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ORIGINAL PAPER

Evaluation of HLA-B*05, *07, *08, *27 and *51 Allele Expression in Adults with Immune Thrombocytopenic Purpura

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ABSTRACT

Background: *Immune thrombocytopenic purpura (ITP) is an immune mediated acquired disease characterized by isolated thrombocytopenia. Since there is no specific and sensitive biomarkers to guide treatment of ITP patients, this study aimed to evaluate the possible application of human leukocyte alleles HLA-B5, 7, 8, 27 and 51 and their association with patients' laboratory data and clinical findings.*

Methods: *Thirty-one adult patients with chronic ITP were included in the present study. Human leukocyte antigen (HLA) typing was done using the standard lymphocytotoxicity HLA typing method. Moreover, patients' medical records were used to collect data about disease presentations, platelet count at diagnosis.*

Results: *Our study included 31 patients (25 females and six males) with a mean age of 40.23±17.06 years. Among all participants, HLA-B5 (25.8%) and HLA-B51 (22.6%) alleles were the most prevalent alleles, followed by HLA-B7 (9.7%) and HLA-B8 (9.7%), HLA-B27 (3.2%). The mean platelet count significantly was lower in patients with HLA-B5 (16.13^{x}103/μL* versus *36.63^{x}10^{3}/μL) (P=0.01) and HLA-B51 (14.14^{x}10^{3}/μL* versus *36.24^{x}10^{3}/μL) (P=0.04). Also, epistaxis and gingival bleeding were observed in patients with 11.5^{x}10^{3}/μL mean platelet count (P=0.04). In addition, lymphocyte and neutrophil cell counts were significantly associated with the expression of HLA-B*05 and HLA-B*51 antigens (P <0.05).*

Conclusion: *According to the present study results, it seems that HLA-B5 and HLA-B51 alongside complete blood count test parameters may have a positive relationship in ITP patients.*

Keywords: human leukocyte antigen, immune thrombocytopenic purpura, platelet.

INTRODUCTION

Immune thrombocytopenic purpura (ITP) is an immune mediated acquired disease characterized by isolated thrombocytopenia (1). There is a lack of data about the prevalence and incidence of ITP worldwide (2). However, an incidence of 1.6 to 4 *per* 100,000 adults *per* year and a prevalence of up to 10 *per* 100,000 adults were reported in different studies (3-5). Diagnosis of ITP is made by excluding other conditions associated with thrombocytopenia (1, 6, 7). Therefore, ITP is classified into two categories, in-

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cluding primary (no known cause) and secondary (all causes of isolated autoimmune thrombocytopenia) (1, 3, 6, 7). Also based on disease duration, ITP is divided into three phases: acute (first three months), persistent (three to 12 months) and chronic (more than 12 months) (1, 3, 6). In adults, almost 80% of cases have primary ITP (3, 4) and about 70% chronic ITP (5). The main presentation of ITP is represented by bleeding, including petechiae, purpura, mucosal bleeding, etc (4). Fatal bleeding is rarely reported in 5% of cases (3, 5). The pathogenesis of ITP is complex and not exactly clear. Generally peripheral destruction of platelets and reduced platelets production are the leading causes of ITP (7). Production of autoantibodies against platelets and megakaryocytes, impaired regulation of immune cells, and change of cytokines profile play roles in ITP pathogenesis. Since a detectable autoantibody is founded in 60% of ITP patients, it seems T cell mediated mechanisms are involved too (3, 4). Besides, both genetics predisposing and environmental factors participate in the pathogenesis of autoimmune diseases, and HLA genes are known to contribute to autoimmunity (4, 8). In addition, treatment of ITP is still controversial, especially in patients with lower platelet counts and no bleeding (9). Thus, introducing biomarkers are essential to help our clinical judgment and guide our treatment (5). Hence, in this study we aimed to evaluate the expression of HLA-B*05, *07, *08, *27 and *51 in adult ITP patients in order to determine their value as a biomarker. ❑

MATERIALS AND METHODS

Selection of patients

Data of the present study has been collected from 31 adults with chronic ITP by using patients' medical records who were referred to our hematology clinic (Shahid Baghaei Hospital in Ahvaz, Southwestern Iran) for treatment follow-up from 2017 to 2020, and included information about patients' age at diagnosis, gender, first presentation of disease and complete blood count (CBC) at diagnosis.

Informed consent was obtained from all participants prior to the commencement of the study. This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1398.252).

Isolation of lymphocytes

Ten milliliters of blood into heparin anticoagulant were collected from each patient. After samples defibrination, 4 mL of Hanks solution were added to six mL blood. After adding Ficoll solution, samples were centrifuged at 1500 rpm for 20 minutes and then washed three times with Hanks's solution. Using a Hamilton syringe, one microliter (µL) of suspension and one µL HLA anti-serum was added to each well of plates. Plates were incubated at room temperature (RT) for 30 minutes. Complement was added to wells and plates incubated at RT for 60 minutes. Also, 5% eosin dye and 37% formaldehyde were added to wells. Finally, plates were evaluated using an inverted microscope (Figure 1).

