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JAPCH ISSN 2689-9817 JOURNAL OF ADVANCED PEDIATRICS AND CHILD HEALTH

Research Article

Evaluation of influence of *IL-6* C-572G gene polymorphism and clinical factors on positive platelet antibody test

Jeong-Shi Lin[1-3]*, Li-Hsuan Lee[1], Hsueng-Mei Liu[1], Ying-Ju Chen[1] and Tzeon-Jye Chiou[1-4]*

[1]Division of Transfusion Medicine, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan

[2]Division of Hematology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan

[3]National Yang-Ming University School of Medicine, Taipei, Taiwan

[4]Cancer Center, Taipei Municipal Wanfang Hospital, Taipei, Taiwan

More Information

***Address for Correspondence:** Jeong-Shi Lin, No. 201, Sec. 2, Shih-Pai Road, Division of Transfusion Medicine, Taipei, 11217, Taiwan, Tel: 886-2-2875-7623; Email: jslin@vghtpe.gov.tw

Tzeon-Jye Chiou, No. 201, Sec. 2, Shih-Pai Road, Division of Transfusion Medicine, Taipei, 11217, Taiwan, Tel: 886-2-2875-7623; Email: jslin@vghtpe.gov.tw

Submitted: December 28, 2020
Approved: January 14, 2021
Published: January 15, 2021

How to cite this article: Lin JS, Lee LH, Liu HM, Chen YJ, Chiou TJ. Evaluation of influence of *IL-6* C-572G gene polymorphism and clinical factors on positive platelet antibody test. J Adv Pediatr Child Health. 2021; 4: 006-012.

DOI: 10.29328/journal.japch.1001023

ORCiD: orcid.org/0000-0002-9284-6665

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Keywords: Interleukin-6; Single nucleotide polymorphism; Platelet antibody; Transfusion

Abstract

Background: Interleukin-6 (*IL-6*) promotes antibody production. The objective of this study was to investigate whether *IL-6* C-572G single nucleotide polymorphisms (SNP) and clinical factors are associated with positive platelet antibody test.

Materials and methods: Thirty platelet recipients with platelet antibodies (responders) and 20 platelet recipients without platelet antibodies (non-responders) were randomly selected. The -572 C>G (rs 1800796) SNPs in the promoter region of *IL-6* gene were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. Solid phase red cell adherence assay (SPRCA) was used for platelet antibody detection.

Results: Age, sex, percentage patients with benign diseases, and percentage of patients with homozygotes for the C allele at position -572 of the *IL-6* gene were similar between responders and non-responders. Although the amounts of platelets pheresis transfused to patients with hematologic diseases were higher than those of non-hematologic diseases (47.2 ± 54.2 *vs.* 17.4 ± 13.8 units, p = 0.019), detection rate of platelet antibodies was lower in patients with hematologic diseases than that in patients with non-hematologic diseases (42.3% *vs.* 79.2%, p = 0.01).

Conclusion: There was no association between *IL-6* C-572G gene polymorphism and positive reactivity in solid phase platelet antibody detection method in platelet recipients.

Introduction

Alloimmunization to human leukocyte antigens (HLAs) and to human platelet antigens (HPAs) may occur after exposure to nonself-antigens during transfusion or pregnancy. Platelet alloimmunization is one of several factors that can contribute to the platelet refractory state. Knowledge of predictors of alloimmunization may be useful for the prevention and management of platelet refractoriness.

Rhesus (Rh) antigens are not present on the platelet surface. However, platelet concentrates may contain enough red blood cells (RBCs) to elicit an anti-D antibody (anti-D) response. Cid, et al. found that the risk of D alloimmunization is low in patients with hematologic disease after D-incompatible platelet transfusions using platelet concentrates prepared by the buffy coat method [1].

