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RESEARCH ARTICLE

Evaluation of miR-21 and miR-150 expression in immune thrombocytopenic purpura pathogenesis: a case-control study

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BACKGROUND: Immune thrombocytopenic purpura (ITP) is a common autoimmune disorder diagnosed with thrombocytopenia and bleeding symptoms due to production of autoantibodies (Abs) against platelets. Nowadays, microRNAs are known as novel biomarkers for diagnosis of diseases. The aim of this study was to investigate the expression levels of miR-21 and miR-150 in ITP patients and determine the role of these miRNAs in ITP pathogenesis.
MATERIALS and METHODS: Thirty newly diagnosed patients with acute ITP and 30 healthy subjects(age and sex matched) as controls were enrolled in this study. The expression level of miR-21 and miR-150 was investigated using Real-time-PCR. Comparison of demographic characteristics of the cases was done using independent *t*-test and chi-square test. Comparison of the expression level of miR-21 and miR-150 with the related parameters was done using independent *t*-test or Mann–Whitney and Kruskal–Wallis test. Spearman rho correlation coefficient was used to investigate the relationship between the expression of miR-21 and miR-150 with demographic characteristics.
RESULTS: The expression of miR-21, 150 in the patients was not different compared with the control group in general. A significant relationship between the expression of miR-21 with hemoglobin, hematocrit and red blood cell hemoglobin concentration was observed.
DISCUSSION: Expression of miR-21 and miR-150 is not associated with pathogenesis of acute ITP and can involve the synergistic role of other miRNAs. Investigation of miR-21 and miR-150 expression along with other miRNAs and cytokines can be helpful in diagnosis and pathogenesis of ITP.

Keywords immune thrombocytopenic purpura, miR-21, miR-150

Introduction

Immune thrombocytopenia (ITP) is an acquired autoimmune disease in children and adults that is associated with impaired immune function and thrombocytopenia. Production of disease-causing autoantibodies (Abs) against platelet-specific antigens such as glycoprotein Ib/IX and IIb/IIIa is an important factor in development of this disease (Ku et al., 2013). These autoantibodies not only bind platelets but cause their destruction through different mechanisms like antibody-mediated and T cell-mediated platelet lysis as well as inhibiting the function of megakaryocytes (MKs) and reducing their survival, which results in decreased platelet production (Ku et al., 2013; Khodadi et al., 2016) . Acute ITP is initially started with an infection in children and spontaneous remission is achieved in the majority of cases within 6 months. In contrast, chronic ITP in adults has a typically insidious start and is not associated with infection or a history of underlying diseases (Heyns et al., 1986; Rank et al., 2010). New advances in epigenetics have detected molecular mechanisms such as microRNA in a variety of disorders and malignancies and their role has been demonstrated both in normal physiology and pathogenesis (Shah et al., 2009).

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MicroRNAs are a family of non-coding, small, 18-25 nucleotide, single-stranded RNA molecules regulating gene expression in two ways: targeting and degenerating mRNAs and affecting post-translation mechanisms in proteins (Anindo and Yaqinuddin, 2012; Zhu et al., 2013). MiRNAs play a vital role in megakaryopoiesis, platelet production, regulation of immune mechanisms and autoimmune diseases (Edelstein and Bray, 2011; Bay et al., 2014). MiRNA level specifically affects the expression of genes and proteins in ITP, indicating that this level of expression can play an important regulatory role in the absence of immune tolerance in ITP (Bay et al., 2014). MiRNAs can function both as oncogenes and tumor suppressors (Gordon et al., 2013).

Investigation of miRNAs involved in the immune system and autoimmune diseases may indicate that these miRNAs can cause antibody production by targeting the genes involved in pathogenesis and immune response in ITP patients. In fact, these antibodies are produced against platelets and MKs in ITP patients, and thus destroy them during differentiation into later stages (Fig. 1) (Stasi, 2012).

