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Influence of genetic polymorphisms in P2Y12 receptor signaling pathway on antiplatelet response to clopidogrel in coronary heart disease

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Abstract

Backgrounds: Remarkable interindividual variability in clopidogrel response is observed, genetic polymorphisms in P2RY12 and its signal pathway is supposed to affect clopidogrel response in CHD patients.

Methods: 539 CHD patients treated with clopidogrel were recruited. The platelet reaction index (PRI) indicated by VASP-P level were detected in 12–24 h after clopidogrel loading dose or within 5–7 days after initiation of maintain dose clopidogrel. A total of 13 SNPs in relevant genes were genotyped in sample A (239 CHD patients). The SNPs which have significant differences in PRI will be validated in another sample (sample B, 300 CHD patients).

Results: CYP2C19*2 increased the risk of clopidogrel resistance significantly. When CYP2C19*2 and CYP2C19*3 were considered, CYP2C19 loss of function (LOF) alleles were associated with more obviously increased the risk of clopidogrel resistance; P2RY12 rs6809699C > A polymorphism was also associated with increased risk of clopidogrel resistance (AA vs CC: $P = 0.0398$). This difference still existed after stratification by CYP2C19 genotypes. It was also validated in sample B. The association was also still significant even in the case of stratification by CYP2C19 genotypes in all patients (sample A + B).

Conclusion: Our data suggest that P2RY12 rs6809699 is associated with clopidogrel resistance in CHD patients. Meanwhile, the rs6809699 AA genotype can increase on-treatment platelet activity independent of CYP2C19 LOF polymorphisms.

Keywords: Genetic polymorphisms, P2Y12, Coronary heart disease

Introduction

Atherosclerosis thrombosis can lead to the development of acute coronary syndrome (ACS) and acute myocardial infarction, which is a severe threat to human health. The number of deaths caused by coronary atherosclerosis alone accounts for one-seventh of all-cause deaths worldwide [1]. Because the platelet activation plays an essential role in the formation of thrombus in atherosclerosis thrombosis, antiplatelet therapy has established as a cornerstone in the treatment of coronary heart disease (CHD). Clopidogrel, a P2Y12 receptor antagonist, is recommended to be widely used in patients suffered from acute coronary syndrome (ACS) and post percutaneous coronary intervention (PCI) to prevent future thrombotic

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events. However, the evidence shows that about 5–44% of patients treated with the standard dose of clopidogrel failed to display an adequate antiplatelet aggregation response [2]. As a result, patients with clopidogrel resistance (CR) may show an increased risk of recurrent adverse cardiovascular events [3]. The variability in clopidogrel response is explained by multiple independent factors including genetic polymorphisms [4].

Clopidogrel is a prodrug that requires two steps of bioactivation via cytochromes P450 (CYP) to form the active thiol derivative. CYP2C19 plays a crucial role in its bioactivation. Genetic polymorphisms that result in remarkable interindividual variability in CYP2C19 activity have been observed. Especially, the *CYP2C19* loss-of-function (LOF) variants, such as *CYP2C19*2* and *CYP2C19*3*, can decrease the AUC of the clopidogrel active metabolite, and patients carrying these variant alleles show higher on-treatment platelet activity and increased risk of atherothrombotic events [5–7]. The American Food and Drug Administration (FDA) even announced a black boxed warning on clopidogrel about *CYP2C19**2 and *CYP2C19**3.

The active thiol derivative metabolite of clopidogrel acts through competing with the soluble platelet agonist adenosine 5-diphosphate (ADP) for the platelet P2Y12 receptor. The inhibition of the P2Y12 receptor will lead to the inhibition of the integrin glycoprotein IIb/IIIa (GPIIb/IIIa) complex on the platelet surface, which is called integrin "inside-out" signaling process [8]. Activation of the integrin αIIbβ3 stimulates platelet adhesion and aggregation and triggers "outside-in" signaling, resulting in platelet spreading, additional granule secretion, stabilization of platelet adhesion and aggregation, and clot retraction [9]. Several proteins are involved in the P2Y12-integrin αIIbβ3 activation pathway. Upon P2Y12 activation, the P2Y12-coupled Gi protein can activate phosphatidylinositol-3-kinase (PI3K, encoded by *PIK3CA*) in platelets, which in turn activates the small GTPase Rap1, a critical mediator of integrin GPIIb/IIIa activation [10–12]. Calcium and diacylglycerol-guanine nucleotide exchange factor 1 (CalDAG-GEF1) is responsible for the conversion of Rap1 from the inactive GDP-bound form to the active GTP-bound form, the latter could interact with the Rap1-GTP-interacting adaptor molecule (RIAM) [13]. Talin, encoded by the *TLN1* gene, is a ~ 270 kDa cytoskeleton adaptor protein contains a globular head region that directly links β-integrin. The binding of talin with integrin is the necessary final step for integrin activation [14]. While RIAM, encoded by the gene *APBB11P,* functions as a scaffold that connects the membrane targeting sequence in Rap1 to talin, thereby recruiting talin to the plasma membrane and activating integrins [15]. A study on an inherited platelet disorder in siblings using whole-exome sequencing has identified a culprit mutation (cG742T) in *RASGRP2*, the gene coding for CalDAG-GEFI, to be causative [16]. Platelets from individuals with the mutation showed reduced ability to activate Rap1 and improper αIIbβ3 integrin inside-out signaling [16]. The aIIb subunit (GPIIb) and the β3 (GPIIIa) are encoded by *ITGA2B* and *ITGB3*, respectively. Single nucleotide polymorphisms (SNPs) in *ITGA2B* and *ITGB3* were found to be associated with indexes of platelet and coagulation hemostasis in healthy Chinese people [17]. Similarly, our previous study in healthy Chinese subjects has also demonstrated that the *ITGA2B* rs5911 polymorphism can increase the effect of ticagrelor on ADP-induced platelet aggregation [18]. Moreover, the associations between polymorphisms *P2RY12* polymorphisms (T744C, G52T) and platelet response are also reported [19, 20].

