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SINGLE NUCLEOTIDE POLYMORPHISMS OF THE INFLAMATORY CYTOKINE GENES: INTERLEUKIN-1B, TUMOR NECROSIS FACTORS-A AND TUMOR NECROSIS FACTOR-B IN ADULT PATIENTS WITH IMMUNE THROMBOCYTOPENIA

Marica Pavkovic[1], Rosica Angelovic[1], Marija Popova-Simjanovska[1], Oliver Karanfilski[1], Slobodanka Trpkovska-Terzieva[1], Tatjana Sotirova[1], Lidija Cevreska[1], Aleksandar Stojanovic[1]

[1] University Clinic for Hematology, Medical Faculty, Skopje, R. Macedonia

Corresponding Author: Marica Pavkovic, MD, PhD, University Clinic for Hematology, Faculty of Medicine, Skopje, ul. Vodnjanska 17, 1000 Skopje, R. Macedonia, Tel: +389 70 344 709; Fax: +389 (0)2 3 21 70 61; E-mail:pavkovicm@yahoo.com

Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by thrombocytopenia due to platelet autoantibodies, causing an accelerated clearance of opsonized platelets by phagocytes. The etiology of ITP remains unclear, both genetic and environmental factors may have a role in the disease development. The aim of our study was to investigate a possible association of three single nucleotide polymorphisms (SNP) in the genes for interleukin beta (IL1B-511C/T), tumor necrosis factor beta (TNF+252G/A) and tumor necrosis factor alpha (TNFA-308G/A) with ITP. We have analyzed 125 adult patients with ITP and 120 healthy matched controls. Genotyping was performed by using PCR- RFLP methods.

Our results demonstrated significantly different genotype distributions and allele frequencies for TNFB+252G/A in patients with ITP, $p = 0.005$ and $p = 0.009$ with Yates correction. We did not find any significant differences in the genotype distribution or allele frequencies for the other two genes. We have found significantly different genotype distribution and allele frequencies for TNFA-308G/A between patients with unresponsive and responsive ITP patients, $p = 0.016$ and $p = 0.009$. There were no significant differences in genotype distribution and allele frequencies for ILB-511C/T and TNFB+252G/A polymorphisms between those two groups of patients. We did not find any significant differences in genotype distribution and allele frequencies for all three polymorphisms between splenectomized and unsplenectomized ITP patients.

The obtained data indicate that the A allele of TNFB+252G/A is more frequent in these patients than in the controls and that this polymorphism may play a significant role in disease susceptibility. The A allele of TNFA-308G/A was more frequent in patients with unresponsive ITP, indicating that this gene polymorphisms may contribute to therapy resistance.

Key words: immune thrombocytopenia, thrombocytopenia, cytokine genes, single nucleotide polymorphisms.

Introduction

Immune thrombocytopenia also known as idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease of unknown etiology, characterized by thrombocytopenia due to the presence of platelet autoantibodies specific for platelet membrane glycoproteins, such as GPIIb/IIIa, GPIb/IX and GPIa/IIa [1]. These autoantibodies cause an accelerated destruction of autoantibody-opsonized platelets by FcγReceptor bearing phagocytic cells in the reticuloendothelial system, or inhibition of platelet production [2]. The etiology of ITP remains unclear, but both genetic and

environmental factors are thought to play role in the development of the disease. Several genes involved in immune system regulation like cytokine genes [3, 4, 5, 6], Fc gamma receptor genes [4, 7], CTLA-4 gene [8, 9], and HLA genes [10], as well as some infective agents like hepatitis C virus, HIV (Human Immunodeficiency Virus), and helicobacter pylori [11, 12, 13] have been associated with susceptibility to ITP in several studies.

Two forms of disease exist: one form is acute ITP that predominantly occurs in children and chronic form which is commonly seen in adults. The acute form of ITP is most common in children. That form of the disease is often selflimiting within a 6-month period and is commonly associated with infection. In adults, ITP is more often chronic disease, spontaneous remissions are rare and therapy with corticosteroids and splenectomy is commonly required [14, 15].

