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ASSOCIATION OF POLYMORPHISMS IN HUMAN PLATELET ANTIGENS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA IN MACEDONIANS

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A b s t r a c t: Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease characterized by thrombocytopenia due to the presence of platelet autoantibodies specific for platelet membrane glycoproteins, such as GPIIb/IIIa, GPIb/IX and GPIa/IIa. These autoantibodies cause an accelerated clearance of opsonized platelets by phagocytes and inhibition of platelet production.

Human platelet antigen (HPA) systems HPA-1, HPA-2, HPA-3 and HPA-5 are components of platelet GP complexes GPIIb/IIIa, GPIb/IX and GPIa/IIa. The HPA system consists of more than 12 bi-allelic antigen polymorphisms in which a base-pair substitution leads to change in an amino acid sequence of a membrane glycoprotein expressed on the platelet surface.

The aim of this study was to examine the association of HPA-1, HPA-2, HPA-3 and HPA-5 polymorphisms with idiopathic thrombocytopenic purpura.

We performed genotyping of HPA-1, HPA-2, HPA-3, and HPA-5 systems in 60 patients with ITP and 120 healthy participants. Genotyping of HPA-1, -2, -3, and -5 alleles were performed by PCR and RFLP methods by using specific primers and restriction enzymes.

Allele and genotype frequencies of HPA-1, HPA-3, and HPA-5 were not significantly different between patients and healthy participants. After Bonferroni adjustment a significant association in ITP patients with HPA-2 alleles (P = 0.015, OR = 1.923, CI = 1.126-3.284) was found. Allele frequencies for HPA-2a were 0.852 in healthy participants and 0.750 in patients, and for HPA-2b 0.148 and 0.250 respectively.

These results suggests that HPA-2b allele was more frequent in patients with ITP and may be involved in the formation of a specific autoepitope.

Key words: idiopathic thrombocytopenic purpura, human platelet antigen systems, restrictiction fragment length polymorphisms.

Introduction

Idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease 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 clearance of opsonized platelets by phagocytes 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 a role in the development of the disease. Several genes involved in immune system regulation such as cytokine genes, Fc gamma receptor genes and HLA genes [3, 4], as well as some infective agents like hepatitis C virus, HIV virus, and helicobacter pylori [5–7] have been associated with susceptibility to ITP in several studies.

It was shown that platelet membrane glycoprotein (GP) complexes, particulary GPIIb/IIIa and GPIb/IX, were the main targets (autoantigens) for the autoantibodies in patients with ITP [8, 9], while GP Ia/IIa was a minor target. Human platelet antigen (HPA) systems HPA-1, HPA-2, HPA-3 and HPA-5 are components of platelet GP complexes GPIIb/IIIa, GPIb/IX and GPIa/IIa. The HPA system consists of more than 12 bi-allelic antigen polymorphisms in which a base-pair substitution leads to change in an amino acid sequence of a membrane glycoprotein expressed on the platelet surface. Due to these polymorphisms, human platelet membrane glycoproteins can be recognized as allo- or autoantigens and can cause different clinical conditions such as post-transfusion refractoriness to platelets, post-transfusion thrombocytopenic purpura and foetomaternal alloimmune thrombocytopenia [10]. There are studies that analyse the possible role and association of HPA polymorphisms with ITP and report a higher incidence of some HPA alleles in patients with ITP. Thude et al. [11] reported in their study a difference in allele frequencies for the HPA-2 system, while others [12, 13] described a significant difference in the allele frequencies for the HPA-5 system in patients with ITP.

The aim of this study was to analyse the frequencies of human platelet (HPA) polymorphisms HPA-1, HPA-2, HPA-3, and HPA-5 in Macedonian patients with ITP and to clarify potential associations between those HPA polymorphisms and the development of autoimmune thrombocytopenia.

Material and methods

Investigated Groups

The total studied sample consisted of 180 examinees composed of two different groups: healthy individuals and patients with idiopathic thrombocytopenic purpura.

Healthy participants (n = 120), 70 female and 50 male, aged 40.7 ± 11.3 years, attending the Institute of Immunobiology and Human Genetics for DNA donation. Inclusion of healthy individuals was random, if a medical doctor declared their health as acceptable (on the basis of medical documentation, completed interview, and physical examination). Individuals with a family history of blood diseases were excluded from the investigation.