Interleukin-6 (*IL-6*) is produced by many different types of cells, including monocytes, lymphocytes, mesenchymal cells, fibroblasts, endothelial cells, keratinocytes, mesangial cells, and endometrial cells [2,3]. *IL-6* is involved in regulation of B cell differentiation into antibody (Ab)-producing plasma cells and immunoglobulin production [4]. *IL-6* promotes antibody

production by promoting the B cell helper capabilities of CD4+ T cells through increased IL-21 production. *IL-6* could therefore be a potential coadjuvant to enhance humoral immunity [5]. *IL-6* inhibits TGF-β-induced regulatory T (Tregs) differentiation [6]. Tregs cells play a major role in restraining immune responses to maintain immune homeostasis [7]. A reduced Treg activity was observed in transfused mice that make alloantibodies (responders) [8].

IL-6 gene, located in 7p21, spans 5kb. *IL-6* C-572G mutation in the promoter region is that cytosine (C) being substituted by guanine (G). The single nucleotide polymorphisms (SNP) with the presence of G at position -174 (rs1800795) within the 5' region of the *IL-6* gene was found to be associated with increased donor-specific HLA antibody production after renal transplantation [9]. The -174C allele of the *IL-6* promoter region was reported to be associated with systemic onset juvenile idiopathic arthritis [10], and susceptibility to rheumatoid arthritis in Europeans [11]. *IL-6* -174C allele is rare in Chinese population [12,13]. Pan, et al. studied 232 individuals and found that 231 carried the wild type of -174G/G; only 1 carried the GC genotype, and the CC genotype was not detected at all [12]. We previously studies 149 individuals and found that all individuals had GG at -174 position of *IL-6* gene without any diversity [13]. But there are no published studies that have examined the association between *IL-6* C-572G SNP and platelet antibody production.

Materials and methods

Study subjects

The total number of healthy subjects and patients is 100: 50 healthy subjects, 30 with platelet antibody (responders) and 20 without platelet antibody (non-responders).

In 30 responders, there were 11 patients (36.7%) with hematologic diseases (immune thrombocytopenia n = 7, aplastic anemia n = 1, acute leukemia n = 3), and 19 patients with non-hematologic diseases (septic shock n = 4, liver cirrhosis n = 4, uremia n = 1, pneumonia n = 1, breast cancer n = 2, ovarian cancer n = 1, lung cancer n = 1, hepatoma n = 2, prostate cancer n = 1, bladder cancer n = 1, renal cancer n = 1).

Of 20 non-responders, 15 patients (75%) had hematologic diseases (immune thrombocytopenia purpura n = 1, aplastic anemia n = 3, myelodysplastic syndrome n =2, acute leukemia n = 4, lymphoma n = 4, multiple myeloma n = 1), and 5 patients had non-hematologic diseases (hepatitis B n = 1, arthritis n = 1, hepatoma n = 1, lung cancer n = 1, ovarian cancer n = 1).

In 50 healthy subjects (21 women and 29 men; median age: 32.5 years, range: 20-58 years), they had no any active medical problem, or evidence of abnormal hepatic or renal function from screening laboratory test results in routine physical examination.

Cumulative amounts of platelets pheresis transfused in responders were calculated until the platelet antibody test. Blood components transfused to patients with hematologic diseases or malignancies were leukoreduced, but blood components for patients with non-hematologic diseases or non-malignant diseases were non-leukoreduced respecting the regulation of the reimbursement of leukoreduced components in Taiwan.

The study protocol was approved by the institutional review board of Taipei Veterans General Hospital. All subjects provided written informed consents.

Determination of platelet antibodies

The solid phase red cell adherence assay technique with the MASPAT kit (Sanquin, Amsterdam, The Netherlands) was used to detect antiplatelet antibodies (against HLA class I and HPAs) in patient serum. A positive or weak positive reaction indicates the presence of platelet and/or HLA specific antibodies in the serum.

Genomic DNA extraction and genotyping of *IL-6*

Genomic DNA was extracted from blood samples with Puregene DNA isolation kit (Gentra System, Minneapolis, MN, USA). The -572 C>G (rs 1800796) SNPs in the promoter region of *IL-6* gene were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method as previously described [13,14].