The expression of miR-21 as an oncogenic miRNA is increased in a large number of cancers, including breast and prostate cancers, leukemia, lymphoma and autoimmune diseases. This miRNA molecule plays a variety of roles in the cell (Dai et al., 2007; Rossi et al., 2010). Studies have shown that increased miR-21 expression is associated with poor prognosis in patients and is essential for malignant cell growth and proliferation pathways as well as cell survival. Other studies have also indicated its role in drug resistance in solid tumors (Rossi et al., 2010; Bai et al., 2011). Several miRNA types have been recognized in megakaryopoiesis and their important role in differentiation and lineage commitment of MKs has been indicated (Li et al., 2011). MiR-150 is a tumor suppressor miRNA specific for lymphoid series (B, T) that is expressed in mature B-and T cells causing increased activity, growth, and proliferation of these cells. This miRNA has several functions, including proliferation inhibition of malignant cells and induction of apoptosis in them. Reduced miR-150 expression in malignant cells has been observed in a large number of leukemias, indicating its tumor suppressor role (Ghisi et al., 2011). Studies show that miR-150 causes MK differentiation with a negative impact on C-myb protein (Li et al., 2011).

Increased or decreased expression of a large number of miRNAs has been identified in different cancers, including hematologic malignancies such as leukemia and lymphoma, which addresses miRNAs as important targets in diagnosis, prognosis and treatment of diseases (Babashah et al., 2012).

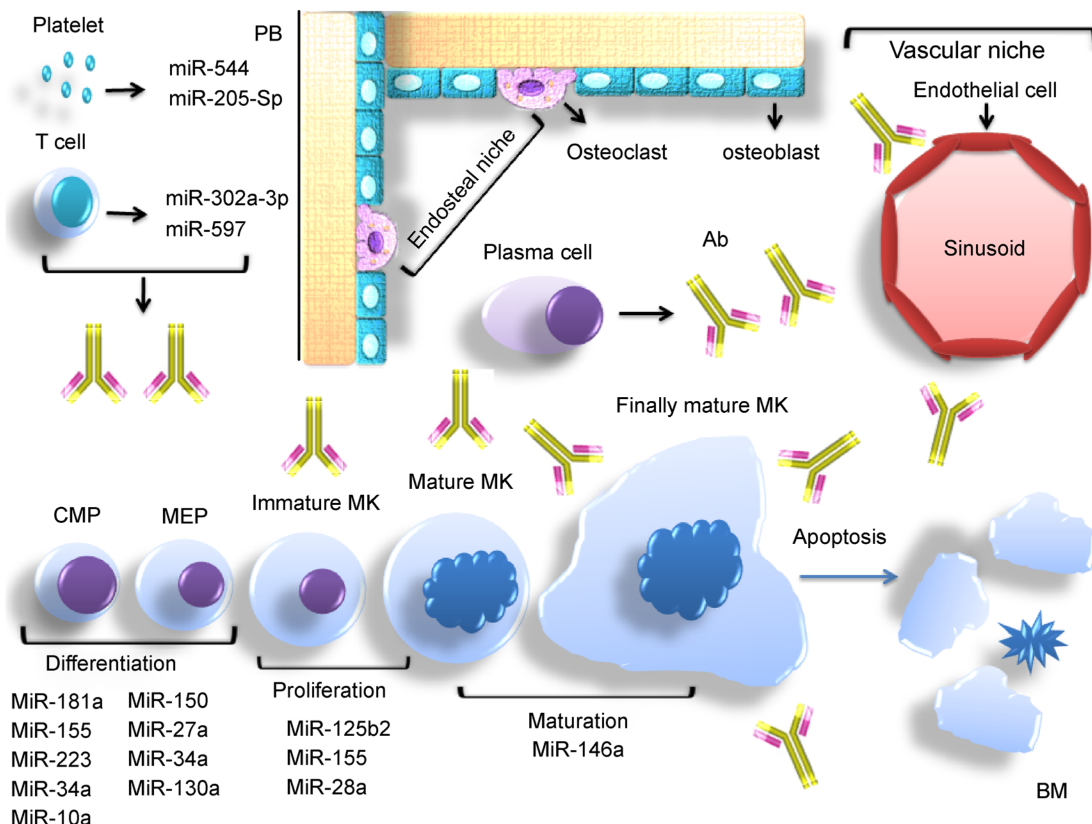

Figure 1 MiRNAs involved in pathogenesis and Megakaryopoiesis of ITP. MiRNAs can affect immune system to produce antibodies in ITP. Antibodies disturb MKs in BM and this leads to apoptosis of immature and mature MKs as well as thrombocytopenia in ITP patients. Abbreviations: IL, Interleukin; MK, megakaryocyte; CMP, common myeloid progenitor; MEP, MK-erythroid precursor; Ab, antibody; BM, bone marrow; PB, peripheral blood.

