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Original article

Assessment of IL-1B31 and IL1 RA Gene Polymorphism in Immune Thrombocytopenia

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Abstract:

Background: Primary immune thrombocytopenia (ITP) is one of the most common autoimmune diseases characterized by low platelet count resulting from increased platelet destruction and impaired platelet production. The pathophysiology of ITP remains understood, and the study of polymorphism in different cytokine coding genes is important to understating the pathophysiology of the disease. This study aimed to evaluate the association between IL1β-31 and IL-1RA gene polymorphism in ITP. **Subjects and methods:** this is a case-control study conducted on 50(30 children and 20 adults) ITP patients and 60 age and sex-matched healthy controls. Genotyping of IL1β-31and IL-1RA gene polymorphism was performed using Restriction Fragment Length Polymorphism (RFLP-PCR) and detection of variable number tandem repeats. **Results:** the present study showed a significant association between IL1β-31 and IL-1RA gene polymorphism and ITP development. The mutant homozygous and heterozygous IL1β-31 and IL-1RA genotype and the mutant allele showed higher frequency compared to the control group and are associated with ITP susceptibility (odd ratio >1), p-value (<0.05) for both. In addition, our results showed a significant association between IL1β-31and IL-1RA gene polymorphism and ITP severity as well as resistance to steroid treatment. However, no association was found with the onset of the disease. **Conclusion:** IL1β-31and IL-1RA gene mutant types are associated with an increased risk of ITP susceptibility. Additionally, they were associated with a severe type of disease and no response to steroid treatment.

Keywords: Primary immune thrombocytopenia; IL1β-31; IL-1RA polymorphism; PCR-RFLP.

Introduction

Immune thrombocytopenia (ITP) is an acquired thrombocytopenia that carries a risk of bleeding. It is also defined as an isolated peripheral low platelet count less than 100 × 10^9/L without any obvious secondary cause. It might have no symptoms or present with a varying degree of bleeding that ranged from generalized skin purpura to serious mucosal or internal bleeding. Its pathogenesis is related mostly to platelet destruction in addition to decreased bone marrow platelet production (1-3).

Most ITP cases are related to autoimmune processes but also secondary causes due to malignancy or

connective tissue disease association were described and termed secondary ITP (4).

Both innate and adaptive immune systems share in platelet destruction in primary ITP cases. It has been well known that humoral mechanisms with autoantibodies and cytokines play the main role in ITP pathogenesis. Macrophages clear the autoantibodies' opsonized platelets, so take the most fundamental step in platelet destruction. Recently cell meditated immune system was discovered to share in megakaryopoiesis impairment (5).

Several cytokines (interleukin IL-1, IL-2, and IL-17 were explored to exert a role in the ITP pathogenesis. So, pro-inflammatory and anti -inflammatory cytokine gene polymorphism may be an added element in clinical characteristics and bleeding risk in ITP patients. Therefore, polymorphism analysis is important to be evaluated and researched (6-9).

IL-1 is related to platelet function and number. The IL-1 family is composed of IL-1A, IL1β-31, and IL-1RA, and each exerts a role in ITP pathogenesis (10, 11).

IL1β-31and IL-1RA (Receptor antagonist) is located on chromosome 2q14. Macrophages release IL-1β as a pro-inflammatory mediator in autoimmune disorders like ITP and inflammatory conditions. While IL-1RA is an anti-inflammatory factor, it acts as a natural antagonist to interleukin 1 itself (12, 13).

Several studies explored the association between the promoter region of the gene at position -31 (C / C, rs1143627) allelic variations and IL1β-31. Some of them conclude diseases susceptibility (14, 15).

In 1992, early studies investigated IL-1RA gene polymorphism at intron 2 site and five allele polymorphism was discovered as copies of 86-bp sequence. IL-1RA I allele with 410 bp had the higher frequency with 4 tandem repeats in the 70 studied unrelated healthy individuals (16) it is likely that the variable number of tandem repeats (VNTR) may influence gene transcription and protein synthesis (17).