Statistics

Deviation from Hardy-Weinberg equilibrium was tested by using the chi-square test for goodness of fit. For comparison of categorical variables between groups, the Pearson's chi-square test with Fisher's exact test was performed. The Mann-Whitney U test was used to compare numeric variables between groups. The differences between groups were considered significant if p - values of two-tails were less than 0.05. Statistical analysis was performed using IBM SPSS Statistics, Version 20 (IBM Corp. Armonk, NY, USA).

Results

Patient characteristics

Age, sex, and percentage of patients with malignant diseases in responders were similar to those in non-responders. Percentage of patients with hematologic diseases in responders was lower than that in non-responders (36.7% *vs.* 75%, p = 0.01, Table 1).

Percentage of malignancies and hematologic malignancies in responders

In 30 responders, there were 12 patients (40%) with malignant diseases (hematologic malignancies n = 3, non-hematologic malignancies n = 9). Of 20 non-responders, 12 patients (60%) had malignant diseases (hematologic malignancies n = 9, non-hematologic malignancies n = 3). The

percentage patients with malignancies in responders was similar to that in non-responders (40% *vs.* 60%, $p = 0.248$), Table 1.

The ratio of hematologic malignancies to non-hematologic malignancies in responders is lower in responders than that in non-responders (1:3 *vs.* 3:1, $p = 0.039$).

Frequencies of genotypes and alleles of –572C/G SNPs of *IL-6* gene

The distribution of genotypes for *IL-6* –572C>G (rs1800796) polymorphism did not differ from Hardy-Weinberg proportion in either patients or heathy volunteers. The frequencies of genotypes and alleles of *IL-6* –572 C>G polymorphism was similar between patients and healthy subjects (Table 1).

Association between *IL-6* -572 SNP and platelet antibody production

As shown in table 1, the frequency of G allele of –572 *IL-6* gene SNP in responders was similar with that in non-responders (26.7% (16/60) *vs.* 22.5% (9/40), $p = 0.637$).

In the responder group, percentage of the recipients with a G-positive (CG + GG) genotype was the same with that of the non-responder group, (40% *vs.* 40%, $p = 1.0$) (Table 1).

Age, sex, percentage of patients with benign disease, percentage of patients with hematologic disease, and amounts of platelet transfused did not differ between the CC genotype and G-positive genotype of –572 *IL-6* gene promotor (Table 2). Compared to patients with a CC genotype, the relative risk of platelet antibody production in patients with G-positive genotype of –572 *IL-6* gene promoter SNP was 1.0 (95% CI: 0.63-1.587). No association was observed between the –572 *IL-6* G-positive genotype and the production of platelet antibodies.

Association between hematologic diseases and platelet antibody production

No significant differences regarding baseline characteristics, such as age, sex percentage of patients with malignant disease, or percentage of patients with G positive genotype at position -572 of the *IL-6* gene was noted between the hematologic and the non-hematologic group, Table 3. Total number of transfused platelets pheresis were higher in patients with hematologic diseases than those of non-hematologic diseases (47.2 ± 54.2 *vs.* 17.4 ± 13.8, $P = 0.019$) (Table 3), but detection rate of platelet antibodies was lower in patients with hematologic disease than that in patients with non-hematologic diseases (42.3% *vs.* 79.2%, $p = 0.01$) (Table 1). Compared to patients with hematologic diseases, patients with non-hematologic diseases had 1.871 (95% CI: 1.142 - 3.065) fold relative risk of platelet antibody production.

Discussion

Our data found that percentage of patients with hematologic diseases in responders (with platelet antibody production) was lower than that in non-responders. Whereas

Table 1: Patient characteristics and the frequency of genotypes and alleles of -572 polymorphism in *IL-6* promotor grouped by platelet alloimmunization and healthy subject.

