Int J Clin Exp Pathol 2016;9(1):275-281
www.ijcep.com /ISSN:1936-2625/IJCEP0018094

Original Article

Polymorphism of platelet collagen receptor glycoprotein VI is associated with aspirin response in patients with unstable angina

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Received October 18, 2015; Accepted November 28, 2015; Epub January 1, 2016; Published January 15, 2016

Abstract: Background: Polymorphisms of the major platelet collagen receptor, glycoprotein (GP) VI have been reported to affect signal transduction upon engagement of GPVI ligands. The aim of study is to evaluate the effect of GPVI polymorphisms (A22630T and C22644A) on aspirin or clopidogrel response in patients with unstable angina (UA). Methods: 84 consecutive patients with UA were enrolled in this study and took antiplatelet medications, including 75 mg/d clopidogrel and 100 mg/d aspirin for consecutive 7 days. Arachidonic acid (AA) or ADP-induced platelet aggregation was performed to assess the response of aspirin or clopidogrel. PCR amplification was used for GPVI sequencing. Results: Our results showed the maximum platelet aggregation induced by AA or ADP was 17.73 ± 10.40% or 61.46 ± 14.90%, respectively. Of 84 UA patients, percentage of resistance of aspirin and clopidogrel was 21.4% (18/84) and 22.6% (19/84). In addition to known haplotypes, three novel haplotypes of GPVI were found, including a new 'broken haplotype' due to non-linked inheritance of different combinations at positions 22630 and 22644. Multivariate logistic regression analysis showed T carriers of GPVI A22630T (P = 0.022) and hyperlipidemia (P = 0.028) were associated with responders to aspirin but not to clopidogrel. Due to lower prevalence, analysis of C22644A polymorphism was not evaluated. Conclusion: T carriers of GPVI A22630T were associated with aspirin responsiveness in UA patients. If these findings are confirmed in large cohort clinical studies, they could ultimately enable evaluation of aspirin response in UA patients.

Keywords: Glycoprotein VI, polymorphisms, unstable angina, aspirin response, clopidogrel response

Introduction

Platelets play an important role in thrombosis and homeostasis. Following vascular injury, platelets roll, adhere and firmly attach to subendothelial matrix via membrane receptors such as glycoprotein (GP) VI, that binds collagen and GPIb-IX-V which binds von Willebrand factor (VWF), leading to platelet aggregation [1, 2]. As a major platelet collagen receptor, GPVI is a member of immunoglobulin (Ig) superfamily, and contains two extracellular Ig domains, a transmembrane domain and a cytoplasmic tail. It forms a non-covalent complex with Fc receptorg y chain (Far) which is required for GPVI surface expression [3, 4]. In response to ligand binding, phosphorylation of the Immunoreceptor Tyrosine Activation Motif (ITAM) within FcRγ by Lyn leads to recruitment and assembly of Syk, and activation of adaptor proteins, such as SLP-76 and LAT, ultimately resulting in the activation of phospholipase Cγ2 (PLCγ2). PLCγ2 causes elevation of cytosolic Ca^{2+} leading to activation of the integrin αIIbβ3, which binds fibrinogen and mediates platelet aggregation and activation [5].

Three common haplotypes of GPVI: GPVIa (aa allele), GPVIb (bb allele) and GPVIab (ab allele) are identified [6-8]. These alleles involve 10 base substitutions, 5 resulting in amino acid changes which are T13254C (S219P), A19871G (K237E), A21908G (T249A), A22630T (Q317L) and C22644A (H322N) with homozygotes of