The aim of our study was to investigate a possible association of three single nucleotide polymorphisms (SNP) within three genes for inflammatory cytokines, interleukin 1-beta (IL-1β, IL1B-511 C/T), tumor necrosis factor beta (TNF-β, TNFB+252 G/A) and tumor necrosis factor alpha (TNF-α, TNFA -308 G/A) with chronic ITP in adult patients in Republic of Macedonia.

Materials and Methods

We have analyzed 125 unrelated adult patients with chronic ITP (35 men and 90 women) with median age of 47 (range 18–83 years) and 120 healthy, age and sex, matched controls (Table 1). The diagnosis of ITP was based on thrombocytopenia (platelet count < 100 × 109/L), normal or increased bone marrow megakaryocytes without morphological elements of dysplasia, and exclusion of other diseases that could be a cause for thrombocytopenia like underlying autoimmune disorders like SLE (Systemic Lupus erythematosus), hipersplenismus, hepatitis C, HIV [15]. The median follow up of the patients was 44 months (12–384 months). Clinical information recorded for every patient were sex, age at diagnosis, platelet count at diagnosis and last control, bleeding symptoms, treatment modality and response to treatment (Table 2).

Table 1

Patients' and controls characteristics

Characteristics	Patient n = 125	Controls n = 120
Age (years), median (range)	47 (18–83)	49 (18–82)
Gender		
Male	35 (28%)	32 (26%)
Female	90 (72%)	88 (73%)
Platelet count at diagnosis, Median (range)	13×10^9/L (0–98)	213×10^9/L (138–435)

Thirty three patients (26.4%) were asymptomatic, 82 (65.5%) had minor skin or mucosal bleeding and 14/125 (11.2%) had bleeding from gastrointestinal or genitourinary system. None of the patients had severe, life threatening bleeding symptoms. The median platelet count at diagnosis was 13 × 109/L (range: 0–98 × 109/L) and 178 × 109/L (range: 2–687 × 109/L) at the last control. Numbers of patients with severe, moderate or mild thrombocytopenia at diagnosis and at the last control are shown in Table 2. Treatment modalities and responses are also summarized in Table 2. Corticosteroids were initial treatment for 95% of patients, 38 patients were splenectomized, 22 patients were treated with intravenous gamma globulins (IVIG), while 5 pts did not received any specific treatment due to mild, asymptomatic thrombocytopenia. Complete response according to the criteria by Rodeghiero et al. [15] to initial treatment (prednisone±splenectomy) was achieved in 38%, partial response in 52% and no response in 12/120 (10%) of patients.

Refractory ITP was defined as failure to achieve at least a response or loss of response after splenectomy [2, 15]. Fourteen

patients had refractory form of ITP, while 28 patients had ITP unresponsive to one or more agents. "Unresponsive ITP patients" are unsplenectomized patients not responding to medical treatments. All together, 42(34%) patients had unresponsive or refractory form of ITP according to new definition criteria by Rodighiero et al. [15].

