Original Article

Association between ICOS polymorphisms and immune thrombocytopenia in an Iranian population

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Abstract

Context: Immune thrombocytopenia (ITP) is an autoimmune disease that is caused by the dysregulation of immune system, in which the circulating platelets (Plt) are destroyed by reticuloendothelial system, leading to hemorrhagic manifestations in patients. **Aims:** Recent studies have indicated that polymorphisms can contribute to ITP susceptibility and outcome. Given the importance of follicular helper T (Tfh) cells in ITP, we evaluated polymorphism in Inducible T-cell Costimulator (ICOS) as a Tfh surface marker among ITP patients to find a likely prognostic factor. **Subjects and Methods:** We recruited 54 ITP patients and 46 persons with no history of thrombocytopenia to conduct this case–control study. In addition to routine laboratory parameters, three polymorphisms, namely rs10932036, rs4404254, and rs10932037 of ICOS gene, were assessed by polymerase chain reaction. **Statistical Analysis:** Mann–Whitney, Kruskal–Wallis, and Chi-square tests were employed to compare and evaluate the data, and $P < 0.05$ indicated a statistically significant association. **Results:** The findings of our study showed that allele and genotype frequencies of all three polymorphisms in question were similar between case and control with no significant difference. However, our assessment indicated higher mean Plt counts in RS4404254 CC genotype than other genotypes under investigation. **Conclusions:** It seems that rs10932037, rs4404254, and rs10932036 polymorphisms of ICOS gene are not involved in susceptibility to ITP. Nevertheless, we found that those carrying RS4404254CC polymorphism have better prognosis due to higher Plt counts both in acute and chronic ITP patients.

Keywords: *Autoimmune, immune thrombocytopenia, inducible costimulator, platelet disorder, polymorphism*

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder that results from a defect in peripheral tolerance where the circulating platelet (Plt) counts reach $<100 \times 10^9$/L.[1,2] The incidence of ITP ranges 4–10 per 100,000 people and bleeding in the skin and mucosa is the most frequent manifestation of it.[2-6] The most distinguished classification of ITP relates to acute and chronic disease, which is based on evaluating the course of disease as well as patient's age and is crucial for therapeutic approaches.[4,7] ITP can also be secondary to other conditions such as infection (human immunodeficiency virus, hepatitis C virus, and *Helicobacter pylori*) or primary immune deficiency (autoimmune lymphoproliferative syndrome and common variable immune deficiency). However, 80% of ITP cases are primary, which is more difficult to treat than secondary ITP.[3,4,6]

Until now, foreign studies on ITP patients from Iran have not addressed the prevalence of ITP in this country; however, we investigated Persian researches stating that ITP prevalence in Iran is similar to international studies. In addition, corticosteroids and intravenous immunoglobulin are commonly used for acute patients and immunosuppressive drugs such as rituximab for chronic patients.[8]

Previous studies indicate that cellular immune dysfunction, which occurs due to imbalance and disruption of T-cells, plays a central role in ITP drive.[1,6,9] CD28 family receptors are involved in costimulatory signaling of T-cells, which is essential for the activation and proliferation of T-cells.[10] A prominent member of this family is CD28, the downregulation of

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which has been shown to cause T-cell dysfunction.[10] Studies have also revealed that blocking the interaction of CD28 family members leads to glycoprotein-specific T-cell tolerance.[6,9]

Inducible T-cell costimulator (ICOS) or CD278 (2q33) is a member of CD28 family that is extensively associated with the structure and function of CD28.[11] Various subsets of T-cells express ICOS, including follicular helper T (Tfh) with phenotypic feature of $CD3^+$ $CD5^+$ $CXCR5^+$ $ICOS^+$ $PD\text{-}1^+$.[7,12] By releasing Interleukin (IL)-21, Tfh has a potential role in the production of Auto-antibody against common Plt surface glycoproteins (e.g., GP IIb/IIIa).[3,13,14] Cell surface ICOS of T_{FH} interacts with its ligand (CD275 or ICOS-L) on B-cell surface, prolonging cell–cell contact through Ab-affinity maturation, B-cell survival, and plasma cell (PC) differentiation.[11,15,16] A study by Xie *et al*. showed that Tfh from ITP patients is of $ICOS^{high}$ type. Moreover, the quantity of $ICOS^{high}$ Tfh cells of ITP patients is greater than healthy controls (HC). In addition, their research detected a positive correlation between $ICOS^{high}$ and/or $PD\text{-}1^{high}$ with serum levels of IL-21 in patients.[12] Similar assessments on humans and mice revealed that the absence of ICOS was associated with a severe decrease in $CXCR5^+$ $CD4^+$ Tfh cells in germinal centers and peripheral blood.[17] On the other hand, studies conducted on other autoimmune diseases (AID) such as systemic lupus erythematosus (SLE), autoimmune thyroid disease, rheumatoid arthritis (RA), and Sjögren's syndrome have indicated that high expression of ICOS along with $CXCR5^+$ and $CD4^+$ as circulating Tfh cell markers is closely linked to Auto-antibody production in these AIDs.[12]