Characteristics and Type	Responder ($n = 30$)	Non-responder ($n = 20$)	P^1	Healthy subject ($n = 50$)	P^2	P^3
Age (years)			0.804		< 0.001	< 0.001
Median	62.5	64.0		32.5		
Range	23-90	15-89		20-58		
Sex			0.103		0.325	0.351
Male	14(46.7%)	14(70%)		29(58%)		
Female	16(53.3%)	6(30%)		21(42%)		
Disease (benign or not)			0.248			
Benign	18(60%)	8(40%)				
Malignant	12(40%)	12(60%)				
Disease (Hema or not)			0.010			
Hematologic	11(36.7%)	15(75%)				
Non-hematologic	19(63.3%)	5(25%)				
Genotype			0.574		0.234	0.962
CC	18(60%)	12(60%)		29(58%)		
CG	8(26.7%)	7(35%)		19(38%)		
GG	4(13.3%)	1(5%)		2(4%)		
Allele			0.637		0.601	0.949
C	44(73.3%)	31(77.5%)		77(77%)		
G	16 (26.7%)	9(22.5%)		23(23%)		
Genotype group			1.0		0.860	0.878
CC	18(60%)	12(60%)		29(58%)		
CG+GG	12(40%)	8(40%)		21(42%)		
Plt pheresis(units)			0.026			
Mean±SD	18.9 ± 14.6	54.0 ± 59.8				
Median	17.5	31.5				

P^1 = non-responders compared with responders; P^2 = healthy subjects compared with responders; P^3 = healthy subjects compared with non-responders; Comparison of age and platelets pheresis transfused was performed by the Mann-Whitney U test; Comparisons of sex, disease, autoantibody, genotype, allele and genotype group were performed by chi-square test and Fisher's exact test, Plt = platelets.

Table 2: Patient characteristics and platelet requirements grouped by CC genotype and G positive genotype (CG + GG) of -572 polymorphism in *IL-6* promotor.

Characteristics	CC (*n* = 30)	CG + GG (*n* = 20)	*p* - value
Age (years)			
Median	64.0	62.0	0.903
Range	15-88	26-90	
Sex			0.773
Male (*n* = 28)	16(53.3%)	12(60%)	
Female (*n* = 22)	14(46.7%)	8(40%)	
Disease (benign or not)			1.0
Benign (*n* = 26)	16(53.3%)	10(50%)	
Malignant (*n* = 24)	14(46.7%)	10(50%)	
Disease (Hema or not)			0.565
Hema (*n* = 26)	17(56.7%)	9(45%)	
Non-Hema (*n* = 24)	13(43.3%)	11(55%)	
Platelets pheresis(units)			
All patients (*n* = 50) Responders (*n* = 30)	41.4 ± 51.9 (*n* = 30) 19.7 ± 16.2 (*n* = 18)	20.3 ± 17.2 (*n* = 20) 17.6 ± 12.4 (*n* = 12)	0.242 0.983
Non-responders (*n* = 20)	73.8 ± 69.1 (*n* = 12)	24.3 ± 22.9 (*n* = 8)	0.069

Haema = hematologic disease, Non-hema = non-hematologic disease; Comparisons of sex and disease were performed by chi-square test and Fisher's exact test; Comparisons of age and platelets pheresis transfused were performed by the Mann-Whitney U test.

Table 3: Patient characteristics and platelet requirements grouped by hematologic disease and non-hematologic disease.

Characteristics	Hema disease (*n* = 26)	Non-hema disease (*n* = 24)	*p* - value
Age (years)			0.943
Median	64.5	62.5	
Range	15-89	29-90	
Sex			0.569
Male (*n* = 28)	16(61.5%)	12(50%)	
Female (*n* = 22)	10(38.5%)	12(50%)	
Disease (benign or not)			1.0
Benign (*n* = 26)	14(53.8%)	12(50%)	
Malignant (*n* = 24)	12(46.2%)	12(50%)	
Platelet antibody			0.01
Present (*n* = 30)	11 (42.3%)	19 (79.2%)	
Absent (*n* = 20)	15 (57.7%)	5 (20.8%)	
Genotype			0.565
CC (*n* = 30)	17 (65.4%)	13 (54.2%)	
CG+GG (*n* = 20)	9 (34.6%)	11 (45.8%)	
Platelets pheresis(units)			
All patients (*n* = 50)	47.2 ± 54.2	17.4 ± 13.8	0.019
Responders (*n* = 30)	19.7 ± 15.5	18.4 ± 14.4	0.767
Non-responders (*n* = 20)	67.4 ± 63.6	13.8 ± 11.4	0.019