Few studies have been conducted to date to examine the expression level of miRNAs in ITP patients. So, in this study we examined the expression level of miR-21 and miR-150 in patients with acute ITP to evaluate the role of these miRNAs in ITP pathogenesis.

Materials and methods

Study group: patients and samples

Acute ITP diagnosis was based on guidelines of ITP International Working Group, as reported by Rodeghiero et al. (2009). ITP patients were diagnosed by thrombocytopenia (Peripheral blood platelet count < 100 × 10^9/L) and hyperplasia or normoplasia of megakaryocytes in BM and the absence of other causes of thrombocytopenia. After morphologic, clinical and bone marrow examinations on 30children with acute ITP 1 to 14 years, they were enrolled in this study before starting the treatment protocol. Five mL peripheral blood (PB) sample was collected from each patient in tubes containing EDTA anticoagulant. ITP patients included 17 males (56.66%) and 13 females (43.33%) (1-14 years old, median age: 3.07 years). All patients signed a written consent form according to declaration of Helsinki. Thirty age and sex-matched healthy control subjects with no morphologic and clinical disorders were also enrolled in this study. Patients were treated based on ITP protocol in Shafa Hospital in Ahvaz. All demographic, clinical and laboratory features of patients incorporated in this study, including WBC, RBC, PLT, hemoglobin (Hb), MCV, MCH, MCHC, reticulocyte, BUN, Cr, age, and sex are listed in Table 1. All PB samples were taken within four months with written informed consent was obtained from parents of all patients and normal individuals. This study was approved by the local ethics committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS.REC.1393.379) and was conducted within six months.

MiRNA extraction, cDNA synthesis and real-time PCR assays

After drawing blood samples and isolation of peripheral blood mononuclear cells (PBMC) by a Ficoll-Hypaque centrifugation gradient, mRNA was extracted according to isolation protocol of RIBO-Prep kit (Toronto, Canada). The quantification of extracted miRNAs was performed by measuring the absorbance at 260nm. cDNA synthesis was performed as follows: first, 1.5 µL of specific primer, 3 µL RNA and 15.5 µL distilled water were mixed. Then, the mixture was placed in ABI step one plus PCR instrument for 5 min in 95 °C and 10 min in 70 °C. After completion of this step, 3µL RT buffer, 3µL dNTP, 1µL RT enzyme and 3µL distilled water was added to this mixture for 15 min at 25 °C, 15 min at 37 °C, 60 min in 42 °C and 10 min at 75 °C in the instrument, respectively. MiRNA was quantified by realtime polymerase chain reaction (PCR) with 8 µL Fermentas SYBR Green Mastermix (Fermentas Life Sciences, St Leon-Rot, Germany) in a Rotor-Gene 6000 system (Corbett, Concorde, NSW, Australia) according to manufacturer's instructions. Then, 2 µL of template, 2 µL target specific stem loop primers and 8µL of distilled water were added. The reactions were performed as follows: initial polymerase activation at 95 °C for 15min. For miRNA quantification, 40 amplification cycles at 60 °C for 20 s, and 72 °C for 20 s with fluorescence detection. The test was performed in duplicate for each sample. In addition, we followed the minimum information for publication of quantitative Real-Time PCR experiments (MIQE) guidelines to achieve more reliable results (Bustin et al., 2009). The expression of target miRNA was relatively quantified using the comparative cycle threshold (CT) method. The raw data were presented as relative quantity of target miRNA, which was normalized with respect to snord. For miRNA, the data were normalized using the small nucleolar RNA, C/D box (snord) endogenous control. Primers for snord 47 were prepared based on the sequence derived from the study of Naderi and colleagues (Naderi et al., 2015). The primer sequences for miR-21,150 were used for cDNA synthesized synthesis from miRNA as shown in Table 2.

Statistical analysis

Comparison of the demographic characteristics of the cases was done using independent *t*-test and chi-square test. Comparison of expression levels of miR-21 with the related parameters was done using independent *t*-test or Mann–Whitney test. Comparison of expression levels of miR-150

Table 1 Demographic characteristics of ITP patients.