HLA typing and other laboratory parameters

HLA typing was done by the standard lymphocytotoxicity HLA typing method. HISTO TRAY Disease HLA I Class Kit (BAG HEALTHCARE, Lich, Germany) was used to detect HLA alleles.

Statistical analysis

A one-way independent sample T-test was used to evaluate the association between initial platelet count and clinical presentations. Independent sample T-tests were also used to assess the possible association between the most prevalent HLA antigens and laboratory biomarkers. The level of significance was considered as $p<0.05$. Data analysis was performed using SPSS statistics software (V.22. IBM Corporation, Armonk, NY). ❑

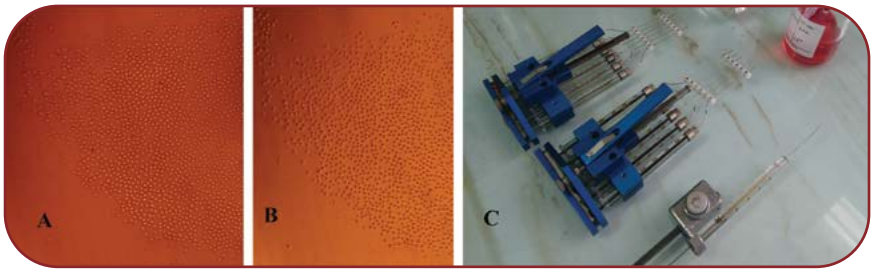

FIGURE 1. Interpretation of HLA expression with invert microscope and Hamilton syringes: A) Live lymphocytes are seen as small colorless spheres, which mean negative expression; B) dead lymphocytes are seen as tiny dark spheres, which means positive expression; C) Hamilton syringes

TABLE 1. Patients' characteristics and laboratory findings

Laboratory characteristics	Primary (N=25)	Secondary (N=6)	Total (N=31)
Hb (mean ± SD) (g/dL)	13.1 ± 1.5	12.3 ± 1.4	12.9 ± 1.5
RBC (mean ± SD) ($\times 10^6/\mu L$)	4.6 ± 0.5	4.3 ± 0.61	4.6 ± 0.57
Hct (mean ± SD) (%)	40 ± 4.3	37.8 ± 4.1	39.5 ± 4.3
Plt (mean ± SD) ($\times 10^3/\mu L$)	29.7 ± 23.7	41 ± 19.5	29.7 ± 23.7
MPV (mean ± SD) (fl)	9.3 ± 1.8	11.8 ± 2.1	9.7 ± 2.1
PDW (mean ± SD) (%)	16.4 ± 2.6	15.3 ± 2.2	16.2 ± 2.5
Lymphocyte (mean ± SD) ($\times 10^3/\mu L$)	2.5 ± 1.7	2 ± 0.7	2.4 ± 1.1
Neutrophil (mean ± SD) ($\times 10^3/\mu L$)	4.6±1.5	6.1 ± 3.6	4.94 ± 2.1
Basophil (mean ± SD) ($\times 10^3/\mu L$)	0.04 ± 0.04	0.01 ± 0.01	0.03 ± 0.04
Monocyte (mean ± SD) ($\times 10^3/\mu L$)	0.5 ± 0.26	0.5 ± 0.2	0.52 ± 0.25
Eosinophil (mean ± SD) ($\times 10^3/\mu L$)	0.09 ± 0.11	0.14 ± 0.15	0.1 ± 0.11
Type of ITP	**Females**	**Malse**	**Total**
✓ Primary	20 (80%)	5 (20%)	25 (80.6%)
✓ Secondary	5 (83.3%)	1 (16.7%)	6 (19.4%)

RESULTS

Thirty-one adult patients with chronic ITP participated in this study, of which 25 (80.6%) individuals had primary type of ITP and six (19.6%) secondary type of ITP due to systemic lupus erythematosus. The mean age of participants at diagnosis was 40.23±17.06 years and 80% of all subjects were females. Laboratory characteristics and types of ITP are presented in Table 1.

HLA antigen expressions and clinical findings

HLA-B5 (25.8%) and HLA-B51 (22.6%) antigens were the most prevalent alleles, followed by HLA-B7 (9.7%) and HLA-B8 (9.7%), HLA-B27 (3.2%) (Figure 2). The frequency of HLA alleles in primary ITP patients were HLA-B5 (33%), HLA-B51(29%), HLA-B7(12.5%), HLA-B8(8.33%) and HLA-B27(0%), and there were no significant differences in terms of frequency (P >0.05).