A study has shown that the use of P2Y12 inhibitor monotherapy, as an alternative approach to DAPT, in patients undergoing coronary revascularization. P2Y12 inhibitor monotherapy was associated with similar risks of death, myocardial infarction, or stroke and lower risks of major bleeding compared with DAPT [21].

However, there was a paucity of studies on other genes in platelet related to the P2Y12 receptor signaling pathway and clopidogrel response. Hence, our study was designed to elucidate the degree of crucial genetic polymorphisms related to the P2Y12 receptor signaling pathway on the clopidogrel resistance in Chinese CHD patients.

Materials and methods

Study subjects

A total of 539 consecutive CHD patients treated with clopidogrel from Xiangya Hospital, Central South University from September 2014 to November 2018 in this prospective clinical study. The age of the patients ranged from 18 to 80 years. These samples were divided into discovery (n = 239) and validation (n = 300) sets. All patients received dual antiplatelet therapy (DAPT) with aspirin and oral administration of 300 mg loading dose (LD) clopidogrel, or 75 mg daily maintaining dose (MD) of clopidogrel for at least 5 days. Venous blood samples were drawn in 6:00–7:00 Am 12–24 h after LD of clopidogrel or on day 5–7 after the initiation of MD of clopidogrel for analysis of platelet reaction index (PRI) and DNA extraction. Subjects were excluded if they had a history of a bleeding disorder, current warfarin use, myelodysplastic or myeloproliferative disorders, chronic liver disease or hypersensitivity to clopidogrel. Subjects were also excluded if they were pregnant, with platelet count less than 10^5 cell/mm^3 (thrombocytopenia), or creatinine clearance less than 25 mL/min, or prior use of GPIIb/

IIIa antagonist before the procedure. Questionnaires and medical records were used to collect family and medical history, age, gender, smoking and alcohol habits, diabetic status and other disease complications, co-medications, platelet count, mean platelet volume (MPV), and physical activities. Patients were followed up by telephone interviewers using standardized questionnaires. The primary endpoint of this study was major adverse cardiac events (MACE), defined as a composite of cardiac death, myocardial infarction (MI), and repeat target vessel revascularization. The study protocols were approved by the Ethics Committee of Central South University (No. CTXY-140002-13) and followed the Declaration of Helsinki. It was also registered on the Chinese Clinical Trial Registry (http://www.chictr.org.cn) (ChiCTR-OPN-15006260). Informed consent was signed by all subjects after explanation on the aims and benefits of this research project.

Vasodilator-stimulated phosphoprotein-phosphorylation (VASP-P) assay

PRI was detected within 24 h after blood is drawn. To avoid platelet activation induced by needle puncture, the initial first blood millimeters were discarded. Blood samples were immediately collected in a vacutainer tube containing 3.8% trisodium citrate, filled to capacity, and analyzed immediately. A standardized flow cytometric assay (Platelet VASP®; Diagnostica Stago, Biocytex, Marseille, France) was used to determine the VASP-P level in whole blood according to the standard protocols [22]. Briefly, 10 µL blood sample was incubated with PGE1 or with PGE1 + ADP for 10 min and fixed with paraformaldehyde, after which the platelets were permeabilized with non-ionic detergent. The cells were labeled with a primary monoclonal antibody against serine 239-phosphorylated VASP (16C2), followed by a secondary fluorescein isothiocyanate-conjugated polyclonal goat anti-mouse antibody. The total duration of the preparation was within 30 min after blood sampling. Analyses were then performed on EPICS XL-MCL flow cytometer (Beckman Coultronics, Margency, France). The platelet population was identified from its forward and side scatter distribution and 10,000 platelets were gated for each sample. Platelet reactivity index (PRI) was calculated from the median fluorescence intensity (MFI) of samples with the formula:

$$\mathrm{PRI}\ (\%) = \left[\left(\mathrm{MFI}_{\mathrm{PGE1}} - \mathrm{MFI}_{\mathrm{PGE1+ADP}} / \mathrm{MFI}_{\mathrm{PGE1}}\right) \times 100\%.\right.$$