No gold standard has been developed for ITP; therefore, several studies have evaluated various factors (including differences in specific genetic backgrounds) and their association with ITP.[1,4] Given the importance of ICOS signaling in ITP pathogenesis[9] and the evaluation of ICOS expression levels in Tfh cells of ITP patients, ICOS gene polymorphisms are likely to affect ITP pathogenesis by changing the expression of ICOS. Nevertheless, to our knowledge, no study has investigated the correlation between ICOS gene polymorphism with ITP to this date. In this research, we aimed to achieve a new diagnostic and prognostic factor in ITP by evaluating three polymorphisms of ICOS gene in a cohort of 100 subjects.

Subjects and Methods

Study sample characteristics

To perform this case–control study, we enrolled 54 patients with ITP (24 males and 30 females) who referred to Baqa'i Hospital of Ahvaz in 2019. Inclusion criteria were physical examination, history, and laboratory results such as Plt counts $<100 \times 10^9$/L plus normal hemoglobin and White blood cell (WBC) counts as well as the absence of concomitant disease with thrombocytopenia such as SLE, current diagnosis of malignancy, and viral disease.[15,18] Furthermore, given the differences in laboratory parameters as well as medication between acute and chronic ITP patients, we subdivided our subjects into acute and chronic groups according to disease duration and age [Figure 1]. In addition, 46 subjects without ITP nor other autoimmune diseases who were matched for age and sex were recruited as HC group. The demographic characteristics of our study population are summarized in Table 1. It should be noted that the Ethics Committee of AJUMS Research Deputy approved this study with IR.AJUMS.REC.1397.676 ethics code.

DNA extraction and genotyping

2 ml of EDTA-anticoagulated blood (SL/Italy) was drawn from the subjects and the DNA was extracted using a commercial kit according to the manufacturer's instructions (Roche/Germany). The appropriateness of the extracted DNA was confirmed by a Thermo Scientific NanoDrop Spectrophotometer (Onec) and the samples were stored at −70°C for later steps. Due to the limited number of studies on ICOS polymorphisms in AID, the polymorphism located in regulatory region of ICOS gene was considered as well as available publications on these polymorphisms.[16,19] Therefore, the three polymorphisms of rs10932036, rs10932037 (c. 1624C > T), and rs4404254 from ICOS gene were selected for assessment in ITP.

In the study plan, a primer pair was designed for polymerase chain reaction (PCR) because of the proximity of polymorphisms to each other. The primer sequence is as follows: Forward: TTCTTTCCTCTGCTGCTCAA and Reverse: GGAGTCTCTCAACCCTGGAA. Our PCR reactions involved 25 λ of reaction mixture. Each tube contained 0.25 λ of each primer (1:2 dilution), 12.5 λ of distilled water, 10 λ of commercial master mix (Amplicon/Denmark), and finally 2 λ of sample. To prepare PCR product, 2x thermocycler (Peqlab Biotechnologie Gmbh) with the cycle of heat lid to 110.0°C, 94.0°C for 5 min, 72°C for min, 60.0°C for 1.0 min, and 94.0°C for 1.0 min 30x start cycle (1.0) was used. To control the work, the samples were loaded onto 2% agarose gel, which formed a band at 500 bp [Figure 2] and no samples were missed during these steps. Finally, the ABI PRISM 3130 × 1 DNA Analyzer was employed using SANGER sequencing [Figure 3].