Patients were assessed based on their first presentation, with 22% of all subjects being asymptomatic and incidentally diagnosed by CBC test results. Among symptomatic patients, cutaneous petechiae and purpura was the most prevalent manifestation (38.7%). None of the participants had fatal or serious bleedings (Table 2). In the evaluation of chronic primary ITP patients, all symptomatic ones had a platelet count under $30^{*}10^3/\mu L$ although the difference was not significant (p value 0.7). Further evaluation showed an association between platelet count and occurrence of epistaxis and gingival bleeding (P=0.04) in this type of ITP patients (Table 3). There was no association between age and primary mean platelet count.

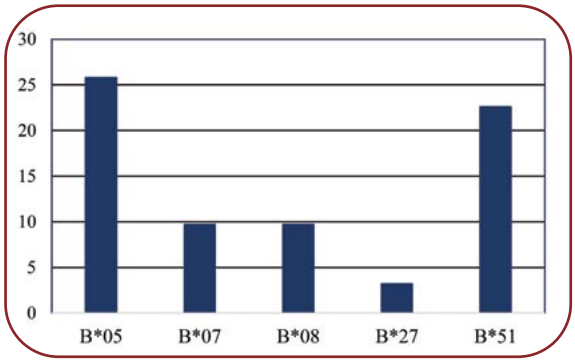

FIGURE 2. Frequency of HLA*B alleles among study participants

TABLE 2. Frequency of first disease manifestations

First manifestation	Primary (25)	Secondary (6)	Total (31)
Cutaneous petechia and purpura	44%	16.7%	38.7%
Ecchymosis	36%	33.3%	35.5%
Epistaxis and gingival bleeding	16%	0	12.9%
Vaginal bleeding	8%	16.7%	9.7%
Asymptomatic	12%	66%	22.5%

*Some patients had more than one symptom

Clinical feature	Results	Count	Mean platelet count	SD	P-value
Cutaneous petechia and purpura	Positive	11	29 ×10³/μL	23.4	0.711
	Negative	13	30 ×10³/μL	25	
Ecchymosis	Positive	9	20 ×10³/μL	17.9	0.106
	Negative	15	35 ×10³/μL	25.4	
Epistaxis and gingival bleeding	Positive	4	11.5 ×10³/μL	8.4	0.04
	Negative	20	33.4 ×10³/μL	24.2	
Vaginal bleeding	Positive	2	15.5 ×10³/μL	13.4	0.284
	Negative	22	31 ×10³/μL	24	
No symptoms	Positive	3	64 ×10³/μL	12.4	0.724
	Negative	21	24 ×10³/μL	10.4	

*Clinical presentation of 29 patients were available. SD=standard deviation

TABLE 3. Association between patients' platelet count and clinical presentations in chronic primary ITP

Association of HLA antigen expressions with hematological findings

The mean platelet count was 29.7±23.7 and 41±19.5 in primary and secondary patients, respectively. It was significantly lower in chronic primary patients with HLA-B5 (16.13×10³/μL *versus* 36.63×10³/μL) (P=0.01) and HLA-B51 (14.14×10³/μL *versus* 36.24×10³/μL) (P=0.04) (Table 4). Further analysis has revealed that the mean neutrophil count was significantly higher in chronic primary patients positive for HLA-B5 (P=0.02) and B51 (P=0.01) (Table 5). By contrast, the lymphocyte count was lower in patients positive for HLA-B5 (P=0.02) and HLA-B51 (P=0.04) (Table 5). ❑

Allele	Result	Number	Mean platelet count	Standard deviation	P-value
HLA-B5	Positive	8	16.13×10³/μL	9.18	0.01
	Negative	16	36.36×10³/μL	26.06	
HLAB51	Positive	7	14.14×10³/μL	67.8	0.004
	Negative	17	36.24×10³/μL	25.29	
HLA-B7	Positive	3	45.33×10³/μL	31.89	0.23
	Negative	21	27.57×10³/μL	22.54	
HLA-B8	Positive	2	32×10³/μL	38.18	0.89
	Negative	22	29.52×10³/μL	22.48	
HLA-B27	Positive	0	-	-	-
	Negative	24	29.79×10³/μL	23.79	

TABLE 4. Association of HLA alleles and mean platelet count in chronic primary ITP

DISCUSSION

Various mechanisms for the contribution of the HLA system to the development of autoimmune disorder have been shown, including, among others, alteration in peptide-TCR-HLA interaction, conversion in HLA expression, and post-translational modification in peptide binding cause (10). Hence, the HLA system has a key role in immune regulation.