SNP selection and genotyping

Genomic DNA was purified from peripheral blood leukocytes by Wizard® Genomic DNA Purification Kit (Promega Corperation). A total of 13 SNPs in 8 genes including *P2RY12* (rs2046934, rs6809699), *PIK3CA* (rs67562832, rs67562832), *RASGRP2* (rs2230414), *APBB1IP* (rs11015149), *TLN1* (rs2295795, rs10814270), *ITGB3* (rs3785873, rs58847127), *ITGA2B* (rs3760364) and *CYP2C19* (rs4244285/*CYP2C19*2*, rs4986893/*CYP2C19*3*) were selected in our study. The SNPs selected were either reported to be clinically relevant or htSNPs indicated by Haploview analysis (www.broad.mit.edu/mpg/haploview/index.php) with a frequency > 5% in the 1000 genomes project for 97 Chinese Han Beijing (CHB) individuals (www.1000genomes.org). Details of the SNPs were shown in Table 1. Method of polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) was used for *CYP2C19*2* and *CYP2C19*3* genotyping as described previously [23]. The other SNPs were genotyped by Sequenom's MassARRAY

Table 1 Information of 13 SNPs selected in this study

Gene	SNP	Chr	Alleles	Functional consequence	MAF
CYP2C19	rs4244285	10:94781859	G > A	Pro227Pro	0.221
	rs4986893	10:94780653	G > A	stop gained	0.014
P2RY12	rs2046934	3:151339854	G > A	intron variant	0.205
	rs6809699	3:151338810	C > A	Gly12Gly	0.089
PIK3CA	rs67562832	3:179173633	A > G	intron variant	0.075
	rs77576241	3:179156079	C > T	intron variant	0.051
RASGRP2	rs2230414	11:64728885	C > A	Gly583Gly	0.36
APBB1IP	rs11015149	10:26523246	C > A	intron variant	0.15
TLN1	rs2295795	9:35712006	G > A	Ser1227Leu	0.278
	rs10814270	9:35704153	C > T	Ala2023Ala	0.4
ITGB3	rs3785873	17:47301872	G > A	intron variant	0.208
	rs58847127	17:47257956	G > C	intron variant	0.142
ITGA2B	rs3760364	17:44390436	T > A	upstream variant	0.011

Table 2 Baseline characteristics of 539 patients in clopidogrel resistance and non-resistance groups

Parameters	Sample A (N = 239)			Sample B (N = 300)				
	Non-CR (N = 90)	CR (N = 149)	*P* value	Non-CR (N = 98)	CR (N = 202)	*P* value	All patients (N = 539)	
Age (x ± SD)	61.67 ± 10.67	61.08 ± 10.02	0.669	61.87 ± 10.81	60.05 ± 9.61	0.143	60.94 ± 10.13	
Male, n (%)	59 (65.6)	97 (65.1)	0.943	70 (71.4)	131 (64.9)	0.256	357 (66.2)	
Diabetes, n (%)	14 (17.9)	29 (25.2)	0.234	15 (20.0)	32 (17.9)	0.691	90 (16.7)	
Hypertension, n (%)	56 (71.8)	79 (66.9)	0.473	46 (61.3)	92 (51.4)	0.147	273 (50.6)	
Dyslipidemia, n (%)	9 (11.4)	25 (21.7)	0.063	16 (21.3)	46 (25.7)	0.460	96 (17.8)	
Smoking, n (%)	31 (39.2)	47 (37.3)	0.781	25 (34.7)	71 (40.3)	0.410	174 (32.3)	
Alcohol use, n (%)	21 (28.4)	32 (28.1)	0.963	20 (27.8)	44 (25.1)	0.668	117 (21.7)	
Co-medication								
PPI, n (%)	47 (56.6)	74 (54.8)	0.794	41 (41.8)	78 (38.6)	0.593	240 (44.5)	
CCB, n (%)	23 (27.7)	28 (20.9)	0.250	6 (6.1)	0 (0.0)	0.000	57 (10.6)	
Statin, n (%)	51 (61.4)	71 (53.0)	0.222	21 (21.4)	0 (0)	0.000	143 (26.5)	
Morphine, n (%)	2 (2.4)	2 (1.5)	0.620	0 (0.0)	0 (0.0)	N/A	4 (0.7)	
Platelet count($\times 10^9$/L)	206.85 ± 77.01	202.28 ± 63.74	0.638	106.27 ± 109.76	177.87 ± 92.43	0.147	177.15 ± 94.81	
300 mg of clopidogrel, n (%)	41 (45.6)	78 (52.3)	0.309	47 (48.8)	105 (52.0)	0.514	271 (50.3)	
MPV (fL)	9.45 ± 3.79	9.29 ± 4.05	0.761	8.06 ± 4.73	9.20 ± 4.02	0.032	9.06 ± 4.15	
PRI (%)	34.14 ± 11.42	67.41 ± 10.89	0.000	29.89 ± 14.33	73.09 ± 10.36	0.000	57.16 ± 21.90	

PPI, proton pump inhibitor; CCB, calcium channel blocker; MPV, mean platelet volume

assay (Sequenom, San Diego, California, USA). Genotypes of 10% of the samples were verified by PCR-based sequencing. All selected SNPs were genotyped in discovery samples (239 CHD patients). The SNPs which have significant differences in PRI will be validated in validation samples(300 CHD patients).