Limited studies have examined polymorphisms in IL-1β and IL-1RA in ITP patients, our aim was to evaluate the association of IL-1β and IL-1RA gene polymorphisms with development in ITP in both children and adults in Egyptian population and its association with severity and treatment response.

Subjects and methods

A total of 110 Egyptian participants were enrolled in this case control study. They included 50 ITP patients: 30 (60%) children and 20 (40%) adults, 22 (44%) males and 28 (56%) females. Our sample ages ranged between 3 to 40 years. Patients were attending the pediatric and internal medicine departments at Benha University Hospitals from May 2020 to December 2021. In addition, 60 age and sex-matched healthy controls were selected.

The local ethical committee at Benha University approved the study (MS 37-1-2020) and the study was conducted according to the declaration of Helsinki. All participants or their guardians have signed a written informed consent that explained the study procedure.

Cases selection was based on the international consensus on ITP definition and classification including: Platelet count less than 100 $x10^9$/L at least for

two consecutive blood tests, bone marrow showed a normal or increased megakaryocytes and exclude other causes of thrombocytopenia (١٨).

The inclusion criterion included patients presented with ITP at any age. The exclusion criteria included patients with a history of autoimmune disorders (SLE) or hemorrhagic diseases, thrombocytopenia related to medications(heparin), viral infection (HCV, HIV), patients presented with organomegaly, and patients with active infection.

All patients were enrolled after history taking, clinical examination then laboratory investigations.

Patients were stratified according to severity based on thrombocytopenia bleeding score (19).

Steroid therapy was the mainstay treatment, with high-dose dexamethasone 40 mg/d for 4 days or prednisone 1.0 mg/kg/d for 4 weeks. Complete response was described when patients' platelet count $\geq 100\times10^9$/L measured on two occasions >7 days apart, without bleeding.

The response is achieved when platelet count rose over 30×10^9/L with at least a 2-fold increase than the baseline count and without bleeding. And no response (NR) was described when patients' platelet counts $<30\times10^9$/L or less than a 2-fold increase in baseline platelet count or bleeding.

Severe ITP is termed when patients presented with bleeding and need treatment or new bleeding requiring additional therapeutic intervention with a different platelet-enhancing agent, or an increased dose.

Blood samples were collected from all enrolled subjects for laboratory investigations.

Blood sampling:

Three milliliters of peripheral venous blood sample were collected inK2-EDTA (1.2mg/mL) containing vacutainer then mixed gently and divided into:

One milliliter for complete blood picture examination using an automated hematology system (Sysmex xs 800) and morphological assessment and the remaining two milliliters were used for DNA extraction using the DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions then stored at -80 C until used for IL1β-31and IL-1RA genotyping using restriction fragment length polymorphism (RFLP-PCR).

Genetic analysis

After DNA extraction was performed, the purity of the DNA was assessed by checking absorption at 260/280 nm and the DNA concentration was calculated in (ng/µL).

The amplification was done for IL1β-31C>T rs1143627 using a set of primers as follows; forward 5′-AGCTTCCACCAATACTCTTTTC CCCTTTCC-3′ and reverse 5′-TACACACAAAGAGGCAGAGAG ACAGA-3′ while the primer sequence for IL-1RA VNTR 86 bp was as follows: 5′-CCCCTCAGCAACACTCC-3′ and reverse 5′-GGTCAG AAGGGCAGAGA-3′.

PCR reaction was performed using 10X PCR buffer containing (2.5 µL) MgCl2, Taq polymerase1 U/µL (1.5 µL) (Invitrogen), 50 ng from genomic DNA, dNTPs 10 mmol (1 µL), 1 µL from each

primer, and 16.5 μL high-performance liquid chromatography-grade water in a 25-μL reaction mixture.

PCR was performed using PCR thermal cycler (MultiGene™ OptiMax Thermal Cycler) (USA). The PCR conditions were as follows: an initial denaturation step at 110°C for 5 min, 35 cycles at 94° C for 45 s, annealing at 60° C for 30 s, and extension at 72° C for 1.5 min, with a final extension at 72o C for 5 min.