Consecutive patients with chronic immune thrombocytopenia (n = 60), 43 women and 17 men, with an average age of 46.8 ± 16.8 years, referred to the University Clinic of Haematology, Faculty of Medicine, Skopje were included. All patients met the diagnostic criteria for ITP a) platelet count below 100×10^9/L in peripheral blood, b) normal or increased megakaryopoesis on bone marrow examination, and c) the absence of clinically apparent associated conditions or causes of thrombocytopenia.

All individuals were of Macedonian origin, and residents of different geographical areas of the Republic of Macedonia. All patients and healthy individuals included in this study signed a written consent to participate in the study.

Genomic DNA isolation and HPA genotyping

Blood samples were collected after written consent and DNA was isolated from peripheral blood leukocytes by the phenol-chlorophorm extraction method or with BioRobot EZ1 workstation (QIAGEN) [14]. The quality and quantity of DNA was analysed by GeneQuant (Pharmacia). Isolated DNA samples were stored in the Macedonian Human DNA Bank (hDNAMKD) [15]. Genotyping of HPA-1, -2, -3, and -5 alleles were performed by PCR and RFLP methods by using specific primers and restriction enzymes [16, 17].

Statistical Methods

The population genetics analysis package, PyPop, developed by the Biostatistics Core for the Workshop [18–20] was used for analysis of the HPA data for this report. Allele frequencies and expected Hardy Weinberg proportions (HWP) for each HPA allele were determined [21]. The exact test for genotype frequency deviation from HWP was calculated using the Arlequin implementation accessed via PyPop [22]. Those alleles that did not fit HWP were evaluated to determine whether there was an excess of homozygotes or hetero-

zygotes, or if any particular genotypes were significantly different from expected frequencies by the chi square test. The Ewens-Watterson homozygozity test of neutrality (EWN) [23] with Slatkin's exact p-values (SEPV) [24, 25] was used to indicate any deviations from the hypothesis of neutral selection for each locus. Pearson's p-values, crude Odds Ratio (OR) and Wald's 95% confidence interval (CI) were calculated for analysis of associations between HPA alleles and idiopathic thrombocytopenic purpura with GraphPad QuickCalcs: free statistical calculators (http: //www.graphpad.com/quickcalcs/) with Bonferroni corrected p-value [26]. P less than 0.05 were taken as significant.

Results

Frequencies of HPA-1, HPA-2, HPA-3, and HPA-5 alleles, test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality), and Slatkin's Exact P Value with P of F statistics for each investigated group is shown in Table 1.

The frequency of HPA-1a, HPA-2a, HPA-3a, and HPA-5a dominate in healthy participants and in ITP patients with the biggest value for the HPA-5a (0.909 and 0.925, respectively) and smallest value for HPA-3a (0.578 and 0.525 respectively). For the all HPA alleles, test of neutrality showed negative value for F_{nd} statistic, with significant P of F statistics for HPA-3 in ITP patients (P = 0.020), which was significant after Bonferroni correction (Table 1).

Table 1

Frequencies of HPA-1, HPA-2, HPA-3, and HPA-5 alleles, test of neutrality with Fnd statistic (Ewens-Watterson test of neutrality), and Slatkin's Exact P Value with P of F statistics for each investigated group

	n*	Alleles			Test of Neutrality (F)	
					EWN†	SEPV‡
		Allele	Number	Frequency	Fnd	P of F
Healthy						
HPA-1	240	HPA-1a	207	0.863	-0.439	0.305
		HPA-1b	33	0.137		
HPA-2	264	HPA-2a	225	0.852	-0.542	0.285
		HPA-2b	39	0.148		
HPA-3	230	HPA-3a	133	0.578	-1.929	0.055
		HPA-3b	97	0.422		
HPA-5	252	HPA-5a	229	0.909	-0.021	0.375
		HPA-5b	23	0.091		

ITP Patients						
HPA-1	120	HPA-1a	106	0.883	-0.131	0.380
		HPA-1b	14	0.117		
HPA-2	120	HPA-2a	90	0.750	-1.141	0.210
		HPA-2b	30	0.250		
HPA-3	120	HPA-3a	63	0.525	-1.881	0.020*
		HPA-3b	57	0.475		
HPA-5	120	HPA-5a	111	0.925	0.272	0.478
		HPA-5b	9	0.075		

*, n = number of participants; †, EWN = Ewens-Watterson test of neutrality; ‡, SEPV = Slatkin's Exact *P* Value; *****, statistically significant after Bonferroni adjustment (p-value x number of alleles) < 0.05.