Statistical analysis

Statistical analysis was performed using SPSS Statistics 19.0 (SPSS Inc., Chicago, USA). Continuous variables were presented as mean ± standard deviation (SD). The unpaired two-sided Student's t-test was used to compare normally distributed continuous data between two groups, and comparisons of difference in PRI among genotypes were carried out by one-way ANOVA test under the co-dominant model. The Benjamini–Hochberg procedure was performed to control the false discovery rate for multiple testing. $\chi 2$-test was used to compare categorical variables between/among groups. Unconditional logistic regression was used to assess the association between genotypes and clopidogrel resistance, odds ratios (OR) and the 95% confidence intervals (CI) were calculated and adjusted by the covariates. The Non-CR group was been defined as control group. Association analyses were conducted under three genetic models, including co-dominant, dominant, and recessive. Given D is the major allele and d is the minor allele for an SNP, the co-dominant model means DD versus Dd versus dd, the dominant model means DD versus Dd + dd, while the recessive model means DD + Dd versus dd. Statistical significance was defined as $P < 0.05$.

Results

Baseline characteristics of study patients and genotyping

From 2014 to 2018, a total of 539 eligible CHD patients with clopidogrel treatment were recruited in this study (Table 2). According to PRI from VASP-P assay, the patients were categorized into clopidogrel resistance (CR, PRI > 50%) and non-CR (PRI≦50%) [24, 25]. Among the patients, 351 (65.12%) were classified as CR, and 188 (34.88%) were classified as non-CR. There was no significant difference between the two groups regarding age, gender, smoking and alcohol administration habits, disease complications (diabetes, hypertension, dyslipidemia), co-medications (proton pump inhibitor, calcium channel blocker, statin, morphine), platelet count and MPV ($P > 0.05$). Meanwhile, the difference between the groups with 300 mg LD or 75 mg/d MD was not also statistically significant ($P > 0.05$, Table 3). So we combined patients with LD and MD as a whole in the subsequent analysis. Patients in the CR group showed significantly higher mean PRI value than the non-CR group ($P = 1.0 \times 10^{-43}$).

Association of candidate SNPs with clopidogrel response

Genotype distribution of the 13 studied SNPs in the CR and non-CR groups were summarized in Table 3. Fitness to Hardy–Weinberg equilibrium was observed for

Table 3 Distribution genotypes and allele frequencies and the candidate SNPs between CR and non-CR patients

Gene/SNP	Genotype	Non-CR	CR	Co-dominat *P* value	Dominant *P* value	Recessive *P* value
*CYP2C19*2*	No. of patients with data	90	149	0.020	0.031	0.000
	*1/*1, n (%)	52 (57.8)	64 (43.0)			
	*1/*2, n (%)	37 (41.1)	74 (49.7)			
	*2/*2, n (%)	1 (1.1)	11 (7.4)			
*CYP2C19*3*	No. of patients with data	87	144	0.227	N/A	0.227
	*1/*1, n (%)	81 (93.1)	127 (88.2)			
	*1/*3, n (%)	6 (6.9)	17 (11.8)			
*CYP2C19**2*3	No. of patients with data	87	144	0.072	N/A	0.072
	*1/*1, n (%)	46 (52.9)	53 (36.8)			
	*1/*2 + *1/*3, n (%)	7 (8.0)	2 (1.4)			
	*2/*2 + *1/*3, n (%)	0 (0.0)	0 (0.0)			
P2RY12 rs2046934	No. of patients with data	87	141	0.945	N/A	0.945
	GG, n (%)	51 (58.6)	82 (58.2)			
	GA, n (%)	36 (41.4)	59 (41.8)			
P2RY12 rs6809699	No. of patients with data	86	141	0.043	0.115	0.021
	CC, n (%)	76 (88.4)	107 (75.9)			
	CA, n(%)	10 (11.6)	30 (21.3)			
	AA, n (%)	0 (0)	4 (2.8)			
PIK3CA rs67562832	No. of patients with data	84	146	0.470	0.629	0.336
	AA, n (%)	64 (76.2)	119 (81.5)			
	AG, n (%)	19 (22.6)	24 (16.4)			
	GG, n (%)	1 (1.2)	3 (2.1)			
PIK3CA rs77576241	No. of patients with data	86	146	0.303	0.442	0.133
	CC, n (%)	84 (97.7)	136 (93.2)			
	CT, n (%)	2 (2.3)	9 (6.2)			
	TT, n (%)	0 (0)	1 (0.7)			
APBB1IP rs11015149	No. of patients with data	86	146	0.024	0.023	0.364
	CC, n (%)	73 (84.9)	117 (80.1)			
	CA, n (%)	10 (11.6)	29 (19.9)			
	AA, n (%)	3 (3.5)	0 (0)			
TLN1 rs2295795	No. of patients with data	85	145	0.326	0.135	0.672
	GG, n (%)	47 (55.3)	76 (52.4)			
	GA, n (%)	36 (42.4)	59 (40.7)			
	AA, n (%)	2 (2.4)	10 (6.9)			
TLN1 rs10814270	No. of patients with data	85	147	0.422	0.346	0.241
	CC, n (%)	21 (24.7)	47 (32.0)			
	CT, n (%)	44 (51.8)	73 (49.7)			
	TT, n (%)	20 (23.5)	27 (18.4)			
ITGB3 rs3785873	No. of patients with data	85	145	0.236	0.634	0.090
	GG, n (%)	61 (71.8)	88 (60.7)			
	GA, n (%)	20 (23.5)	48 (33.1)			
	AA, n (%)	4 (4.7)	9 (6.2)			
ITGB3 rs58847127	No. of patients with data	86	145	0.363	0.440	0.291
	GG, n (%)	71 (82.6)	127 (87.6)			
	GC, n (%)	15 (17.4)	17 (11.7)			
	CC, n (%)	0 (0)	1 (0.4)			
ITGA2B rs3760364	No. of patients with data	86	144	0.853	N/A	0.853
	TT (%)	80 (93)	133 (92.4)			
	TA (%)	6 (7)	11 (7.6)			