Statistical analysis

Mean and SD were used for numerical data, as well as frequency and percentage for qualitative data. The frequencies of alleles and genotypes were obtained by direct counting. Comparing the resulting frequency with the expected one, Hardy–Weinberg equilibrium (HWE) was evaluated by Chi-square and odds ratio and confidence interval was also estimated. Logistic regression was

Table 1: Demographic features of subjects

	ITP		Control	OR (95% CI)	*P*
	Acute (40)	Chronic (14)			
Age	6.66±12.16	36.00±13.0	12.67±12.87	1.007 (0.981-1.033)	0.612
Gender					
Male	19 (47.5%)	5 (35.8%)	20 (43.4%)	0.962 (0.435-2.124)	0.923
Female	21 (52.5%)	9 (64.2%)	26 (56.6%)		
Laboratory result					
RBC	4.36±0.68	4.31±0.92	5.05±0.67	0.203 (0.092-0.449)	<0.001
Hb	10.98±1.63	12.22±2.48	13.16±2.05	0.550 (0.404-0.748)	<0.001
HCT	32.99±4.81	36.87±8.39	38.89±4.26	0.824 (0.744-0.913)	<0.001
WBC	9.57±3.28	7.98±3.32	8.51±2.27	1.082 (0.939-1.247)	0.275
Lymph	3.48±1.77	2.87±1.23	4.29±2.31	0.775 (0.621-0.968)	0.024
PLT	31.50±22.12	29.85±15.07	265.50±64.83	-	<0.001
PDW	17.36±1.90	16.70±0.65	15.34±3.09	1.348 (1.119-1.623)	0.002
MPV	13.10±1.58	13.43±1.91	11.65±1.66	1.738 (1.299-2.325)	<0.001

Table static test: Logistic regression, Mann-Whitney, χ^2. ITP: Immune thrombocytopenia, OR: Odds ratio, RBC: Red blood cell, Hb: Hemoglobin, HCT: Hematocrit, WBC: White blood cell, Lymph: Lymphocyte, PLT: Platelet, PDW: Platelet distribution width, MPV: Mean platelet volume, CI: Confidence interval

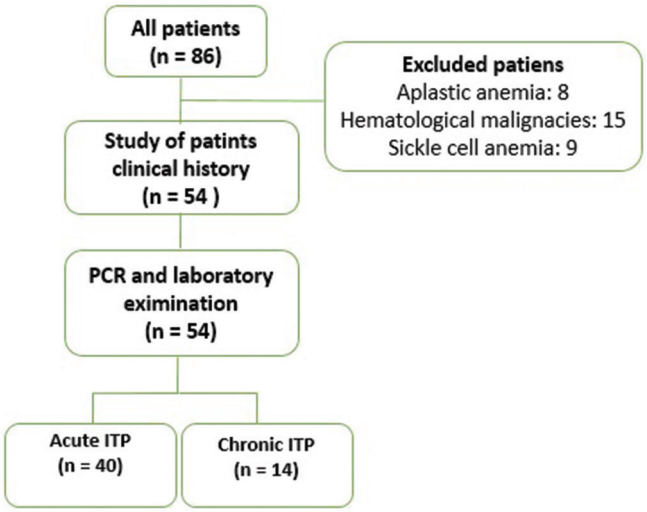

Figure 1: Flow diagram showing patient selection and exclusion, polymerase chain reaction, and laboratory examination. ITP indicates immune thrombocytopenia

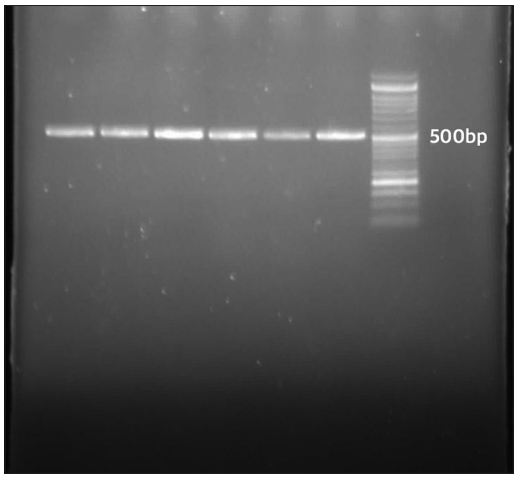

Figure 2: Running ICOS polymorphism samples in agarose gel together with Leder 50. All the samples formed bands at 500 bp region

employed to analyze the values of laboratory parameters in the study population. Mann–Whitney, Kruskal–Wallis, and Chi-square tests were used to investigate the significance of polymorphism association with susceptibility to ITP. All analyses were performed with SPSS (IBM Corporation, Armonk, NY, USA) 25.0 version, and $P < 0.05$ was considered as the significance level.