After PCR amplification, the PCR products were subjected to 37°C overnight digestions using 2 U of Ava I (New England Biolabs, USA) for IL1β-31 electrophoresis was used to separate digestion fragments on 5% agarose with ethidium bromide staining using appropriate commercially available size markers for comparison. Every allele was marked by size.

Genotypes were designated as follows:

For IL1β-31, the presence of two bands of 135 bp and 114 bp indicated the presence of homozygous C/C genotype, single band at 249 bp indicated homozygous T/T genotype. While the presence of three bands at 249, 135, and 114 bp indicated heterozygous C//T genotype.

For IL1Ra II, the alleles were identified by size after the separation of amplified products compared to 100 bp DNA ladder. They were as follows: IL1Ra-I, 410 bp; L1Ra II, 240 bp; IL1Ra III, 500 bp; IL1Ra IV, 325 bp; and IL1Ra V, 595 bp.

To ensure the validity of the test, the gel was assigned by two independent blind investigators, in addition, the samples were run using internal positive and negative controls and 20% of samples were repeated randomly.

Statistical methods

The collected data was revised, coded, and tabulated using the Statistical Package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.).

The allele and genotype frequencies were determined by the counting method. Hardy–Weinberg was calculated in each group to ensure that the study groups are in equilibrium using the following Hardy–Weinberg equilibrium equation (P2 + 2pq + q2 = 1) where *p* is defined as the frequency of the dominant allele and q is defined as the frequency of the recessive allele.

The normality of data distribution was identified using the Shapiro-Wilk test. Mean and standard deviation was used for numerical parametric data, while median and range for non-parametric data.

The Chi-Square test was used to examine the relationship between two qualitative variables and Fisher's exact test was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells. Odds ratios (ORs) and adjusted p-values were analyzed by logistic regression analyses with 95% confidence intervals (95% CIs). Values were considered significant when $p \leq 0.05$.

Results

The clinical characteristics of ITP patients are listed in (Table 1). It shows that 30(60%) of patients had childhood onset while 20(40%) had adult onset. 80% of patients were newly diagnosed, 16% had chronic course while 4% had persistent ITP.

Clinically, 60% of patients had a history of preceding febrile illness, skin manifestations like petechiae, ecchymosis was the most common presenting symptom, as it was present in 41 (82%) of patients. Only 12 (24%) of cases were presented with active bleeding in the form of epistaxis, hematuria, bleeding from gums, and menorrhagia. No one of the patients presented with organomegaly. Severe & very-severe ITP cases were 29 (58%) and 7(14%) respectively, while mild & moderate ITP cases were 5(10%) and 9(18%) respectively.

All ITP patients received steroids; 5 (10%) patients received immunosuppressed drugs while 3(6%) patients received IVIG. Thirty-seven patients (74%) showed a complete response to corticosteroid therapy, 12 patients (24%) showed a response while 1(2%) patient showed no response to corticosteroid therapy.

Our results revealed that the childhood-onset was significantly associated with the newly diagnosed cases, while adulthood onset was significantly associated with chronicity. Additionally, the childhood-onset was significantly associated with preceding febrile illness and active bleeding, higher grade of severity and better response (Table 2).

Table (1) Clinical characteristics of ITP patients.

		Cases N=٥0	
Onset	Childhood onset	30	60%
	Adult onset	20	40%
Course	Newly diagnosed	40	80%
	Chronic	8	16%
	Persistent	2	4%
Clinical presentation	Preceding febrile illness	٣٠	60%
	Skin manifestation	٤١	82%
	Active bleeding	١٢	24%
	Organomegaly	٠	0%
Severity	Mild	5	10%
	Moderate	9	18%
	Severe	29	58%
	Very severe	7	14%
Treatment	ISD	5	10%
	Steroid	50	100%
	IVIG	3	6%
Response	CR	37	74%
	R	12	24%
	NR	1	2%

CR, complete response; R, response; NR, no response

Table (2) Comparison of onset of ITP with other studied parameters.