Observed vs. expected HPA-1, HPA-2, HPA-3, and HPA-5 genotypes for each investigated group, Hardy Weinberg proportions, and Guo and Thompson Hardy Weinberg Output is shown in Table 2.

The most frequent HPA genotypes in both investigated groups were homozygous a/a genotypes (HPA-1a1a, HPA-2a2a, HPA-3a3a, and HPA-5a5a), less frequent were heterozygous a/b genotypes (HPA-1a1b, HPA-2a2b, HPA-3a3b, and HPA-5a5b), and homozygous b/b genotypes (HPA-1b1b, HPA-2b2b, HPA-3b3b, and HPA-5b5b) were very rare. Most of the genotypes in healthy participants and in ITP patients showed a good fit with HWP expectations, except the homozygous genotypes (HPA-1b1b, HPA-2b2b and HPA-5b5b) which could not be calculated because their frequencies were less than 5 (Table 2).

Table 2

Observed vs. expected HPA-1, HPA-2, HPA-3, and HPA-5 genotypes for each investigated group, Hardy Weinberg proportions, and Guo and Thompson Hardy Weinberg Output

Gene	Genotype	Observed number	Expected number	P-value	HWP* P-value	GTHWO† P-value
Healthy						
HPA-1	HPA-1a1a	90	89.3	0.938	0.775	0.461
	HPA-1a1b	27	28.5	0.784		
	HPA-1b1b	3	2.3	‡		
HPA-2	HPA-2a2a	95	95.9	0.928	0.750	0.737
	HPA-2a2b	35	33.2	0.760		
	HPA-2b2b	2	2.9	‡		
HPA-3	HPA-3a3a	41	38.5	0.681	0.330	0.345
	HPA-3a3b	51	56.1	0.497		
	HPA-3b3b	23	20.5	0.573		
HPA-5	HPA-5a5a	104	104.0	0.996	0.982	1.000
	HPA-5a5b	21	20.9	0.983		
	HPA-5b5b	1	1.0	‡		

ITP Patients						
HPA-1	HPA-1a1a	48	46.8	0.863	0.487	0.165
	HPA-1a1b	10	12.4	0.501		
	HPA-1b1b	2	0.8	‡		
HPA-2	HPA-2a2a	33	33.8	0.897	0.733	0.742
	HPA-2a2b	24	22.5	0.752		
	HPA-2b2b	3	3.8	‡		
HPA-3	HPA-3a3a	20	16.5	0.394	0.073	0.073
	HPA-3a3b	23	29.9	0.205		
	HPA-3b3b	17	13.5	0.347		
HPA-5	HPA-5a5a	51	51.3	0.962	0.811	1.000
	HPA-5a5b	9	8.3	0.815		
	HPA-5b5b	0	0.0	‡		

* HWP = Hardy Weinberg proportions; † GTHWO = Guo and Thompson Hardy Weinberg Output; ‡ Cannot be calculated because expected ≤5, $\chi 2$ test.

Association between HPA-1, HPA-2, HPA-3, and HPA-5 alleles and genotypes with idiopathic thrombocytopenic purpura (ITP) with Pearsons P-value, crude odds ratio, and Wald's 95% confidence interval is shown in Table 3.

A significant association in ITP patients with HPA-2 alleles (P = 0.015, OR = 1.923, CI = 1.126–3.284) was found. For all the rest of the HPA alleles and genotypes no significant association in ITP patients was found. Most of the associations with homozygous genotypes (HPA-1b1b, HPA-2b2b and HPA-5b5b) could not be calculated because their frequencies were less than 5 and the chi-square test was not calculated (Table 3).