	Age	RBC	WBC	PLT	Hb	HCT	MCV	MCH	MCHC	Reticulocyte	BUN	Cr
N = 30												
Mean	3.7333	4.5007	9.4860	25.4000	9.8660	31.8633	75.5940	22.9190	31.2747	0.5967	11.7000	0.6667
Std. deviation	3.36240	0.85025	3.34521	14.93572	2.07791	4.43143	8.53696	3.33266	2.19811	0.17711	2.25755	0.08442

Abbreviations: WBC; white blood cell, RBC; Red blood cell , Hb; Hemoglobin, PLT; platelet, HCT; Hematocrit, MCV; mean cell volume, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentration, BUN; blood urea nitrogen.

Table 2 Primersequences of miR-21, 150 and Snord 47

Primer	sequence	Primer length(bp)
miR-21	Forward: 5'- ACGTGTTAGCTTATCAGACTG A-3'	21
	Reverse: 5'- GAGCAGGGTCCGAGGT-3'	16
	RT: 5'- GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACTCAACA-3'	51
miR-150	Forward: 5'- ACATCTCCCAACCCTTGTAC-3'	18
	Reverse: 5'- GAGCAGGGTCCGAGGT-3'	16
	RT: 5'-GGTCGTATGCAGAGCAGGGTCCGAGGTATCCATCGCACGCATCGCACTCATACGA CCCACTGG-3'	64
Snord 47	Forward: 5'- ATCACTGTAAAACCGTTCA-3'	19
	RT: 5'- GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACCACCTC-3'	51

with the related parameters was done using Kruskal–Wallis test. Spearman rho correlation coefficient was used to investigate the relationship between expression of miR-21, miR-150 and demographic characteristics. Relative quantification software (REST) (2009, QIAGEN, Valencia, USA) was used to analyze relative real-time PCR data (Pfaffl et al., 2002; Saki et al., 2014). REST analysis software was used with efficiency correction and the statistical method was as follows: $(Etarget)^{DCP}$ target (MEAN control – MEAN sample)/$(ERef\ index)^{DCP}$ Ref index (MEAN control – MEAN sample). $p < 0.05$ was considered statistically significant.

Results

Expression of miRNAs in ITP samples

Among 30 patients under study, the expression of miR-21 was decreased in 8 patients, whereas it was not different from control group in 22 patients (Fig. 2). Out of 30 patients evaluated, the expression of miR-150 was decreased in 6 patients, while it was increased in 5 patients. The expression level was not different from control group in 19 patients (Fig. 2). Overall, miR-21 and miR-150 expression showed no difference compared with control group (Fig. 3).

Correlation between miRNA expression and demographic, clinical and laboratory data

All patients were positive in terms of antibody presence in peripheral blood as well as direct and indirect Coombs tests. A significant relationship was observed between miR-21 expression in the two groups of low and equal expression with other laboratory parameters such as Hb, HCT and MCHC. However, no significant relationship was observed between miR-21 expression in both groups with other laboratory parameters ($p < 0.05$) (Table 3). Also, No significant correlation was seen between miR-21 expression with other laboratory parameters ($p < 0.05$) (Table 5).

No significant relationship was observed between miR-150 expression in three groups of low, equal and high expression with laboratory parameters ($p < 0.05$) (Table 4). Furthermore, no significant correlation was seen between miR-150 expression with other laboratory parameters ($p < 0.05$) (Table 6).

Discussion

ITP is a heterogeneous disorder associated with reduced platelet count due to accelerated immune-mediated destruction of platelets as well as defective platelet production by MKs (Johnsen, 2012). The causative agent of ITP is unknown, but the involvement of immune system in progression of this disease has been widely accepted (Zhou et al., 2005). Despite substantial progress in understanding the regulatory mechanisms of thrombopoiesis, the signaling pathways triggering and regulating this process are not fully understood (Machlus et al., 2014). MiRNAs play critical roles in megakaryopoiesis, platelet production, regulation of immunological functions and autoimmune diseases and there are several complexities concerning their role in megakaryopoiesis and pathogenesis of ITP (Edelstein and Bray, 2011).