		Children n=30		Adult n=20		*p*
Course	Newly diagnosed	28	93.3%	12	60%	0.003
	Chronic	1	3.3%	7	35%	
	Persistent	1	3.3%	1	5%	
Clinical presentation	preceding febrile illness	29	96.7%	1	5%	<0.001
	Skin manifestation	24	80.0%	17	85%	0.724
	Active bleeding	12	40.0%	0	0%	0.001
Severity	mild	1	3.3%	4	20%	<0.001
	moderate	1	3.3%	8	40%	
	severe	21	70.0%	8	40%	
	very severe	7	23.3%	0	0%	
Response	CR	28	93.3%	9	45%	<0.001
	R	2	6.7%	10	50%	
	NR	0	0.0%	1	5%	

Chi square test was used to get *p* value.

Regarding IL1β-31, CC and C are the reference genotype and allele respectively. This study revealed that homozygous and heterozygous genotypes (TT and CT), and T allele showed significantly higher frequency in cases when compared to control groups (p <0.05 for each) and was associated with ITP susceptibility (OR>1 for each).

Regarding IL-1RA, I/I and I are the reference genotype and allele respectively. This study revealed that homozygous and heterozygous genotypes (I/II, II/II), and II allele showed significantly higher frequency in cases when compared to control groups (p<0.05 for each) and was associated with ITP risk compared to control group (OR>1 for each) (Table 3).

The association of the risk of early disease onset and severity among all studied cases with the IL1β-31and IL-1RA gene polymorphism, were studied. The data from the present study revealed no significant association between both early disease risk and the severity with IL- IL1β-31or IL-1RA genotype and alleles (p>0.05).

In the present study, we evaluated the association of the outcome after treatment among all studied cases with the IL1β-31and IL-1RA gene polymorphism.

Our results showed a significantly higher frequency of IL1β-31homozygous mutant TT genotype, and mutant T allele frequency in non-CR when compared to CR cases (p<0.05 for each), with risk of non-CR susceptibility (OR>1 for each).

Similarly, a significantly higher frequency of IL-1RA, II/II, I/II+II/II genotypes, and II allele frequency in non-CR when compared to CR cases (p<0.05 for each), with a risk of non-CR susceptibility (OR>1 for each) (Table 4).

Regression analysis was conducted for the prediction of ITP susceptibility using gender, IL- IL1β-31 and IL1Ra genotypes as covariates. IL1β-31 CT, TT, IL1Ra I/II, II/II genotypes were considered risky predictors of ITP susceptibility (Table 5).

Regression analysis was conducted for the prediction of non-CR using age, gender, baseline platelet count, severity, IL1β , and IL-1RA genotypes as covariates IL1β-31 TT genotype, IL1Ra II/II genotypes were considered risky predictors of ITP non-CR (Table 6).

Table (3) Association of IL-1B-31, IL-1RA variable number tandem repeat (VNTR) genotypes and alleles with risk of developing ITP.

		Control N=٦0		ITP N=50		*p*	OR (95% CI)
		N	%	N	%		
IL-1β	***CC***	33	55.0	13	26.0		
	CT	20	33.3	27	54.0	**0.005**	2.144(1.266-3.634)
	TT	7	11.7	10	20.0	**0.028**	2.221(1.088-4.535)
	CT+T T	27	45.0	37	74.0	**0.002**	2.165(1.321-3.546)
	C	86	71.7	53	53.0		
	T	34	28.3	47	47.0	**0.004**	1.656(1.171-2.344)
IL-1RA	***I/I***	35	58.3	17	34.0		
	I/II	22	36.7	25	50.0	**0.040**	1.696(1.025-2.807)
	II/II	3	5.0	8	16.0	**0.017**	2.866(1.204-6.823)
	I/II+I I/II	25	41.7	33	66.0	**0.011**	1.863(1.153-3.010)
	I	92	76.7	59	59.0		
	II	28	23.3	41	41.0	**0.005**	1.675(1.167-2.403)

OR Odd ratio; CI, Confidence interval. Logistic regression analysis was used.

Table (4) Association of IL-1β, IL-1RA genotypes and alleles with risk of ITP non-CR (R+NR).