Table 2

Patients' characteristics

Characteristics	Patients n = 125	(%)
Age (years), median (rang)	47	(18-83)
Sex		
Men	35	(28%)
Women	90	(72%)
Clinical presentation		
No bleeding symptoms	33	(26.4%)
Bleeding symptoms	92	(73.6%)
Mucocutaneos bleeding	82	(65.5%)
GIT or urogenital bleeding	14	(11.2%)
Platelet count at diagnosis, median (rang)	13×10^9/L ($0–98 \times 10^9$/L)	
Thrombocytopenia at diagnosis		
Severe (Plt < 30 x 10^9/L)	94	(75.2%)
Moderate (Plt < 30–50 × 10^9/L)	18	(14.4%)
Mild (Plt > 50 × 10^9/L)	13	(10.4%)
Platelet count at the last control, median (rang)	178×10^9/L ($2–687 \times 10^9$/L)	
Thrombocytopenia at the last control		
Severe (Plt < 30 × 10^9/L)	9	(7.2%)
Moderate (Plt < 30–50 × 10^9/L)	13	(10.4%)
Mild (Plt > 50 × 10^9/L)	29	(23.2%)
Normal (Plt > 100 × 10^9/L)	74	(59.2%)
Follow up in months, median (rang)	44 (12–384)	
Treatman		
Prednison	120	(96%)
Splenectomy	38	(30.4%)
IVIG (i.v. imunoglobulini)	22	(17.6%)
Other (Azathioprin, yclophosphamide, rituximab)	44	(35%)
Without therapy	5	(4%)
Response to initial treatment (Prednison±Splenectomy)		
CR	46/120	(38%)
PR	62/120	(52%)
NR	12/120	(10%)
Refractory ITP	14	(11.2%)
Unresponsive ITP	28	(22%)
Refractory & unresponsive ITP	42	(33.6%)

DNA was isolated from peripheral blood mononuclear cells with standard phenol/chloroform extraction. Genotyping was performed by using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method already published [4, 16, 17, 18]. Written informed consent was obtained from all participants.

The distribution of genotypes and allele frequencies were compared between patients and controls using a chisquared test. Differences were consider significant if $p < 0.05$.

Results

Our results demonstrated significantly different distribution of the TNFB genotypes in patients with ITP (n = 125; G/G = 3, A/G

= 38, A/A = 84) comparing with the healthy controls (n = 120; G/G = 16, A/G = 35, A/A = 69), p = 0.005 (Table 3). Allele frequencies for TNFB + 252G/A were also significantly different in patients with ITP (A allele 82.4%, G allele 17.6%) comparing with controls (A allele 72.1%, G allele 27.9%), p = 0.009 with Yates correction (Table 3). We did not found significant differences in the genotype distribution or allele frequencies for two other genes (Table 3).

We divided all ITP patients to two groups: those with refractory or unresponsive form of ITP and those with ITP responsive to initial treatment and compared these three polymorphisms in those two groups of patients. We found significantly different genotype distribution of TNFA-308G/A between patients with unresponsive and responsive ITP, p = 0.016 (Table 4). Allele frequencies for TNFA-308G/A were also significantly different in patients with refractory or unresponsive and responsive ITP (A allele 15.5% versus 4.8%), p = 0.009 with Yates correction (Table 4). There were no significant differences in genotype distribution and the allele frequencies for TNFB+252G/A and the IL1B-511C/T between these two groups of patients (Table 4).

Table 3

Genotype distribution and allele frequencies in patients with ITP and controls

Cytokine SNP	Genotype/Allele	ITP (n = 125) (%)	Controls (n = 125) (%)	P-value (n = 120) (%)
TNF-β	A/A	84 (67.2)	69 (57.5)	***0.005***
(+252 G/A)	A/G	38 (30.4)	35 (29.2)	
	G/G	3 (2.4)	16 (13.3)	
	A	206 (82.4)	173 (72.1)	***0.009****
	G	44 (17.6)	67 (27.9)	
TNF-α	A/A	1 (0.8)	2 (1.6)	0.573
(-308 G/A)	A/G	19 (15.2)	23 (19.2)	
	G/G	105 (84)	95 (79.2)	
	A	21 (8.4)	27 (11.3)	0.363*
	G	229 (91.6)	213 (88.7)	
IL-β	C/C	67 (53.6)	62 (51.7)	0.439
(-511 C/T)	C/T	39 (31.2)	45 (37.5)	
	T/T	19 (15.2)	13 (10.8)	
	C	173 (69.2)	169 (70.4)	0.845*
	T	77 (30.8)	71 (29.6)	

* With Yates' correction

Table 4

Genotype distribution and allele frequencies in patients with responsive ITP and unresponsive ITP