In this study, we examined the expression level of miR-21 and miR-150 in ITP patients. Rossi et al. in 2010 investigated the expression level of miR-21 in CLL patients. They confirmed that the expression of miR-21 as an oncogene was significantly increased in patients, which was associated with poor prognosis and shorter survival (Rossi et al., 2010). Baya et al. in 2010 assessed the role of miR-21 expression in CML patients. They found that the miR-21 (as an oncogene) is significantly increased in CML patients and leads to drug resistance of K562 cells against daunorubicin (Bai et al., 2011). The role of miR-21 has been established in a number of autoimmune diseases. In a study by Dai et al. in 2007, the differences in expression of miRNAs, including miR-21, in autoimmune diseases of systemic lupus erythematosus (SLE) and ITP were discussed. Their study showed that miR-21 expression was decreased in SLE patients while it was not different between ITP patients and the control group (Dai et

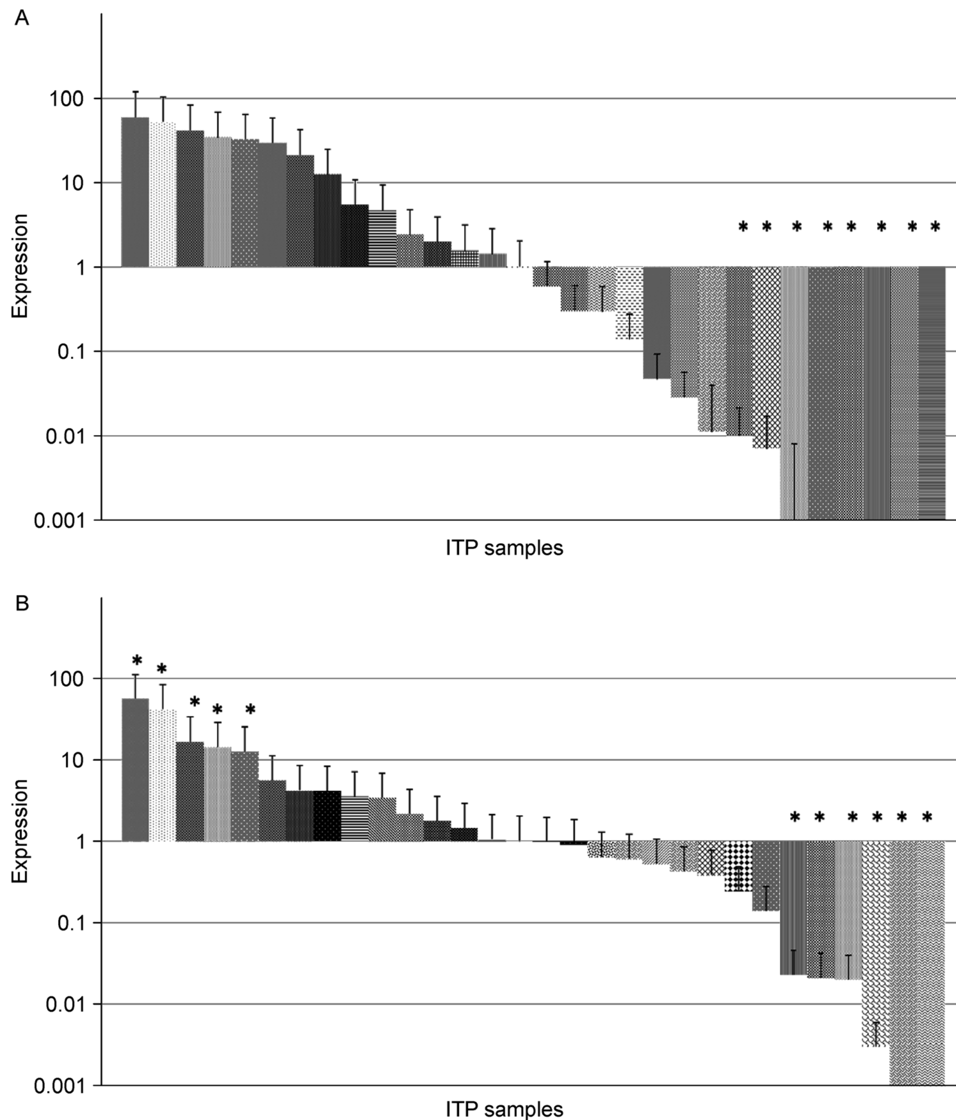

Figure 2 Expression analysis of miR-21 (A) and miR-150 (B) in 30 ITP samples using real-time PCR.