Table 1. Clinical and demographic characteristics of patients

Parameters	Numbers (%)
Age	61 ± 12
Female (%)	33 (39.3)
Hypertension (%)	57 (67.9)
Diabetes (%)	15 (17.9)
Hyperlipidemia (%)	30 (35.7)
History of thrombosis (%)	19 (22.6)
Overweight (%)	30 (35.7)
Smoking (%)	34 (40.5)
Clopidogrel non-response (%)	19 (22.6)
Aspirin non-response (%)	18 (21.4)
GPVI22630 (AT+TT, %)	25 (29.8)
GPVI22644 AC (%)	3 (3.6)

the former sequences being GPVIa, latter being GPVIb and heterozygotes for GPVIab. For GPVIa and GPVIb, there is no significant difference in surface expression and ligand-binding affinity, but significant differences in the binding affinity for the intracellular binding partners, Lyn and calmodulin (CaM) [9]. Likely due to these functional differences, polymorphisms of GPVI were shown to be associated with myocardial infarction [10], coronary thrombosis [11], sticky platelet syndrome [12] or possibly ischemic stroke [13]. In addition, the GPVI polymorphism (T13254C) was reported to be associated with aspirin non-responsiveness in patients with coronary artery disease [14]. As the only known functional differences between the GPVI polymorphisms being the cytoplasmic binding affinity towards Lyn and CaM, whether these two SNP sites (A22630T and C22644A) located proximally to binding regions of Lyn and CaM could influence aspirin responsiveness in patients with unstable angina (UA) remains poorly understood. The aim of this study was to evaluate the effect of GPVI polymorphisms (A22630T and C22644A) on aspirin and clopidogrel responses in UA patients treated with dual antiplatelet agents.

Materials and methods

Patients

84 patients with unstable angina (UA) from the department of cardiology were consecutively enrolled in the study, which was diagnosed based on the guidelines [15, 16]. Briefly, unstable angina was defined as chest pain typical of angina occurring at rest with duration of at least 20 min and requiring hospitalization in patients with known coronary artery disease based on coronary angiogram or a positive stress test. No active ulcer, myelodysplastic syndrome and smocyte dyscrasias were observed in the patients. In addition, none of the patients was administered any antiplatelet agents prior to this study. Antiplatelet therapy included 75 mg/d clopidogrel and 100 mg/d aspirin for consecutive 7 days. Experiments were carried out with the approval of Xiangyang City Central Hospital (Hubei, China) Standing Committee on Ethics in Research involving humans and informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Blood collection and platelet aggregation

On day 8 post administration of aspirin and clopidogrel, blood was drawn from patients after receiving antiplatelet drugs into 3.2% (w/v) tri-sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifuging blood for 10 min at 200×g. Platelet-poor plasma (PPP) was obtained by centrifuging PRP at 3000×g for 15 min. Platelet concentration in PRP was adjusted to 250×10^9/l by using PPP. Platelet aggregation in citrated PRP was carried out in a Helena Aggram (Helena Laboratories, Beaumont, USA). Arachidonic acid (AA, 0.5 mM) and ADP (10 mM), purchased from Helena Laboratories, Beaumont, USA, was used as platelet agonist to evaluate aspirin and clopidogrel response. Aspirin or clopidogrel resistance was defined as maximum platelet aggregation being more than 20% or 70%, respectively.

Genomic DNA extraction and PCR amplification

Venous whole blood was collected into a tube with EDTA as anti-coagulant. Genomic DNA was extracted from whole blood with High Pure PCR Template Preparation Kit (Roche, Germany), according to manufacturer's instructions. The quality and quantity of extracted DNA was determined by agarose gel electrophoresis and spectrophotometry (Model DU800, Beckman Coulter, Brea, CA, USA).

Table 2. Distributions of GPVI allele (A22630T and C22644A) and polymorphisms frequency in patients

A				
GPVI	Genotype	Allele frequency	χ^2	*P*
A22630T	AA: 70.2% (59/84)	A: 83.3% (140/168)	0.274	0.600
	AT+TT: 29.8% (25/84)	T: 16.7% (28/168)		
C22644A	CC: 96.4% (81/84)	C: 98.2% (165/168)	0.028	0.868
	AC: 3.6% (3)	A: 1.80% (3/168)		