Table 3 (continued)

Gene/SNP	Genotype	Non-CR	CR	Co-dominat *P* value	Dominant *P* value	Recessive *P* value
RASGRP2 rs2230414	No. of patients with data	86	143	0.642	0.716	0.478
	CC, n (%)	39 (45.3)	58 (40.6)			
	CA, n (%)	36 (41.9)	69 (48.3)			
	AA, n (%)	11 (12.8)	16 (11.2)			

each of the SNP ($P>0.05$). The significant difference in genotype distribution for the *CYP2C19*2* polymorphism (co-dominant $P=0.020$, recessive $P=0.031$, and dominant $P=0.000$), the *P2Y12* rs6809699 polymorphism (co-dominant $P=0.043$, recessive $P=0.115$, and dominant $P=0.021$) and the *APBB11P* rs11015149 polymorphism (co-dominant $P=0.024$ and recessive $P=0.023$) was observed between CR and non-CR patients (Table 3). However, the Benjamini–Hochberg adjusted *P* values is higher than the false discovery rate (0.05) except the dominant *P* Value of *CYP2C19*2* (data not shown). Carriers of the *CYP2C19*2* allele (57.0% vs 42.2%, CR vs non-CR, $P=0.026$) and the *P2RY12* rs6809699 A allele (24.1% vs 14.0%, CR vs non-CR, $P=0.053$) was obviously over-represented in the clopidogrel CR group. The frequency of carriers of the *CYP2C19*3* allele trended to be increased in clopidogrel CR patients (11.8% vs 6.9%, CR vs non-CR, $P=0.227$), though a significant difference was obtained. No difference in genotype distribution of other SNPs was observed between CR and non-CR groups ($P>0.05$). And there was no association between the genetic polymorphisms and the occurrence of major adverse cardiovascular events (MACE) among the patients has been observed (Additional file 1: Table S1).

Unconditional logistic analysis was carried out for SNPs showed a significant difference in genotype distribution between clopidogrel CR and no-CR patients. After adjusted for dyslipidemia and concomitant use of statins and proton pump inhibitors, our results showed that patients with *CYP2C19*2/*2* genotype showed significantly increased risk of CR (OR 7.406, 95% CI 0.894–61.361; $P=0.063$) as compared with *CYP2C19*1/*1*homozygotes. When both *CYP2C19* LOF alleles (*2 and *3) were considered, CYP2C19 poor metabolizers (PMs, *CYP2C19**2/*2 or *CYP2C19**2/*3 or *CYP2C19**3/*3) showed significantly increased risk of CR (OR 4.599, 95% CI 1.221–17.320, $P=0.024$) as compared with wild-type homozygous for both SNPs. Patients carrying the *P2RY12* rs6809699 CA genotype or the rs6809699 A allele also showed significantly increased risk of CR (CA vs CC genotype: OR 2.270, 95% CI 1.019–5.059, $P=0.045$; CA + AA vs CC genotype: OR 2.636, 95% CI 1.199–5.796; $P=0.016$). The *APBB11P* rs11015149 polymorphism showed no association with clopidogrel response ($P>0.05$). (Table 4).

Combined influence of CYP2C19 LOF and P2RY12 rs6809699 polymorphism on on-treatment PRI

Mean PRI among *CYP2C19* genotypes were shown in Fig. 1. Patients were grouped into EMs, IMs, and PMs according to carrying status of the *CYP2C19**2 and *CYP2C19**3 alleles. In the discovery samples and all samples, PM patients showed significantly higher PRI than IM and EM patients, respectively. The influence of *CYP2C19**2 polymorphism on PRI was observed, which was not found in *CYP2C19*3* polymorphism (Table 5).

For the *P2RY12* rs6809699 genotypes, these patients with mutant homozygous AA (n = 4) were showed significantly higher PRI than the wild-type CC (n = 183) and heterozygous CA (n = 40) genotype groups ($P=0.0081$ and 0.0094, respectively, Fig. 2A). In consideration that the influence of *P2RY12* rs6809699 on clopidogrel response might be affected by *CYP2C19* LOF, stratification analysis according to *CYP2C19* genotypes was further performed. As shown in Fig. 2A, rs6809699 AA homozygotes showed significantly higher PRI than patients carrying both the rs6809699 CC and the rs6809699 CA genotypes ($P=0.0096$ and 0.0036, respectively). Only one patient with the AA genotype in carriers of the *CYP2C19* LOF limited statistical analysis in these patients, but the tendency remained. Then the rs6809699 was validated in all subjects (discovery and validation samples) (Fig. 2).