Table 3

Association between HPA-1, HPA-2, HPA-3, and HPA-5 alleles and genotypes with idiopathic thrombocytopenic purpura with Pearsons P-value, crude odds ratio, and Wald's 95% confidence interval

Gene	Allele or Genotype	ITP*	Healthy	Odds ratio	Wald's 95% CI†	Pearson's P-value
HPA-1	HPA-1a	106 (88.3%)	207 (86.3%)	1.207	0.619–2.353	0.580
	HPA-1b	14 (11.7%)	33 (13.7%)	0.828	0.425–1.615	
	HPA-1a1a	48 (80.0%)	90 (75.0%)	1.333	0.626–2.838	0.455
	HPA-1a1b	10 (16.7%)	27 (22.5%)	0.689	0.309–1.537	0.361
	HPA-1b1b	2 (3.3%)	3 (2.5%)	‡	‡	‡
HPA-2	HPA-2a	90 (75.0%)	225 (85.2%)	0.520	0.304–0.888	0.015*
	HPA-2b	30 (25.0%)	39 (14.8%)	1.923	1.126–3.284	
	HPA-2a2a	33 (55.0%)	95 (72.0%)	0.476	0.252–0.898	0.021
	HPA-2a2b	24 (40.0%)	35 (26.5%)	1.848	0.969–3.522	0.060
	HPA-2b2b	3 (5.0%)	2 (1.5%)	‡	‡	‡
HPA-3	HPA-3a	63 (52.5%)	133 (57.8%)	0.806	0.517–1.256	0.341

	HPA-3b	57 (47.5%)	97 (42.2%)	1.240	0.796–1.933	
	HPA-3a3a	20 (33.3%)	41 (35.7%)	0.902	0.467–1.744	0.760
	HPA-3a3b	23 (38.4%)	51 (44.3%)	0.780	0.412–1.475	0.445
	HPA-3b3b	17 (28.3%)	23 (20.0%)	1.581	0.766–3.262	0.213
HPA-5	HPA-5a	111 (92.5%)	229 (90.9%)	1.239	0.555–2.766	0.601
	HPA-5b	9 (7.5%)	23 (9.1%)	0.807	0.361–1.802	
	HPA-5a5a	51 (85.0%)	104 (82.5%)	1.199	0.515–2.790	0.674
	HPA-5a5b	9 (15.0%)	21 (16.7%)	0.882	0.377–2.063	0.773
	HPA-5b5b	0 (0.0%)	1 (0.8%)	‡	‡	‡

* ITP = idiopathic thrombocytopenic purpura; † CI = confidence interval; ‡ Cannot be calculated because expected ≤5, $\chi 2$ test; * statistically significant after Bonferroni adjustment (p-value x number of alleles) < 0.05.

Discussion

In this manuscript we report HPA-1, HPA-2, HPA-3, and HPA-5 polymorphisms that exist in Macedonians, and possible association with idiopathic thrombocytopenic purpura. After Bonferroni adjustment, only HPA-2 alleles showed a significant association in ITP patients. Several types of multiple testing corrections were used: i) Bonferroni; ii) Bonferroni Step-down (Holm); iii) Westfall and Young Permutation; and iv) Benjamini and Hochberg False Discovery Rate [26, 27]. The methods are listed in order of their stringency, with the Bonferroni being the most stringent, and the Benjamini and Hochberg FDR being the least stringent. The more stringent a multiple testing correction, the less false positive genes are allowed. The trade-off of stringent multiple testing corrections are that the rate of false negatives is very high.

We found negative value for F_{nd} statistic for HPA alleles, with significant P of F statistics for HPA-3 alleles in ITP patients (but not in healthy participants) which indicates balancing selection operating on the alleles at that cluster. We found also that HPA-1, HPA-2, HPA-3 and HPA-5 genotypes are in equilibrium with HWP.

Our results are different from the results reported by other studies. We did not find a significant difference in the allele frequencies for HPA-5 antigen as Kim and Song [12] did in their study in Korean patients with ITP. This can be explaining by the population difference. Our results are also different from the results reported by Thude et al. [11]. In their study the HPA-2b allele was not found in patients with ITP and all patients had only the HPA-2a allele. On the other hand, the HPA-2b allele was found in 8% of controls. This difference in allele frequency for the HPA-2 alleles was statistically significant (p = 0.017) but contrary to our results where the HPA-2b allele was significantly more prevalent in patients with ITP than in controls (25% versus 14.8%, p = 0.015).