B						
	GPVI haplotype		GPVI genotype		UA (n = 84)	
	Q317L	H322N	A22630T	C22644A	No.	%
GPVIa	QQ	HH	AA	CC	59	70.2
GPVIab	QL	HN	AT	CA	1	1.2
GPVIb	LL	NN	TT	AA	0	0
Broken	QL	HH	AT	CC	21	25.0
Broken	LL	HN	TT	CA	2	2.4
Broken	LL	HH	TT	CC	1	1.2

PCR amplification for GPVI sequencing was performed as previously described [17]. Briefly, the paired primer for PCR was designed as follows: 5' GTC CTG CCC GCC AGT ACT ACA CC 3' (Forward); 5' TGA GTC GCC TCC CAT GCC ATG ATC 3' (Reverse). PCR amplification was performed by using Taq polymerase with conditions being 95°C for 3 min, 30 cycles of 95°C for 30 sec; 62°C for 30 sec (annealing) and 72°C for 30 sec, 72°C for 10 min (final extension). The PCR product was analyzed on a 1.5% agarose gel and visualized with SYBR Gold (Molecular Probes-Invitrogen). Purified PCR product was sequenced by using BigDye Terminator v3.1 cycle kit (Applied Biosystems, CA, USA).

Statistical analysis

Data was represented as Mean ± SD. Student t test and Chi square test was used for the analysis of continuous and categorical variables respectively. Hardy-Weinberg equation was used to estimate the frequency of GPVI alleles. By using SPSS 13.0 software, Chi square test was used to analyze the correlation of response of aspirin or clopidogrel with gender, hypertension, diabetes, hyperlipidemia, history of thrombosis, overweight (weight index ≥ 25 kg/m^2), and smoking, GPVI A22630T or C22644A. In addition, other variables with $P < 0.10$ from Chi square test were included for multivariate logistic regression analysis. $P < 0.05$ was considered to be significantly different.

Results

Characteristic of UA patients

Of the included 84 UA patients, there were 51 male (61%) and 33 female (39%), aged from 38-85 with a median age of 61. Further details regarding the background and other characteristic of UA patients are summarized in **Table 1**.

Prevalence of GPVI polymorphism

The frequency of A and T alleles were 83.3% and 16.7% in patients (**Table 2A**). Regarding the distribution of T carriers (AT+TT) of GPVI A22630T, it was 29.8% (25/84) (**Table 2A**). In terms of the prevalence of C22644A, a lower proportion of A carriers was found in UA patients (1.8%).

The three most common haplotypes of GPVI as previously reported were GPVIa, GPVIb and GPVIab. The 5 different amino acids responsible for these haplotypes on each chromosome were thought to be linked inheritance. As shown in **Table 2B**, the frequency of GPVIa and GPVIab was 70.2% and 1.2%. However, GPVIb was not found in patients, consistent with previous studies showing GPVIa and GPVIb being the major and minor haplotypes [8, 9]. Interestingly, we found three novel haplotypes, named as a broken haplotype, due to non-linked inheritance, with either homozygotes or heterozygotes at amino acid position 317 (Q/L) combined with homozygotes or heterozygotes at position 322 (H/H), corresponding to 22630 (A/T) and 22644 (C/C) on gene sequences. More precisely, these three novel haplotypes were Q317L/H322N, L317L/H322N and L317L/H322H. Q317L/H322H seemed to be a prevalence haplotype with a higher frequency observed in patients (25.0%).

GPVI polymorphism and aspirin or clopidogrel response

AA (0.5 mM) or ADP (10 mM) were used as platelet agonists to evaluate platelet respon-

Table 3. Comparative analysis of aspirin response

Parameters	Aspirin response (%)		*P*	OR (*95% CI*)
	Resistance (n = 18)	Responders (n = 66)		
Age	61 ± 13	61 ± 12	0.785	
Female	5 (27.8)	28 (42.4)	0.259	0.522 (*0.167-1.634*)
Hypertension	15 (83.3)	42 (63.6)	0.113	2.857 (*0.750-10.881*)
Diabetes	4 (22.2)	11 (16.7)	0.843	1.429 (*0.395-5.169*)
Hyperlipidemia	11 (61.1)	19 (28.8)	0.011	3.887 (*1.311-11.529*)
History of thrombosis	5 (27.8)	14 (21.2)	0.785	1.429 (*0.435-4.688*)
Overweight	8 (44.4)	22 (33.3)	0.383	1.600 (*0.554-4.3624*)
Smoking	9 (50.0)	25 (37.9)	0.353	1.640 (*0.574-4.683*)
GPVI22630 (AT+TT)	1 (5.6)	24 (36.4)	0.011	0.103 (*0.013-0.823*)
GPVI22644 AC	0 (0)	3 (4.5)	N/A	