Discussion

In this study, we evaluated the effects of genetic polymorphisms in the P2Y12 receptor-mediated signaling pathway and *CYP2C19* on clopidogrel antiplatelet response in Chinese CHD patients. We observed that *CYP2C19**2 and *3 and *P2Y12* rs6809699 polymorphisms were associated with an increased risk of clopidogrel resistance indicated by platelet VASP-P level.

Clopidogrel is a prodrug that needs to be bioactivated in two sequential cytochrome P450-dependent steps before it exerts an inhibitory effect on ADP-induced platelet aggregation. According to the literature, the prevalence of clopidogrel resistance among the Asian

Table 4 Logistic analysis of the association of gene polymorphisms and clopidogrel response

Gene/SNP	Genotype	Non-CR	CR	OR[a] (95% CI)	*P*[a] value
*CYP2C19**2	**1/*1*, n (%)	52 (57.8)	64 (43.0)	1.0 (ref)	N/A
	**1/*2*, n (%)	37 (41.1)	74 (49.7)	1.625 (0.949–2.783)	0.076
	**2/*2*, n (%)	1 (1.1)	11 (7.4)	8.938 (1.117–71.509)	0.015
	Carriers of *2	38 (42.2)	85 (57.0)	1.817 (1.070–3.086)	0.026
*CYP2C19**3	**1/*1*, n (%)	81 (93.1)	127 (88.2)	1.0 (ref)	N/A
	**1/*3*, n (%)	6 (6.9)	17 (11.)	1.807 (0.684–4.774)	0.227
*CYP2C19**2 and *3	**1/*1*, n (%)	46 (52.9)	53 (36.8)	1.0 (ref)	N/A
	**1/*2* + **1/*3*, n (%)	7 (8.0)	2 (1.4)	0.248 (0.049–1.253)	0.072
	**2/*2* + **1/*3*, n (%)	0 (0.0)	0 (0.0)	4.509 (1.387–14.660)	0.012
	Carriers of **2* or **3*, n (%)	7 (8.0)	2 (1.4)	0.248 (0.049–1.253)	0.072
P2RY12 rs6809699	CC, n (%)	74 (86.0)	107 (75.9)	1.0 (ref)	N/A
	CA, n (%)	12 (14.0)	30 (21.3)	1.729 (0.831–3.595)	0.140
	AA, n (%)	0 (0)	4 (2.8)	N/A	0.099
	CA + AA, n (%)	12 (14.0)	35 (24.1)	2.017 (0.982–4.142)	0.053
APBB1IP rs11015149	CC, n (%)	73 (84.9)	117 (80.1)	1.0 (ref)	N/A
	CA, n (%)	10 (11.6)	29 (19.9)	1.809 (0.833–3.931)	0.130
	AA, n (%)	3 (3.5)	0 (0)	N/A	0.030
	CA + AA, n (%)	13 (15.1)	29 (19.9)	1.392 (0.680–2.850)	0.364

[a] Adjusted for use of statins and dyslipidemia

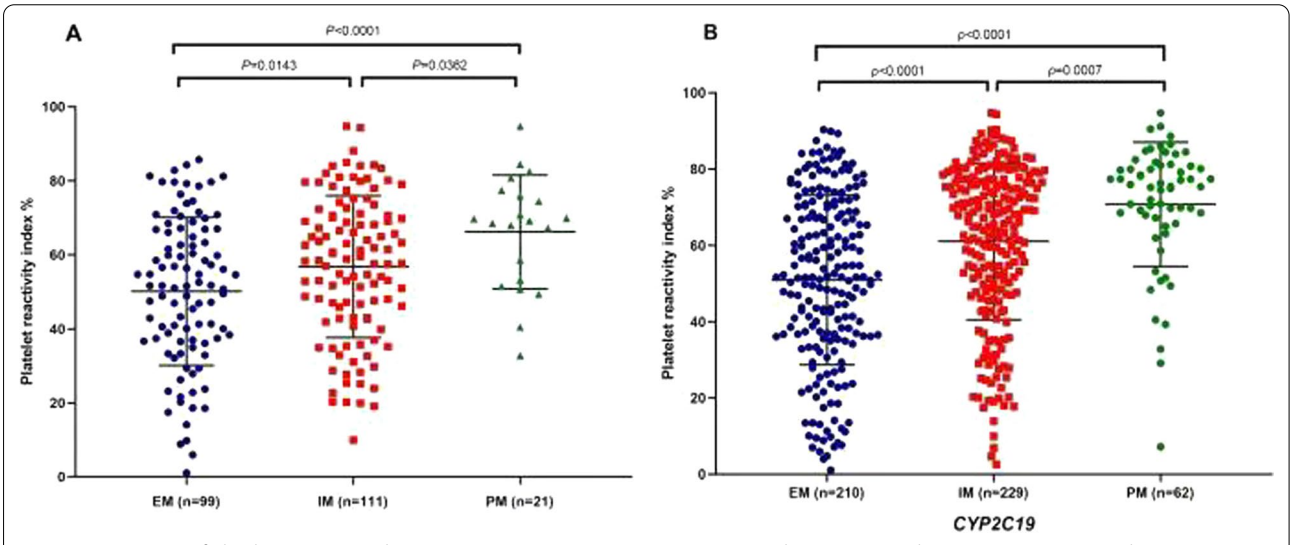

Fig. 1 Comparison of platelet reactivity index (PRI) in CHD patients among *CYP2C19*2* and *CYP2C19*2* and **3* genotype groups in discovery samples (**A**) and all subjects (**B**)