Cytokine SNP	Genotype/Allele	ITP (n = 83) (%)	Unresponsive ITP (n = 42) (%)	P-value
TNF-β	A/A	52 (62.7)	32 (76.2)	0.086
(+252 G/A)	A/G	30 (36.1)	8 (19.1)	
	G/G	1 (1.2)	2 (4.7)	
	A	134 (80.7)	72 (85.7)	0.422*
	G	32 (19.3)	12 (14.3)	
TNF-α	A/A	0 (0)	1 (2.4)	***0.016***
(-308 G/A)	A/G	8 (9.6)	11 (26.2)	
	G/G	75 (90.4)	30 (71.4)	
	A	8 (4.8)	13 (15.5)	***0.009****
	G	158 (95.2)	71 (84.5)	
IL-β	C/C	46 (55.4)	21 (50)	0.74
(-511 C/T)	C/T	24 (29)	15 (35.7)	
	T/T	13 (15.6)	6 (14.3)	
	C	116 (69.9)	57 (67.8)	0.85*
	T	50 (30.1)	27 (32.2)	

* With Yates' correction

We did not found significant differentces in genotype distribution and allele frequtentcies for all three gene polymorphisms between splenectomized and unsplenetctotmitzed ITP patients (Table 5).

Table 5

Genotype distribution and allele frequencies in splenectomized patients and unsplenectomized patients with ITP

Cytokine SNP	Genotype/Allele	ITP (n = 87) (%)	Splenectomized ITP (n = 42) (%)	P-value
TNF-β	A/A	53 (61)	30 (79)	0.125
(+252 G/A)	A/G	32 (37)	7 (18)	
	G/G	2 (2)	1 (3)	
	A	138 (79.3)	67 (88.2)	0.135*
	G	36 (20.7)	9 (11.8)	
TNF-α	A/A	0 (0)	1 (2.6)	0.109
(-308 G/A)	A/G	10 (11.5)	8 (21)	
	G/G	77 (88.5)	29 (76.4)	
	A	10 (5.7)	10 (13.2)	0.08*
	G	164 (94.3)	66 (86.8)	
IL-β	C/C	48 (55.2)	19 (50)	0.66
(-511 C/T)	C/T	25 (28.7)	14 (36.8)	
	T/T	14 (16.1)	5 (13.2)	
	C	121 (69.5)	52 (68.4)	0.97*
	T	53 (30.5)	24 (31.6)	

* With Yates' correction

Discussion

ITP is a heterogeneous clinical disorders characterized by immune mediated platelet destruction. Cellular immunity and cytokine response have important role in the patophysiology of ITP. The cytokine genes are polymorphic, and sometimes these gene polymorphisms are associated with different levels of cytokine production. Cytokine gene polymorphisms have been associated with different autoimmune diseases. Different studies have reported association of the SNP at TNFB+252G/A with various autoimmune diseases, for example G allele was associated with systemic lupus erythematosus [19], Graves's disease [20], and A allele with systemic sclerosis [21].

Our data indicate that the A allele of TNFB+252G/A is more frequent in patients than in controls and that the A allele of TNFA-308G/A was more frequent in patients with refractory & unresponsive ITP comparing to patients with responsive ITP. Both genes (TNFA and TNFB) are arranged within the MHC locus on chromosome 6p, and the G allele of TNFB and A allele of TNFA are associated with increased TNF expression [22, 23]. The TNFB+252G/A polymerphism is located in the first intron and it correlates with a level of TNFB protein production by lymphocytes [22]. Individuals having TNFB+252 G allele are high producers of TNFB, while those possessing the A allele are low producers. TNFB is produced by activated T cells and is involved in the maturation and activation of B-cells [24, 25]. These findings do not correlate with our results, which show higher prevalence of A allele (low producers) in patients with ITP. Our results are similar with previously reported results by Foster et al. [4] that have shown higher prevalence of A allele for TNF-alpha and TNF-beta in Caucasian children with chronic ITP; but different from the results reported by Satoh et al. [3] in Japanese adult patients with ITP. The reason for this difference is unclear. Possible explanation may be the differences in patient's ethnic background, number of samples or that these two gene polymorphisms are in linkage disequilibrium with other neighboring genes.