Results

Comparison of demographic characteristics between patients and healthy controls

This study was conducted on 54 patients whose features are summarized in Table 1. There were 40 acute ITP patients (19 males and 21 females) and the remainder (14 patients) were in the chronic phase (5 males and 9 females). The mean age of patients in chronic and acute group was 36.00 ± 13.00 and 12.16 ± 6.66 years, respectively. The mean Plt counts in acute and chronic groups was 31.50 × 10^9/L and 29.85 × 10^9/L, respectively, and the mean difference in Plt counts of case and control groups (265.50 × 10^9/L) was potentially significant ($P < 0.001$). In addition to Plts, the difference in other relevant laboratory parameters, namely mean platelet volume (MPV) and platelet distribution width (PDW), was also significant between case and control groups ($P < 0.002$ and <0.001, respectively).

Allele frequencies and genotypes distribution

By comparing the genotype distribution obtained in this study and the expected frequency with regard to similar studies, it was found that the frequencies of our study were in agreement with HWE.[11,19,20] The frequency of ICOS gene polymorphisms and alleles is summarized in Table 2.

Table 2: Distribution of ICOS alleles and genotypes

SNP code	Allele/ genotype	Immune thrombocytopenia				Control (%)	OR (95% CI)	*P*
		Acute (%)	Chronic (%)	OR (95% CI)	*P*			
Rs10932037	C	74 (92.0)	26 (92.0)	-	-	85 (92.3)	0.978-0.934	0.955
	T	6 (8.0)	2 (8.0)	-	-	7 (7.6)		
	CC	34 (85)	12 (85.7)	0.944 (0.167-5.329)	0.948	39 (84.8)	0.969 (0.322-2.912)	0.955
	CT	6 (15)	2 (14.3)			7 (15.2)		
	TT	0	0			0		
Rs4404254	T	68 (85.0)	21 (75.0)	-	-	78 (84.7)	0.968-0.363	0.652
	C	12 (15.0)	7 (25.0)	-	-	14 (15.2)		
	TT	31 (77.5)	9 (64.2)	-	0.609	35 (76.1)	-	0.882
	TC	6 (15)	3 (21.4)	1.722 (0.358-8.295)	0.498	8 (17.4)	0.984 (0.343-2.827)	0.977
	CC	3 (7.5)	2 (14.3)	2.296 (0.331-15.931)	0.400	3 (6.5)	1.458 (0.325-6.546)	0.622
Rs10932036	A	74 (92.0)	26 (92.0)	-	-	84 (91.3)	0.774-0.794	0.726
	T	6 (8.0)	2 (8.0)	-	-	8 (8.7)		
	AA	34 (85)	12 (85.7)	0.944 (0.167-5.329)	0.948	38 (82.6)	0.826 (0.283-2.408)	0.726
	AT	6 (15)	2 (14.3)			8 (17.4)		
	TT	0	0			0		

Table static test: Logistic regression, χ^2. SNP: Single-nucleotide polymorphism, OR: Odds ratio, CI: Confidence interval