population was estimated at 17.2–81.6% [26]. In this study, a total of 539 consecutive Chinese patients with coronary heart disease were recruited and found that 65.1% patients had clopidogrel resistance. CYP2C19 activity is reported to be crucial in the metabolism and efficacy of clopidogrel. *CYP2C19* LOF alleles, including *2 and *3 can decrease the plasma concentration and $AUC_{0-24\ h}$ of the active metabolite of clopidogrel, which results in impaired antiplatelet effect of clopidogrel [6, 24]. A recent meta-analysis has concluded that *CYP2C19* LOF is associated with increased risk of adverse clinical events in patients who underwent clopidogrel therapy despite differences in clinical significance according to ethnicity [7]. In support of these previous reports, we observed that patients with the *CYP2C19**1/*1 genotype showed significantly lower PRI than the *CYP2C19**2

Table 5 Comparison of platelet reactivity index (PRI) in CHD patients among *CYP2C19**2 and *3 genotype groups

SNP	Genotype	PRI (Discovery samples)	*P* value	PRI (All samples)	*P* value
CYP2C19*2	GG	51.27 ± 19.69	0.0015	51.94 ± 22.10	< 0.0001
	AG	56.99 ± 19.02		61.74 ± 20.08	
	AA	70.33 ± 13.47		72.18 ± 15.71	
CYP2C19*3	GG	54.21 ± 20.01	0.1719	57.64 ± 21.98	0.1742
	AG	60.15 ± 16.50		61.05 ± 20.17	
	AA	–		82.71 ± 11.00	

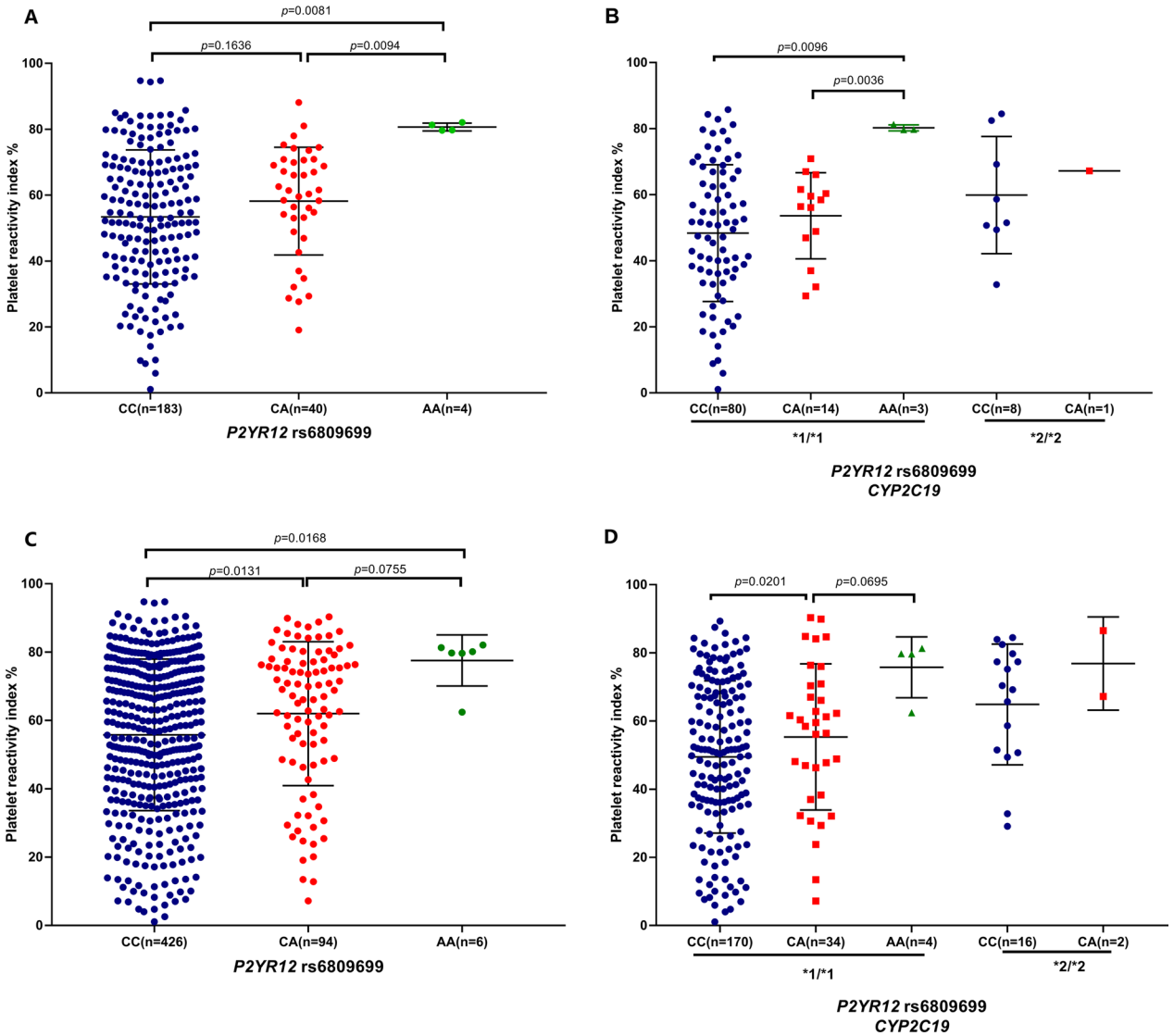

Fig. 2 Comparison of PRI in CHD patients among *P2RY12* rs6809699 genotypes stratified by *CYP2C19* genotypes in discovery samples (**A**, **B**) and all subjects (**C**, **D**)