Table 4. Comparative analysis of clopidogrel response

Parameters	Clopidogrel response (%)		*P*	OR (*95% CI*)
	Resistance (n = 19)	Responders (n = 65)		
Age	61 ± 8	62 ± 13	0.912	
Female	9 (47.4)	24 (36.9)	0.412	1.538 (*0.548-4.315*)
Hypertension	11 (57.9)	46 (70.8)	0.291	0.568 (*0.198-1.633*)
Diabetes	4 (21.1)	11 (16.9)	0.942	1.309 (*0.364-4.705*)
Hyperlipidemia	7 (36.8)	23 (35.4)	0.907	1.065 (*0.368-3.080*)
History of thrombosis	5 (26.3)	14 (21.5)	0.900	1.301 (*0.400-4.234*)
Overweight	4 (21.1)	26 (40.0)	0.129	0.400 (*0.119-1.341*)
Smoking	6 (31.6)	28 (43.1)	0.369	0.610 (*0.206-1.805*)
GPVI22630 (AT+TT)	7 (36.8)	18 (27.7)	0.443	1.523 (*0.518-4.480*)
GPVI22644 AC	1 (5.3)	2 (3.0)	0.666	1.750 (*0.150-20.421*)

siveness to aspirin or clopidogrel, respectively. The maximum platelet aggregation induced by AA or ADP was 17.73 ± 10.40% or 61.46 ± 14.90%. Among patients, percentage of resistance of aspirin and clopidogrel was 21.4% (18/84) and 22.6% (19/84), respectively, consistent with previous study showing that 20.4% (129/634) aspirin resistance was observed in Chinese stroke patients receiving antiplatelet medications [18].

The Chi square test was used to analyze the correlation of variables with the response of aspirin or clopidogrel. As seen in **Table 3**, age, gender, hypertension, diabetes, history of thrombosis, overweight, and smoking did not affect aspirin response ($P > 0.05$). Whereas, hyperlipidemia was associated with aspirin non-response ($P = 0.011$), suggesting increased platelet reactivity in patients with hyperlipidemia, consistent with previous study showing the association of hyperlipidemia with an enhance platelet thrombus formation on an injured artery leading to increased propensity for acute thrombosis [19]. Meanwhile, T carriers of GPVI 22630 (AT+TT) was also found to affect aspirin response and was associated with aspirin responders ($P = 0.011$). Due to lower prevalence of GPVI C22644A polymorphism, the statistical analysis was not applicable (N/A). In terms of clopidogrel response (**Table 4**), none of the selected parameters were found to be associated with clopidogrel response ($P > 0.05$), even for T carriers of GPVI 22630, which was associated with aspirin responders.

Apart from the univariate analysis of the association of each variable with aspirin response, variables with $P < 0.1$ from **Table 3** (hyperlipidemia and T carriers of GPVI 22630) were chosen for multivariate logistic regression analysis. Consistent with univariate analysis, T carriers of GPVI 22630 (95% CI = 0.008-0.686, $P = 0.022$) and hyperlipidemia (95% CI = 1.165-13.817, $P = 0.028$) were found to be associated with aspirin response (**Table 5**).