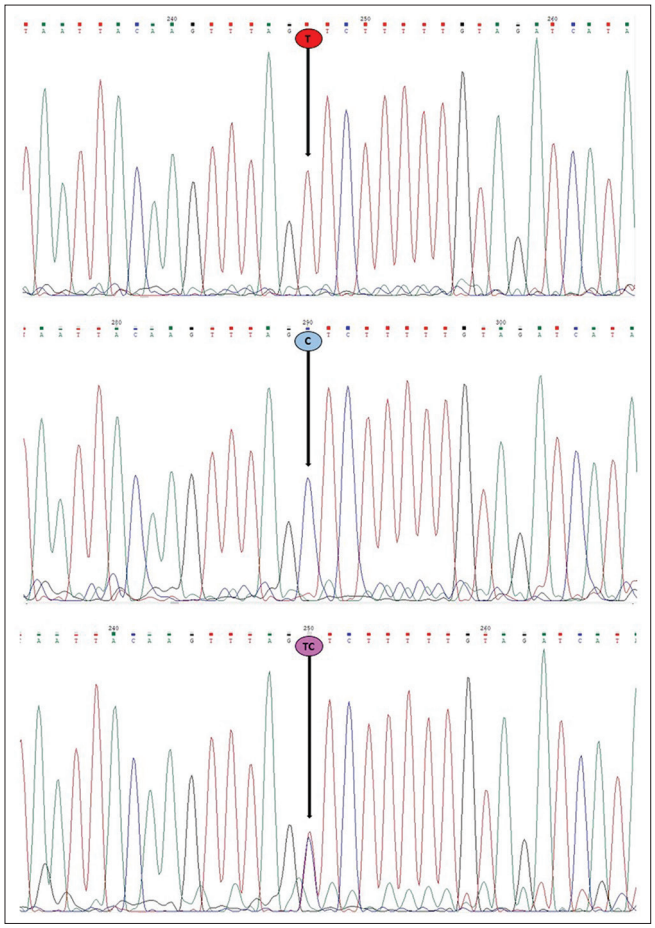

Figure 3: Sequence of Rs4404254T>C polymorphisms. (a-c) Homozygous major, heterozygous, and homozygous minor alleles in Rs4404254T>C polymorphism, respectively

Investigation of rs10932037C >T polymorphism revealed that the expression pattern of genotype in acute and chronic groups was similar to HC group and was not statistically significant (P = 0.955). Moreover, no subject in either populations was a homozygous carrier of RS10932037C >T minor alleles. rs10932037C >T polymorphism alleles also had a similar distribution in case and control groups without a significant difference (P = 0.955).

Assessment of rs4404254T >C indicated the presence of all three genotypes of this polymorphism in case and control groups. The genotype distribution pattern showed a difference between chronic and acute groups, which was not statistically significant (P = 0.609). There was no significant difference in the frequency of case and HC genotypes (P = 0.882), either. Besides, the frequency of T and C alleles of Rs4404254 polymorphism was similar in the studied groups without significant difference (P = 0.652).

Investigation of rs10932036A > T polymorphism revealed the lack of TT (minor homozygous allele) genotype carriers in the population under study. The frequency of AA and AT genotypes was similar in case–control as well as in acute and chronic groups and was not statistically significant (P = 0.726 and 0.948, respectively). Moreover, the frequency of A and T alleles did not show any significant difference between groups (P = 0.726).

Relationship between polymorphisms and laboratory indices at diagnosis

According to Table 3, we examined the association of routine laboratory parameters with genotype of each polymorphism in our study groups. No significant difference was found in the majority of indices such as red blood cells, hematocrit, MPV, and PDW; however, the results of Plt counts showed that carriers of rs4404254CC genotype had higher mean counts than the other two genotypes of this polymorphism, which was statistically significant (P = 0.028). Higher Plt counts have been

observed in rs10932036AT and rs10932037CT as well as rs4404254CC; nonetheless, their statistical analysis did not indicate a significant difference (P = 0.083 and 0.054, respectively). MPV and PDW were also separately considered in acute and chronic groups as two important parameters of ITP [Table 4]. Although none of the correlations was significant, Rs4404254CC and RS10932036AT in acute group, respectively, showed the highest mean PDW and lowest mean MPV.

Association of polymorphisms with platelet counts before and after treatment

After the patients achieved remission, the association of each polymorphism with Plt counts at diagnosis and remission was investigated separately in both acute and chronic groups [Table 5]. Assessment of mean values indicated a higher Plt count before and after treatment in acute patients with RS10932037CT genotype; however, the difference was not statistically significant (P = 0.089 and 0.092, respectively). The difference in Plt counts before and after therapy was not significant for other genotypes under study, either in acute or chronic groups.