HLA molecules are located on the short arm of chromosome 6p21 and encode the major histocompatibility complex. By presenting the peptide antigens to the immune cells, the HLA system causes an immune response. One of the most important factors contributing to stimulation of the immune system to produce antibodies is the

TABLE 5. Assessment of association between most prevalent HLA antigens with possible complete blood count test biomarkers

Parameter	HLA-B5	Mean count (SD)	P-value	HLA-B51	Mean count (SD)	P-value
Neutrophil count (%)	Positive	68.41±10.08	0.02	Positive	68.31±11.04	0.01
	Negative	55.94±14.04		Negative	56.67±13.97	
Lymphocyte count (%)	Positive	24.48±7.80	0.02	Positive	24.35±8.53	0.04
	Negative	34.74±11.61		Negative	34.22±11.48	

groove peptide allele sequencing. More recent attention has been focused on providing the role of HLA system in ITP pathogenesis. Detailed examination of the association between HLA and ITP by Olmsted Kim *et al* showed that HLA-A, B, and C increased in Caucasian pediatrics patients with chronic ITP (11). Glycoprotein IIb/IIIa is well known as the recognizing target by the HLA system. In this regard, to better understand the mechanism of HLA in ITP development, Hall *et al* found that peptide immunotherapy suppressed antibody in ITP treatment. They showed that the combination of glycoprotein IIIa peptides aa6-20 and aa711-725 caused the suppression response in ITP patients (12). Also, to evaluate the role of HLA in immune thrombocytopenia Angénieux *et al* reported a higher expression of MHC class 1 in young platelets of human subjects (13). Although many investigations have been conducted, the exact pathogenesis of HLA in association with autoimmune diseases is not well known – for example, HLA-B27 has a diagnostic association with ankylosing spondylitis, while HLA-B51 does not have a diagnostic value, despite a strong correlation with Behçet's disease, and is mostly associated with Behçet's cutaneous manifestations (14, 15). Besides, the distribution of HLA is entirely dependent on race and does not have the same distribution in different regions according to ethnicity (16).

The current survey aims to investigate the association between HLA-B5, 7, 8, 27, and 51 with ITP development and manifestations. Our findings revealed that HLA-B5 and HLA-B51 had the highest prevalence, which was in line with a previous report that found a higher frequency of HLA-B5 and HLA-B51 in pediatric patients with acute ITP in the same region (17).

Further analysis has demonstrated that HLA-B5 and HLA-B51 correlated with lower platelet count in adults with chronic primary ITP (p value 0.01 and 0.004, respectively), and in patients who were positive for these two alleles, the mean platelet count was lower than $20^{x}10^{3}/\mu L$. Based on ASH guideline, although the bleeding manifestations of ITP do not directly depend on platelet count, patients with platelet count lower than $20^{x}10^{3}/\mu L$ are more prone to serious bleedings and should receive treatment to reach a safe platelet count (6).

Although the findings of the present study may suggest a correlation between platelet count in chronic primary ITP and HLA-B5 and HLA-B51 alleles, more studies in different populations with larger sample sizes are demanded to identify whether HLA-B51 could be a predictive biomarker or not.

According to our findings, epistaxis and gingival bleedings occur in lower platelet count (p value 0.04). One of the explanations could be the lower rate of detection of minor bleedings, especially in patients with gingival bleeding. This improves the view of some experts about the necessity of treating ITP patients to achieve a safe platelet count.

Further analysis has demonstrated that higher neutrophil cell counts were significantly associated with the expression of HLA-B5 and HLA-B51 alleles in chronic ITP patients. Xu *et al* suggested that neutrophils were associated with ITP development and neutrophil count was elevated in ITP patients (18). Similarly, the evaluation of HLA-B51 pathogenicity in Behçet's disease suggested that HLA-B51 could cause excessive activity of neutrophils (14). However, further studies need to investigate the interaction between HLA alleles and neutrophils.

Finally, it should be noted that we encountered some limitations, such as the small sample size in this study, which limited our certainty to conclude about obtained results. However, we achieved probable relationships and valuable insights to add knowledge from ITP patients, suggesting further studies in this area. ❑

CONCLUSIONS

Overall, the current study indicated a potential role of HLA-B5 and 51 in predisposition to ITP development. Also, it demonstrated that patients with HLA-B5 and 51 alleles had lower platelet counts, which was accompanied by poor prognosis. In this regard, more research evaluating the role of HLA in chronic ITP patients is strongly recommended. ❑

Conflicts of interest: none declared.

Financial support: none declared.

Research involving human participants and/or animals: All procedures performed in studies involving human participants were in accordance with the ethical standards of the local ethics

committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1398.252) as well as the 1964 Declaration of Helsinki.

Informed consent: Written informed consent was obtained from all participants.

Authors' contributions: AE conceived the manuscript and NYR, AE, TV, NS and SMSP wrote it and prepared tables and figures.

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