survival in Megakaryoblast. Also CXCR4 lead to migration and maturation of megakaryocytes and ultimately production of platelets via NF-KB activation

complete response. Considering these results, lack of correlation between this chemokine and disease prognosis can be justified in this study. G protein-coupled receptors (GPCRs) family members are mediators of vital signaling pathways inside the cell and are crucial target drugs in pharmacology [29]. The drugs used in this study cause increased platelet count via decreased platelet clearance in peripheral blood. With regard to the role of CXCR4 as a member of GPCR family in megakaryopoiesis and increasing platelet production, so the use of agonists of this chemokine in combination with current treatments may develop more durable and appropriate treatment protocols for ITP patients in future. Among our patients, there was only one patient resistant to treatment in whom CXCR4 level before treatment was not different from control but was increased subsequent to treatment. According to the results of this study and considering the inverse relationship between CXCR4 level and platelet count in the study of Jiaan et al., low levels of this chemokine after treatment in all patients and its increase in a patient resistant to treatment can be justified. Analysis of the data from this study and results of previous studies indicating increased CXCR4 expression in chronic ITP patients, further studies seem to be necessary to introduce this gene as a biomarker for prognosis as well as detection of patients resistant to treatment with a high likelihood of disease chronicity.

In conclusion, as a result, better understanding of molecular factors involved in megakaryopoiesis raises them as proper prognostic factors for evaluating the effectiveness of treatment and likelihood of disease progress to chronic ITP. In addition, with regard to the presence of CXCR4 on all platelet precursors and the role of this chemokine in production and release of platelets into the bloodstream as well as its role in resistance to apoptosis

in patients with chronic ITP, it can be concluded that better recognition of this molecular marker can contribute to identification of patients resistant to treatment and probability of disease progress to chronic form.

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Conflict of interest The authors declare that they have no conflict of interest.

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