		CR n=37		non-CR n=13		*p*	OR (95% CI)
		N	%	N	%		
IL-1β	***CC***	11	29.7	2	15.4	-	
	CT	23	62.2	4	30.8	0.959	0.995(0.825-1.201)
	TT	3	8.1	7	53.8	0.001	1.473(1.180-1.840)
	CT+TT	26	70.3	11	84.6	0.259	1.124(0.917-1.378)
	C	45	60.8	8	30.8	-	
	T	29	39.2	18	69.2	0.009	2.085(1.203-3.612)
IL-1RA	***I/I***	16	43.2	1	7.7	-	
	I/II	20	54.1	5	38.5	0.125	1.133(0.966-1.33)
	II/II	1	2.7	7	53.8	<0.001	1.771(1.448-2.165)
	I/II+II/II	21	56.8	12	92.3	0.006	1.288(1.076-1.541)
	I	52	70.3	7	26.9	-	
	II	22	29.7	19	73.1	<0.001	2.974(1.688-5.239)

CR, complete response; Non CR, non-complete response ; OR, Odd ratio; CI, Confidence interval. Logistic regression analysis was used.

Table (5). Regression analysis for prediction of ITP susceptibility.

		Univariable		Multivariable	
		p	OR (95% CI)	*p*	OR (95% CI)
Gender		0.805	0.942(0.586-1.514)		
IL-1β	***CC***				
	CT	**0.005**	2.144(1.266-3.634)	**0.010**	2.03(1.187-3.472)
	TT	**0.028**	2.221(1.088-4.535)	**0.043**	1.956(1.194-4.281)
IL-1RA	***I/I***	-		-	
	I/II	**0.040**	1.696(1.025-2.807)	**0.035**	2.271(1.216-5.628)
	II/II	**0.017**	2.866(1.204-6.823)	**0.049**	1.681(1.001-2.822)

OR Odd ratio; CI, Confidence interval. Logistic regression analysis was used.

Table (6) Regression analysis for prediction of response to treatment.

		Univariable		Multivariable	
		p	OR (95% CI)	*p*	OR (95% CI)
Age		0.182	1.029(0.996-1.063)		
Gender		0.408	0.727(0.342-1.546)		
Baseline platelet count		0.160	0.97(0.94-1.301)		
Severity		0.644	1.224(0.52-2.883)		
IL-1β	***CC***				
	CT	0.959	0.995(0.825-1.201)	0.834	0.886(0.287-2.742)
	TT	**0.001**	1.473(1.180-1.840)	**0.039**	2.667(1.703-10.125)
IL-1Ra	***I/I***	-			
	I/II	0.125	1.133(0.966-1.33)	0.225	2.043(0.644-6.483)
	II/II	**<0.001**	1.771(1.448-2.165)	**0.003**	2.603(1.131-4.285)

OR Odd ratio; CI, Confidence interval. Logistic regression analysis was used.

Discussion:

The pathogenesis of ITP is multifactorial with antibody-mediated and cytokine activity considered as the most prevalent mechanisms (20, 21).

IL-1 superfamily consists of 11 different cytokines, 7 of them are the pro-inflammatory agonists that are involved in autoimmune disease pathogenesis while the rest are antagonists to the inflammatory processes (22).

The aim of this study was to assess pro-inflammatory IL1β-31 and IL-1RA receptor antagonist polymorphism and its association with the risk for ITP in the Egyptian population. In our age and sex matching case-control study, 110 participants were enrolled, (50 ITP patients and 60 control healthy controls), and patients were selected and followed the recommended steroid treatment.

Our study demonstrated that IL1β-31 heterozygous mutant CT and homozygous mutant TT genotypes and the mutant T allele were significantly associated with the risk of ITP susceptibility. Our results agreed with the results of another study that revealed that genotypes of IL1β-31 CT, TT genotypes, and T allele were significantly associated with the susceptibility to ITP (12).