al., 2007). In this study, we examined the expression of miR-21 in ITP as an autoimmune disease. The patients were divided to two groups in terms of miR-21 expression: low and equal expression. Out of 30 patients evaluated, miR-21 expression was decreased in 8 patients, whereas it was not different from controls in 22 patients (Fig. 2). Then, the expression level of this miRNA was compared with other laboratory parameters of patients and a significant relationship was observed between the expression of miR-21 in the two groups of low and equal expression with laboratory parameters of Hb, HCT and MCHC (Table 3). Therefore, it can be stated that according to our study and Dai et al. study in 2007 on miR-21, the expression of this miRNA in autoimmune diseases like acute ITP is not altogether different from the control group (Fig. 3). Of course, miR-21 expression was decreased in 8 patients and may indicate a synergistic role of other factors on its expression level, including miRNAs, genes and cytokines and more investigation is needed for understanding of this synergistic role. The relationship between the expression level of miR-21 in the two groups with Hb, HCT and MCHC can indicate the expression role of this miRNA in increased hemolysis and reduced red blood cells due to presence of antibodies in these patients. Further investigation is required for understanding of this relationship.

Wang et al. in 2008 dealt with miRNA analysis (including miR-150) in CLL patients. They found that the expression level of miR-150 as a tumor suppressor was significantly reduced in these patients compared with the control group (Wang et al., 2008). In 2009, Husseinia et al. examined the expression level of miR-150 and miR-222 in MDS patients and found that miR-150 expression (as a tumor suppressor) was decreased and caused reduced proliferation of malignant cells and improvement of disease in these patients (Hussein et