Haema = hematologic disease, Non-hema = non-hematologic disease; Comparisons of sex, disease, platelet antibody and genotype were performed by chi-square test and Fisher's exact test; Comparisons of age and platelets pheresis transfused were performed by the Mann-Whitney U test.

patient age, female sex, and *IL-6* C-572G gene polymorphism are not risk factor for platelet antibody production in platelet recipients. In our study, age of healthy control subjects was younger than patients (responders and non-responders), but age of responders and non-responders was similar (p = 0.804). So age had no impact on the data analysis for platelet antibody production.

The prevalence of HLA class I antibodies was 2.27% (2/88) in donors without pregnancy or transfusion history [15]. Schnaidt, et al. tested sera of 500 female blood donors after pregnancy for platelet-specific antibodies (HPA-1, 3, 5) using the monoclonal antibody immobilization of platelet antigens (MAIPA) assay. Twenty-one sera (4.2%) were found to be positive: four anti-HPA-1a, one anti-HPA-5a and 16 anti-HPA-5b. Sera were positive up to 30 years after the last pregnancy [16]. Koerner and colleagues analyzed serum antibodies to platelet-specific glycosphingolipids (GSL) which antibodies were detected only in sera of immune thrombocytopenic purpura (ITP) patients. They found one half (6/12) of patients with ITP had antibodies bound to platelet-specific GSL. Sera from none of 10 patients with nonimmune thrombocytopenia, and only 1 of 18 normal subjects gave positive reactions with the platelet-specific GSL group [17].

Cytokines are important regulators of immune responses against foreign antigens. The response to foreign antigens is mainly regulated by $CD4^+$ T-helper cells (Th), which can be subdivided into the distinct subsets Th1 and Th2 based on the cytokines they produce. The Th2 subset help B cells to proliferate and differentiate and is associated with humoral-type immune responses. *IL-6* promotes Th2 differentiation

[18]. *IL-6* plays a major role in inflammatory responses and antibody production [19]. IL-6 was found to have a function in restricting early Friend virus replication [20]. We have previously found that the *IL-6* C-572G gene polymorphism is significantly associated with anti-E antibody (anti-E) production, with the allele G as a risk allele [13].

Friedman, et al. found a high rate of alloimmunization to both RBC (30%) and platelet antigens (85%) in transfused sickle cell disease (SCD) patients, but detected no significant association between RBC and platelet alloimmunization. This suggests that responders to RBC antigens are not more likely to respond to the antigenic determinants responsible for platelet alloimmunization [21]. Verduin, et al. showed that women with sickle cell disease were at a higher relative risk (27.0%) of developing RBC alloimmunization compared to men with the same disease. In patients without hemoglobinopathy, the risk of RBC alloimmunization appeared to be similar among men and women [22]. This is likely explained by more exposure to immunizing events through pregnancy and/or transfusions in females with sickle cell disease. In our study, female patients tend to have an increased relative risk on platelet alloantibodies compared with men (53.3% *vs.* 30%, $p = 0.103$). Sharpe, et al. found that the HLA-alloimmunization rate was different according to gender: 22% for males (4/18) *vs*. 67% for females (10/15) [23].

In this study, total platelet requirements in hematologic patients were higher than those in non-hematologic patients. This may be explained by that hematologic patients often develop thrombocytopenia as a consequence of the underlying disease itself and therapy, and platelet transfusion is required for the prevention or treatment of hemorrhagic manifestations in patients with thrombocytopenia. Wang, et al. found that anti-HLA and anti-HPA alloantibodies were found in 114 of 204 (55.88%) patients with platelet refractoriness, including 110 (96.49%) with anti-HLA alloantibodies only, 2 (1.75%) with anti-HPA alloantibodies only and 2 (1.75%) with both anti-HLA and anti-HPA alloantibodies (anti-HPA-3a and anti-HPA-5b) [24]. Kiefel, et al. studies serum samples from 252 patients with hematologic or oncologic diseases, and platelet antibodies were detected in the sera of 113 patients (44.8% of 252) [25]. In the present study, the detection rate of platelet antibodies in hematologic patients was 42.3%, which was lower than that of patients with non-hematologic diseases (79.2%, $p = 0.01$).