Conclusion

The obtained data indicate that the A allele of TNFB+252G/A is more frequent in patients than in controls and that this poly-

morphism may play role in disease susceptibility. The A allele of TNFA-308G/A gene is associated with increased TNF expression and it was more frequent in patients with refractory & unresponsive ITP, indicating that this gene polymorphisms may contribute to therapy resistance.

This study supports the idea that genetics factors contribute to the pathogenesis of ITP, but that multiple other genetic and environmental factors play role in the etiopathogenesis of immune thrombocytopenia. Further studies of cytokine polymorphisms on larger samples are needed to determine there role in etiology of ITP.

Declaration of Interest

We disclose any financial relationship with any biotechnological or pharmaceutical company and there is no conflict of interest.

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Р е з и м е

ЕДИНЕЧНИ НУКЛЕОТИДНИ ПОЛИМОРФИЗМИ ВО ЦИТОКИНСКИТЕ ГЕНИ: ИНТЕРЛЕУКИН-1Б, ТУМОР НЕКРОЗИС ФАКТОР-А И ТУМОР НЕКРОЗИС ФАКТОР-Б КАЈ ПАЦИЕНТИ СО ИМУНА ТРОМБОЦИТОПЕНИЈА

Марица Павковиќ, Росица Ангеловиќ, Марија Попова-Симјановска, Оливер Каранфилски, Слободанка Трпковска-Терзиева, Татјана Сотирова, Лидија Чевреска, Александар Стојановиќ

Универзитетска клиника за хематологија,
Медицински факултет, Скопје,
Р. Македонија

Имуната тромбоцитопенија (ИТП) е автоимуно заболување кое се карактеризира со намален број тромбоцити како резултат на присуство на антитромбоцитни антитела. Етиологијата на ова заболување е нејасна. Различни генетски и фактори од околината може да имаат улога во појавата на ова заболување. Цел на оваа студија е да ја испита можната поврзаност на три полиморфизми во цитокинските гени: интерлеукин бета (IL1B-511C/T), тумор некрозис фактор бета (TNF+ 252G/A) и тумор некрозис фактор алфа (TNFA-308G/A) со ИТП. Анализиравме 125 возрасни пациенти со ИТП и 120 контролни индивидуи. Генотипизацијата беше направена со методата на PCR-RFLP.

Нашите резултати демонстрираа значајна разлика во генотипската дистрибуција и алелната фреквенција за полиморфизмот TNFB+252G/A кај болните со ИТП, p = 0.005 и p = 0.009 во споредба со контролната група. Таква разлика не беше детектирана за останатите два полиморфизма. Сигнификантна разлика во генотипската дистрибуција и алелната фреквенција најдовме за TNFA-308G/A помеѓу болните со рефрактерна и нерефрактерна форма на ИТП, p = 0.016 и p = 0.009. Немаше сигнификантна разлика во генотипската дистрибуција и алелната фреквенција помеѓу овие две групи болни за останатите два полиморфизма, ILB-511C/T и TNFB+ 252G/A. Не најдовме значајна разлика ниту во генотипската и алелната фреквенција на овие три полиморфизми помеѓу спленектомираните и неспленектомираните болни со ИТП.

Овие податоци укажуваат на можна улога на А-алелот на TNFB+252G/A во етиологијата на болеста, додека почестата појава на А-алелот на TNFA-308G/A кај болните со рефрактерна форма на ИТП укажува на можната поврзаност на овој алел со тераписка резистенција кај болните со ИТП.

Клучни зборови: имуна тромбоцитопенија, цитокински гени, единечни нуклеотидни полиморфизми.