On the contrary, the association of IL1β (rs1143627) gene polymorphism and the risk for ITP development in 89 adult ITP patients in the Uzbek population was evaluated and that study revealed no statistically significant association between IL-1β (rs1143627) gene polymorphism with the risk of ITP development. The T allele and C allele prevalence was a bit different between the ITP patients and controls (75.8% versus 83.3% and 24.2 % versus 16.7 %, $\chi 2 = 1.82$; p = 0.18; OR = 1.53; 95% CI: 0.82-2.86 respectively) (23). These contradictory results could be explained by the difference in ethnicity and heterogeneity of the studied population.

Regarding IL-1RA genotypes, the homozygous and heterozygous mutant genotypes I/II, II/II, and the mutant II allele showed significantly higher frequency in ITP cases when compared to control groups (p<0.05 for each), with an increased possibility of association with risk of ITP susceptibility (OR>1 for each).

Many studies have explored the link between IL-1RA polymorphism and ITP risk in different populations (12,24). One of them studied the association between VNTR IL1Ra polymorphism and risk of ITP in 218 Indian participants, the results of their study showed that the presence of allele-II of IL-1RA led to an increased risk of ITP development. They explored that VNTR IL1Ra polymorphism is linked to ITP pathogenesis and severity. In addition, both the II/II (mutant) and the hetero type of IL- 1Ra VNTR polymorphism had higher percentages among the ITP studied cases (OR=4.48, 95% CI=1.17–17.05, P =0.0230 and OR=1.80, 95% CI=1.03–3.14, P =0.0494) (12).

In Egyptian children, IL-1RA gene polymorphism was studied in a case-control study. They found that mutant I/II IL-1RA VNTR was prevalent in nearly half of ITP cases compared to the control group which was less than a third of studied healthy participants with a significant p value of 0.039. In addition, the frequency of IL-1RA II allele was significantly higher in ITP cases than in controls (p<0.05) (25).

By contrast, the IL-1RA gene polymorphism was studied on 50 Iraqi children with ITP and their controls; their results revealed that the IL-1RA I(412bp) allele variant had been prevalent in both ITP cases and controls. IL-1RA II (240bp) allele variant was found to be prevalent in controls, while IL-1RA I II (548bp) allele variant has been prevalent significantly in ITP studied cases (26). The heterogeneous cases selection and disease course difference between children and adults may explain these differences between studies' results (27).

Regarding the association of IL1β, IL-1RA genotypes, and alleles with risk of early disease onset, no significant association was found between IL1β or IL-1RA genotype and alleles with the

age of onset. Some found opposing results as they found that the IL-1RA gene polymorphism was associated with childhood ITP, but they did not find that association regarding IL1β (24).

Our study found no significant association between IL1β or IL-1RA genotype and alleles with the severity of ITP which disagreed other study that reported that IL1β-31 polymorphism had severe ITP patients especially homozygous mutant and variant alleles (12)**.**

Regarding the response to treatment in our study, we found that 37 cases achieved CR, while 13 cases had inferior outcome with non-CR, IL-1B, TT genotype, and T allele showed significantly higher frequency in non-CR when compared to CR cases, with risk of non-CR susceptibility. Similarly, IL-1RA, II/II, I/II+II/II genotypes, II allele showed significantly higher frequency in non-CR when compared to CR cases, with risk of non-CR susceptibility. Some authors found that polymorphisms in IL-1B-31, and IL-1RA showed no association with the response to steroids in ITP patients (12).

Moreover, regression analysis was conducted for the prediction of inferior response, ILIB TT, and IL-1RA II/II genotypes were considered risky predictors of ITP non-CR. While other risk factors were not associated with the risk of inferior response such as age and gender.

Concerning the gender effect on ITP prognosis and natural history, while an article stated that the male gender carries a double risk for relapse even with an initial complete response (28). Some authors revealed no significant statistical differences regarding patient characteristics between the female and male which is matching with our results (29).

One limitation of this study was the relatively small sample size used for polymorphism analysis and heterogeneity of studied ITP cases. Thus, these results must be verified in multicentric studies with larger samples and populations of different ethnicities to mask some of these discrepancies between the above-mentioned studies.

To conclude, IL1β-31 and IL-1RA gene polymorphisms may have a significant impact on the susceptibility and response to treatment of ITP, but not associated with the severity or age of onset of ITP in the Egyptian population.

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