Platelets express HLA class I antigens, but platelets do not express HLA class II antigens [26]. Primary HLA alloimmunization is dependent on recognition of both HLA Class I and Class II antigenic differences. The presence of viable cells bearing Class II antigens such as lymphocytes and antigen-presenting cells is necessary to stimulate primary HLA alloimmunization. If ways were found either to remove the Class II-carrying leukocytes physically or to inactivate them, HLA alloimmunization could be prevented. This method will not, however, prevent the formation of platelet-specific alloantibody, which is due to transfusion of incompatible platelet antigens [27].

In a meta-analysis, Vamvakas combined the results of 8 randomized controlled trial and found that a statistically significant benefit from white cell reduction in preventing HLA-alloimmunization [28]. Seftel, et al. reported that universal prestorage leukoreduction reduces platelet alloimmunization in multiply transfused patients, patients undergoing stem cell transplantation as well as acute leukemia patients receiving aggressive chemotherapy [29]. In our study blood components for patients with hematologic diseases were leukoreduced, leukoreduction might be responsible for a lower rate of alloimmunization in patients with hematologic disease as compared with patients with non-hematologic disease.

Friedman, et al. [21]. suggested that leukodepletion of blood products might be less efficacious in immunocompetent SCD patients than in cancer and BMT patients who are immunocompromised for prevention of platelet alloimmunization. Intensive chemotherapy may suppress the production of antibody. Schonewille, et al. found that patients who underwent intensive chemotherapy formed antibodies at a much lower rate than other patients [30]. Reinhardt, et al. found that intensive chemotherapy in children with malignancies causes partial immune deficiency, including long-term impairment of humoral immunity [31]. Asfour, et al. found that transfusion of RhD-positive blood components to Rh-negative patients with hematologic cancers, who have received RhD-negative bone marrow and/or peripheral blood stem cells, are at low risk of developing RhD antibodies [32]. Schonewille, et al. studied alloimmunization after blood transfusion in patients with hematologic and oncologic diseases and found that patients who underwent intensive chemotherapy formed antibodies at a much lower rate than other patients [30]. Arora, et al. found that the rate of alloimmunization was higher in patients who had solid cancers (22.6%) or myelodysplastic syndrome (23%) compared with those who had other hematologic malignancies (7%) [33]. The intensity of chemotherapy for patients with hematologic malignancies was higher than that for patients with non-hematologic malignancies. In our study, the percentage of patients with malignancies were similar between responders and non-responders (40% *vs.* 60%, $p = 0.248$), but the ratio of hematologic malignancies to non-hematologic malignancies were lower in responders than non-responders (1:3 *vs.* 3:1, $p = 0.039$). Because blood components transfused to patients with hematologic diseases and malignant diseases were leukoreduced, we can assumed that higher intensity of chemotherapy might contribute more than leukoreduction on reducing platelet antibody formation.

In conclusion, our finding suggest that there is no association between *IL-6* C-572G gene polymorphism and positive reactivity in solid phase platelet antibody

detection method in platelet recipients. Higher intensity of chemotherapy might contribute more than leukoreduction on reducing platelet antibody formation.

Acknowledgement

The authors thank all staffs in Taipei Veterans General Hospital for supporting this study. This study was supported by grants from the Medical Research Fund of Taipei Veterans General Hospital (V106C-179).

Authorship contributions

All of the authors contributed to the conception and design of the study.

Jeong-Shi Lin analyzed and interpreted the data, and wrote and revised the manuscript.

Li-Hsuan Lee designed the study, performed the experiments, and reviewed the manuscript.

Hsueng-Mei Liu designed the study, performed the experiments, and reviewed the manuscript.

Ying-Ju Chen prepared all reagents and instruments, and reviewed the manuscript.

Tzeon-Jye Chiou reviewed and approved the manuscript.

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