Discussion

ITP is a autoimmune disorder in which T-cell dysfunction (especially Tfh) plays a major role.[12] ICOS or CD278 is a Tfh surface marker that is essential in the pathogenesis of autoimmune diseases such as ITP due to its involvement in PC differentiation process and isotype switching.[20,21] Studies have suggested the association of ICOS polymorphisms with susceptibility to autoimmune diseases such as type I diabetes, RA, and systemic sclerosis.[22-24] Nevertheless, to the best of our knowledge, the relationship between ICOS gene polymorphisms with ITP and related indices has not been investigated so far. We assessed three ICOS polymorphisms in a 100-person cohort of Iranian population, assuming that ICOS gene polymorphisms could affect ITP through altering protein level. The polymorphisms selected in our research are located in 3'-untranslated region (3'-UTR). 3'-UTR may affect mRNA stability, degradation, and Post-transcriptional regulation because of its importance in binding of the regulatory element.[25,26]

One of the investigated polymorphisms is Rs10932037C >T, which was previously shown in two independent studies to correlate with the level of ICOS mRNA because of its location in miRs binding site regulating the ICOS expression level. Conversely, our research did not show a significant difference between genotype frequency with RS10932037C >T alleles in ITP patients and controls. In this case, the assumption that rs10932037C >T polymorphism is involved in ITP is rejected. Moreover, the results of RS10932036A >T do not show any association with ITP; however, a previous study confirmed the correlation of this polymorphism with RA.[22]

RS4404254T >C polymorphism is also a non-coding single-nucleotide polymorphism that does not affect the amino acid sequence.[27] Our results showed that the frequency of genotype and allele distribution of Rs4404254 was similar in case and control groups, a finding rejecting the assumption of RS4404254T >C involvement in ITP.

Table 3: Relationship between laboratory parameters with genotype of each polymorphism in patient group

Index	Rs10932037			Rs4404254				Rs10932036		
	CC	TC	PV	TT	TC	CC	PV	AA	AT	PV
RBC	4.30±0.68	4.59±1.01	0.205	4.33±0.66	4.42±0.78	4.31±1.33	0.667	4.32±0.67	4.51±1.09	0.334
Hb	11.23±1.80	11.73±2.69	0.503	11.38±1.82	11.46±2.12	10.38±2.65	0.654	11.29±1.72	11.37±3.05	0.733
HCT	33.74±5.68	35.45±8.47	0.526	34.18±5.74	33.90±6.03	32.74±9.87	0.826	33.93±5.42	34.38±9.62	0.715
WBC	9.43±3.42	7.62±2.38	0.105	9.11±3.00	10.41±4.66	7.28±2.82	0.359	9.30±3.38	8.37±3.12	0.421
Lymph	3.43±1.59	2.72±2.05	0.136	3.49±1.57	2.32±1.52	3.80±2.24	0.056	3.39±1.57	2.95±2.18	0.288
PLT	28.71±18.16	44.62±28.02	0.054	28.57±17.46	28.00±21.30	56.60±27.20	0.028	28.73±18.15	44.50±28.14	0.083
PDW	17.19±1.69	17.17±1.73	0.475	17.21±1.81	16.81±0.80	17.64±2.00	0.742	17.08±1.56	17.67±2.21	0.917
MPV	13.21±1.74	13.08±1.31	0.870	13.17±1.81	13.46±0.95	12.88±1.19	0.805	13.31±1.72	12.65±1.32	0.259

Table static test: Kruskal-Wallis. Mann-Whitney. RBC: Red blood cell, Hb: Hemoglobin, HCT: Hematocrit, WBC: White blood cell, Lymph: Lymphocyte, PLT: Platelet, PDW: Platelet distribution width, MPV: Mean platelet volume

Table 4: Relationship of inducible costimulator polymorphism with mean platelet volume and platelet distribution width among patients

Subgroup	Index	Rs10932037			Rs4404254				Rs10932036		
		CC	CT	p-v	TT	TC	CC	p-v	AA	AT	p-v
Acute	MPV	13.51±1.66	12.86±1.29	0.543	13.09±1.75	13.43±0.95	12.53±1.32	0.658	13.28±1.61	12.28±1.13	0.080
	PDW	17.37±1.91	17.33±199	0.412	17.14±2.03	16.80±0.85	18.13±2.56	0.568	17.22±1.76	18.00±2.50	0.963
Chronic	MPV	13.37±2.04	13.75±1.62	0.587	13.41±2.30	13.55±1.34	13.40±1.31	0.946	13.37±2.04	13.37±1.62	0.587
	PDW	16.70±0.68	16.70±0.70	0.914	16.61±0.62	16.85±0.91	16.90±0.98	0.892	16.70±0.68	16.70±0.70	0.914