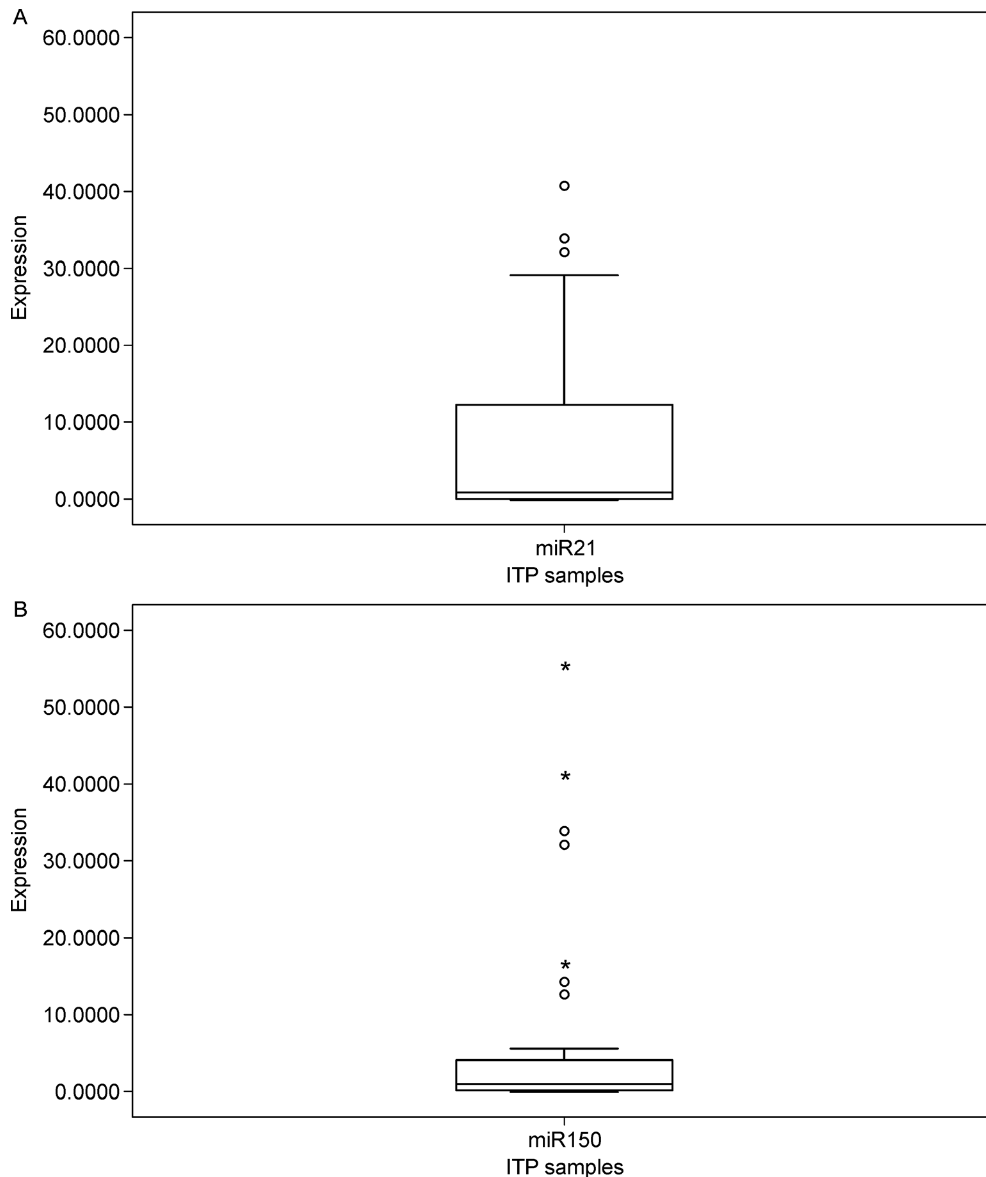

Figure 3 Relative expression levels of miR-21 (A) and miR-150 (B) in 30 ITP samples.

al., 2010). MiR-150 expression also plays an important role in differentiation of MK cells and platelet production (Li et al., 2011). In this study, we investigated the expression of miR-150 as an effective miRNA in megakaryopoiesis. Jernas et al. in 2013 analyzed the miRNAs involved in chronic ITP pathogenesis, including miR-150. They showed that the expression of miR-150 in patients was significantly decreased compared with the control group (Jernås et al., 2013). In this study, patients were divided to three groups in terms of miR-150 expression: decreased, increased and equal expression. Among the 30 patients evaluated, miR-150 expression was decreased in 6 patients, while increased expression of it was observed in 5 patients. The expression level was not different in 19 patients compared with controls (Fig. 2). Then, this miRNA was compared with other laboratory parameters of patients and no significant relationship was found between miR-150 expression with laboratory parameters. Therefore, compared to the survey conducted by Jernas et al. in 2013, the expression of this miRNA in our study was decreased only in 6 patients but was not different with controls in more than half of patients. As a result, different expression levels of this miRNA may indicate a synergistic role of other factors, including miRNAs involvement, immune system and other genes and cytokines in acute ITP patients.

The miRNA molecules involved in MK maturation are probably those affected through destruction of MKs by antibodies in final maturation stages of these cells in BM, causing reduced MK maturity in ITP patients. Therefore, we can say that their expression level is increased in ITP patients. Basically, these antibodies are effective upon final stages of MKs differentiation and cause their lysis (Stasi, 2012). So, it can be stated that the expression of miR-150, which causes

Table 3 Demographic characteristics in ITP patients according to miR-21 expression

miR-21		Age	RBC	WBC	PLT	Hb	HCT	MCV	MCH	MCHC	Reticu-locyte	BUN	Cr
DOWN regulation N = 8	Mean	4.0000	4.9625	8.3750	21.7500	11.5500	34.9750	74.4013	23.4838	32.8025	0.5875	11.1875	0.7125
	Std. deviation	2.26779	0.47352	2.27706	8.86002	1.72378	2.96106	7.83233	4.18101	2.33557	0.09910	1.30760	0.08345
ND N = 22	Mean	3.6364	4.3327	9.8900	26.7273	9.2536	30.7318	76.0277	22.7136	30.7191	0.6000	11.8864	0.6500
	Std. deviation	3.72310	0.90154	3.61717	16.58221	1.87054	4.37791	8.91380	3.05697	1.90825	0.20000	2.51629	0.08018
	p-value	0.19	0.13	0.13	0.99	0.003	0.021	0.42	0.34	0.045	0.70	0.34	0.11

Abbreviations: WBC; white blood cell, RBC; Red blood cell , Hb; Hemoglobin, PLT; platelet, ND; No difference, HCT; Hematocrit, MCV; mean cell volume, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentration, BUN; blood urea nitrogen.