Discussion

The platelet receptor GPVI plays a crucial role in collagen-induced platelet aggregation and acti-

Table 5. Multivariate logistic regression analysis of aspirin response

Parameters	B	S.E.	*P*	EXP (B)	95% CI
GPVI 22630 AT+TT	-2.633	1.151	0.022	0.072	0.008-0.686
Hyperlipidemia	1.390	0.631	0.028	4.013	1.165-13.817

vation. There are three common haplotypes of GPVI, named GPVIa, GPVIb and GPVIab. Previous studies demonstrated that GPVI could be a novel indicator for UA [20] and its polymorphism (T13254C) was associated with aspirin resistance in UA patients [14]. Although the functional differences of GPVI polymorphisms may be related to the different binding affinity towards Lyn and CaM within the cytoplasmic tail of GPVI [9], whether these two SNP sites (A22630T and C22644A) located proximally to these regions could influence aspirin response remains unclear. In this study, we found three novel forms of GPVI haplotype without linkage inheritance, named broken haplotypes, with homozygotes or heterozygotes at 22630 (A/T) combined with homozygotes or heterozygotes at 22644 (CC). In addition, T carriers of GPVI 22630 were found to be correlated with aspirin responders in UA patients treated with dual antiplatelet agents.

Hyperlipidemia, meaning high blood cholesterol, has been demonstrated to be associated with chronic kidney disease [21], deep vein thrombosis [22], ischemic heart disease [19] and other cardiovascular diseases [23] Lowering blood cholesterol levels reduces the incidence of coronary events in individuals with or without coronary disease [24, 25]. Meanwhile, lowering cholesterol level has been demonstrated its capability to slow the progression of coronary atherosclerosis and even induce regression [26]. Previous studies showed that platelets become hyperactive in the presence of hypercholesterolemia. In addition, low density lipoprotein is revealed to be able to activate platelet and increase the production of thromboxane B2 *in vitro* [27]. In this study, we found a higher percentage of hyperlipidemia in patients with unstable angina, which could possible contribute to the thrombotic events in these patients. Furthermore, after multivariate logistic regression analysis, hyperlipidemia was found to be associated with aspirin resistance, possible due to the association of increased thromboxane production with hyperlipidemia, consistent with a previous study demonstrating the association of hyperlipidemia with aspirin resistance in renal transplant recipients [28].

Due to the existence of GPVI different functional haplotypes, polymorphism of GPVI was demonstrated to be associated with many diseases, such as myocardial infarction [10], and thrombosis [11]. Meanwhile, GPVI polymorphism (T13254C) was reported to be associated with aspirin non-responsiveness as evaluated by PFA-100 in patients with coronary artery disease [14]. In this study, apart from previously reported GPVI haplotypes, we found three novel haplotypes of GPVI, named a “broken” haplotype, due to unlinked inherence, which is homozygotes or heterozygotes at amino acid position 317 (Q/L) combined with homozygotes or heterozygotes at position 322 (H/H), corresponding to 22630 (A/T) and 22644 (C/C) on gene sequences. In addition, our study also revealed that T carriers of GPVI 22630 (AT+TT) were associated with aspirin responders. The possible reason for that could be the lower signaling transduction of GPVI resulting from T carriers of GPVI 22630, which was belonged to GPVI b allele, with a decreased binding affinity to Lyn, leading to attenuated Syk phosphorylation in response to ligand binding compared to GPVI an allele. However, the exact mechanism by how GPVI haplotypes affect aspirin response was not investigated and requires further studies.

In conclusion, novel haplotypes of GPVI were found, named broken haplotypes, with homozygocity or heterozygocity at 22630 (A/T) combined with either homozygous or heterozygous 22644 (C/A). T carriers of GPVI A22630T appeared to be associated with responsiveness to aspirin. However, due to the limited number of patients enrolled in this study, large cohort clinical studies are required to confirm these findings.

Acknowledgements

This research was supported by National Natural Science Foundation of China (Grant No. 81400082), Natural Science Foundation of Jiangsu province (Grant No. BK20140219), China Postdoctoral Science Foundation funded

project (project no. 2015M570479), Natural Science Foundation of Hubei Province (Grant No. 2008CDZ067) and Science and Technology Guiding Project of Xiangyang City (Hubei Province) (Grant No. 200812).

Disclosure of conflict of interest

None.

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