Table static test: Kruskal-Wallis, Mann-Whitney. PDW: Platelet distribution width, MPV: Mean platelet volume

Table 5: Relationship of inducible costimulator gene polymorphisms with platelet counts before and after treatment

Subdivide	Treatment	Rs10932037			Rs4404254				Rs10932036		
		CC	CT	p-v	TT	TC	CC	p-v	AA	AT	p-v
Acute	Before	28.70±19.18	47.33±32.17	0.089	28.61±18.30	29.50±24.24	65.33±34.53	0.129	28.73±19.17	47.16±32.33	0.137
	After	214.44±96.84	303.66±131.45	0.092	218.35±94.93	279.50±168.28	222.33±53.00	0.549	222.61±98.35	257.33±149.09	0.705
Chronic	Before	28.75±15.69	36.50±12.02	0.400	28.44±15.16	25.00±18.02	43.50±2.12	0.490	28.75±15.69	36.50±12.02	0.400
	After	119.63±37.07	153.00±14.14	0.195	115.73±38.86	127.66±32.19	158.50±6.36	0.146	119.63±37.07	153.00±14.14	0.195

Table static test: Kruskal-Wallis, Mann-Whitney

Nonetheless, patients carrying the CC genotype in our research showed higher mean Plt counts (both acute and chronic) at the time of diagnosis. Therefore, carriers of this genotype appear to be associated with a better prognosis compared to two other genotypes, which is a finding difficult to justify and may be related to lower WBC counts in carriers of this CC genotype [Table 3]. Given the role ICOS plays in the activation and proliferation of T-cells,[18] we assume that the CC genotype decreases the number and function of Tfh-cells by reducing the ICOS function, which, in turn, reduces Plt degradation. In agreement with our results, Haimila *et al*. suggested that the presence of minor T allele in RS4404254T >C polymorphism is related with lack of or delayed graft function, indicating a higher level of immunity in the presence of T allele.[27] A survey of an immune disease called IgA nephropathy revealed a significant association with T allele carriers (TT and CT).[28] Nevertheless, the study of RS4404254T >C polymorphism in nonsegmental vitiligo and RA Immune disease revealed no association between this polymorphism with these diseases nor their associated indexes.[11,21] The inconsistency of the results can be attributed to the influence of parameters such as other polymorphisms and the complex etiology of autoimmune diseases. We also observed a higher mean Plt count in rs10932037 CT that was not significant [Table 3]. According to the comparison between carriers of CC and CT genotypes, it seems that the decrease in ICOS function and attenuation of Tfh are related to the T allele. In contrast to our hypothesis, however, Haimila *et al*. showed that the presence of T allele (CT and TT) in kidney transplant recipients is associated with lower survival of grafted tissue.[27] Wu *et al*. showed that the TT genotype is associated with a lower overall survival in patients subjected to hematopoietic stem cell transplantation.[29] Indeed, both of these studies indicate a more pronounced immune reaction in carriers of minor T allele (RS10932037). On the other hand, no association was found between rs10932037 polymorphism with IgA deficiency.[30] Evaluation of other ITP indices such MPV and PDW did not show any association with genotypes of this study [Table 4]. However, there were a number of limitations in our research. First, due to the prevalence of ITP and the short period of the study, the sample size was low, especially in the chronic subgroup. Second, because of the absence of rs10932036 TT and rs10932037 TT genotypes in our study population, it was not possible to assess its effect on ITP. Third, few studies have addressed the polymorphisms with which we were concerned. Therefore, further studies with larger sample sizes are required to draw conclusions.

Conclusion

Although our investigation of rs10932037, rs4404254, and rs10932036 polymorphisms of ICOS gene had no significant correlation with the chance of ITP among an

Iranian population, we do note that people carrying the heterozygous rs4404254CC polymorphism have higher Plt counts at diagnosis both in the chronic and acute groups, suggesting better prognosis in them.

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Conflicts of interest

There are no conflicts of interest.

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