This difference in results could be caused by the difference in selected patients and by the difference in distribution of HPA-2 alleles in Macedonian and German populations (14.8% versus 8%). Thude et al. analyzed only German patients with chronic refractory ITP, while we analysed 60 consecutive patients with ITP referred to our department.

an interesting observation in this study is the highest prevalence of the HPA-2b allele (0.148) in Macedonians among European studies and the highest prevalence of this allele in patients with ITP (0.25). We have already referred to the highest prevalence of the HPA-2b allele (0.148) in the Macedonian population in Europe [17] compared with 0.086 in Germans [28], 0.006 in French [29], 0.082 in Austrians [30], and 0.075 in the UK [31]. These results indicate that this allele may be a specific autoepitope in some patients with ITP or a common alloantigen that causes alloimunization in the HPA system. It would be interesting to correlate these findings with the incidence and prevalence of ITP in Macedonia compared with the incidence of ITP in other European countries. But due to insufficient data for the incidence and prevalence of ITP in the Republic of Macedonia relevant conclusions are not possible. At this moment, we have only data for ITP patients diagnosed and treated in our hospital, but it is a tertiary centre and probably significant numbers of patients with ITP are not referred to our hospital. Idiopathic thrombocytopenic purpura is a condition that could have mild asymptomatic forms, very often does not need treatment and patients are often not referred to our department for diagnosis or treatment. We need a population-based study from a defined medical region to determine the real incidence and prevalence of ITP in Macedonia and to compare it with other European countries in relationship with the frequency of HPA-2b allele.

In summary, our results have shown no significant difference in allele and phenotype frequencies for HPA-1, HPA-3, and HPA-5 systems between patients with chronic ITP (n = 60) and healthy participants (n = 120). We have found a significant difference in allele frequency for the HPA-2 system ($p = 0.015$) between patients and healthy participants. These results suggests that the HPA-2b allele was more frequent in patients with ITP and may be involved in the formation of a specific autoepitope.

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Резиме

АСОЦИЈАЦИЈА НА ПОЛИМОРФИЗМИТЕ ВО ХУМАНИТЕ ТРОМБОЦИТНИ АНТИГЕНИ И ИДИОПАТСКАТА ТРОМБОЦИТОПЕНИЧНА ПУРПУРА КАЈ МАКЕДОНСКОТО НАСЕЛЕНИЕ

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Идиопатската тромбоцитопенична пурпура (ИТП) е автоимуно заболување кое се карактеризира со појава на тромбоцитопенија која се должи на присуството на антитромбоцитни автоантитела специфични за тромбоцитните мембрански гликопротеини, како GPIIb/IIIa, GPIb/IX и GPIa/IIa. Овие автоантитела предизвикуваат зголемен клиренс на опсонизираните тромбоцити по пат на фагоцитоза и инхибиција на тромбоцитната продукција.

Хуманите тромбоцитни антигени (ХТА) ХТА-1, -2, -3 и ХТА-5 се делови од тромбоцитните комплекси GPIIb/IIIa, GPIb/IX и GPIa/IIa. ХТА системот се состои од 12 би-алелни антигенски полиморфизми кај кои промената во една нуклеотидна база доведува до аминокиселинска супституција во мембранскиот гликопротеин.

Цел на оваа студија беше да се испита асоцираноста на ХТА-1, -2, -3, и -5 полиморфизми со идиопатска тромбоцитопенична пурпура.

Анализирани беа 60 болни со ИТП и 120 здрави контролни индивидуи. Генотипизацијата беше изведена со помош на PCR и RFLP методата со користење на специфични олигонуклеотиди и рестрикциони ензими.

Алелната и генотипската фреквенција на ХТА-1, ХТА-3, и ХТА-5 не се разликуваше значително помеѓу пациентите и здравите контроли. По Bonferroni израмнувањето, сигнификантна разлика во алелната фреквенција за ХТА-2 беше најдена меѓу пациентите со ИТП и контролите (P = 0,015, OR = 1,923, CI = 1,126–3,284). Алелната фреквенција за ХТА-2а беше 0,852 кај здравите контроли и 0,750 кај болните со ИТП, и 0,148 за ХТА-2б кај контролите наспроти 0,250 кај болните со ИТП.

Овие резултати укажуваат на почеста фреквенција на ХТА-2б алелот кај болните со ИТП и можноста тој да учествува во создавањето на специфичен автоепитот.

Клучни зборови: идиопатска тромбоцитопенична пурпура, хумани тромбоцитни антигени, рестрикциона анализа на генски полиморфизми.

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