Table 4 Demographic characteristics in ITP patients according to miR-150 expression

miR-150		Age	RBC	WBC	PLT	Hb	HCT	MCV	MCH	MCHC	Reticu-locyte	BUN	Cr
DOWN regulation N = 6	Mean	3.6667	5.1100	8.9667	23.0000	11.3500	34.9167	73.2900	22.3650	32.2767	0.5667	11.0833	0.7333
	Std. deviation	1.75119	0.45660	2.18967	4.89898	1.91807	3.36120	8.79344	4.18236	2.42765	0.10328	1.49722	0.08165
ND N = 19	Mean	3.8947	4.3368	9.1568	27.5789	9.8526	31.4211	76.8989	23.2305	31.1884	0.5632	11.8158	0.6632
	Std. deviation	4.10819	0.90387	3.85675	18.12094	1.51010	4.39578	8.32818	3.00683	2.21861	0.08951	2.35237	0.06840
Up regulation N = 5	Mean	3.2000	4.3920	11.3600	20.0000	8.1360	29.8800	73.4000	22.4000	30.4000	0.7600	12.0000	0.6000
	Std. deviation	1.30384	0.77777	1.70088	6.48074	3.06553	4.63325	9.87623	4.08840	1.73638	0.37148	2.91548	0.10000
	p-value	0.35	0.14	0.14	0.89	0.08	0.16	0.43	0.97	0.48	0.4	0.37	0.055

Abbreviations: WBC; white blood cell, RBC; Red blood cell , Hb; Hemoglobin, PLT; platelet, ND; No difference, HCT; Hematocrit, MCV; mean cell volume, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentration, BUN; blood urea nitrogen.

Table 5 Correlation between miR-21 expression and demographic characteristics of ITP patients

Log expression	Age	RBC	WBC	PLT	Hb	HCT	MCV	MCH	MCHC	Reticulo-cyte	BUN	Cr
Correlation coefficient	-0.082	0.042	0.248	-0.203	-0.267	-0.140	-0.323	-0.226	-0.169	0.371	0.147	-0.447
p-value	0.666	0.827	0.187	0.283	0.154	0.461	0.082	0.230	0.371	0.043	0.437	0.013

Abbreviations: WBC; white blood cell, RBC; Red blood cell , Hb; Hemoglobin, PLT; platelet, HCT; Hematocrit, MCV; mean cell volume, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentration, BUN; blood urea nitrogen.

Table 6 Correlation between miR-150 expression and demographic characteristics of ITP patients

Log expression	Age	RBC	WBC	PLT	Hb	HCT	MCV	MCH	MCHC	Reticulo-cyte	BUN	Cr
Correlation coefficient	0.025	0.127	0.124	-0.139	-0.190	-0.052	-0.348	-0.326	-0.290	0.421	0.161	-0.374
p-value	0.897	0.504	0.515	0.465	0.315	0.748	0.059	0.078	0.120	0.020	0.395	0.042

Abbreviations: WBC; white blood cell, RBC; Red blood cell , Hb; Hemoglobin, PLT; platelet, HCT; Hematocrit, MCV; mean cell volume, MCH; mean cell hemoglobin, MCHC; mean cell hemoglobin concentration, BUN; blood urea nitrogen.

differentiation in MK lineage, is not changed in most ITP patients since antibodies do not affect MKs at this stage of megakaryopoiesis (Fig. 1). As a result, the expression of miR-21 and miR-150 is not associated with acute ITP pathogenesis and study of these miRNAs along with other factors such as cytokines as well as other miRNAs and cytokines can be helpful in diagnosis and pathogenesis of ITP (Tavakoli et al., 2016). Furthermore, correlation between the miR-21expressions with laboratory parameters involved in RBC lineage can show the role of this miRNA in the reduction of these parameters. Therefore, it can be helpful along with other related factors in diagnosis of acute ITP patients.

Future perspective

Due to unpredictable course of ITP in patients and the possibility of its transformation to refractory ITP, the choice

of a proper biomarker to evaluate prognosis as well as diagnosis of refractory patients is of utmost importance. Awareness of the role of miRNAs in megakaryopoiesis and platelet production and their role in pathogenesis of ITP patients can lead to application of miRNAs as appropriate diagnostic and therapeutic targets for these patients. Also, detection of miRNAs intracellular targets and cytokines involved in ITP pathogenesis can result to more production of MKs and platelets. This can lead to better patient management and decision-making in order to choose the most suitable therapeutic method, which demands follow-up of their expression levels at diagnosis, during treatment, or after recurrence. Future studies are thus suggested to achieve these goals.

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Compliance with ethics guidelines

Authors declare that they have no conflict of interest. All the procedures performed in the studies involving human participants were in accordance with the ethical standards of local ethics committee of the Ahvaz Jundishapur University of Medical Sciences (AJUMS. REC.1393.379), as well as 1964 Helsinki declaration. Written informed consent was obtained from all patients and normal subjects.

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