heterozygous and homozygous genotypes. Besides, we found that carriers of any of the *2 and *3 alleles showed increased clopidogrel resistance. Our findings further confirmed the pivotal role of *CYP2C19*2* and *3 as pharmacogenomics markers for clopidogrel response. In the 2013 updated Clinical Pharmacogenetics Implementation Consortium Guidelines for *CYP2C19* Genotype and Clopidogrel Therapy, *CYP2C19* genotype-guided clopidogrel therapy was recommended to ACS patients underwent PCI [27]. Standard dosing of clopidogrel is warranted among ACS/PCI patients with a predicted CYP2C19 extensive metabolizer phenotype (*1/*1). If genotyping identifies a patient as a CYP2C19 weak metabolizer phenotype (*2/*2, *2/*3 and *3/*3), the use of an alternative antiplatelet agent (e.g., prasugrel or ticagrelor) is recommended if not clinically contraindicated.

ADP is an essential activator of platelet and acts via P2Y1 (Gq-coupled) and P2Y12 (Gi-coupled) receptors. The Gq-coupled P2Y1 receptor is vital in Ca^{2+} mediated platelet shape change, while the Gi-coupled P2Y12 receptor is required for ADP-induced platelet activation [28]. The active metabolite of clopidogrel binds to the P2Y12 receptor irreversibly and inhibits ADP-mediated platelet activation and aggregation. The role of the *P2RY12* genetic polymorphisms in clopidogrel response has been assessed previously [19, 20, 29–32]. Evidence shows that the *P2RY12* T744C (rs2046934) polymorphism is associated with enhanced platelet aggregation and increased risk of atherothrombosis [19, 30]. However, Thomas et al. failed to replicate this observation with platelet activity assessed by either ADP-Ag ($P=0.39$), or PRI VASP-P ($P=0.97$), or P-selectin expression ($P=0.62$) in 597 NSTE ACS patients [27]. Other studies also come to negative findings [31, 32]. In agreement with the latter investigators, we did not find any association between the *P2RY12* T744C and clopidogrel resistance either.

The *P2RY12* G52T (rs6809699) was also shown to be associated with increased risk of clopidogrel resistance and cardiovascular events in Chinese ACS patients after PCI [20]. In support of this report, we observed that CHD patients with the *P2RY12* rs6809699 CA genotype or carriers of the rs6809699 A allele showed an increased risk for clopidogrel resistance with an OR of 1.729 and 2.017, respectively. After stratification by *CYP2C19*2* and *3 carrying status, the *P2RY12* rs6809699 polymorphism remained to be associated with increased platelet activity. As the rs6809699 polymorphism is a synonymous SNP (Gly12Gly) does not result in amino acid change, the exact function of this SNP deserved further investigation.

Abnormality in GPIIb/IIIa complex is reported in Glanzmann's thrombasthenia patients with impaired platelet aggregation and increased bleeding [33]. The *ITGB3* PlA1/A2 polymorphism (rs5918) results in a leucine (PlA1) to proline (PlA2) substitution in exon2 was observed [34]. This SNP has been extensively studied and is shown to be associated with both antiplatelet drug resistance and increased cardiovascular events [35, 36]. Because the prevalence of the PlA2 allele is low in the Chinese population, the SNP was not included in our study. Two other SNPs, including rs3785873 and rs58847127 at the *ITGB3* locus were investigated in our study. However, no significant findings were obtained for these two SNPs. A healthy subjects study showed that *ITGA2B* rs3760364 were related to bleeding time [17], but we failed to find the association between *ITGA2B* rs3760364 and platelet activity.

In our study, we also observed that the *APBB11P* rs11015149 A allele was significantly over-represented in CR than non-CR patients, but this difference was disappeared after adjusted for statins use and dyslipidemia. The other 6 selected SNPs in genes in the P2Y12-mediated signaling pathway (*PIK3CA* rs67562832 and rs67562832, *RASGRP2* rs2230414, *APBB1IP* rs11015149, *TLN1* rs2295795, and rs10814270) also showed no association with clopidogrel resistance. It remains unknown whether genetic factors in other alternative pathways playing compensatory roles in GPIIb/IIIa inside-out signaling could affect clopidogrel response.

Although the *CYP2C19* genotyping had been widely recommended when considering clopidogrel for cardiovascular indications, it remains undetermined that *P2RY12* polymorphisms associated with clopidogrel resistance. In our study, we reconfirmed the impact of *CYP2C19*2, *3* and *P2RY12* rs6809699 polymorphisms on impaired antiplatelet effects of clopidogrel in Chinese CHD patients. It suggested that *P2RY12* genetic polymorphisms may serve as biomarkers for clopidogrel response. Meanwhile, we found the increased risk of clopidogrel resistance in *CYP2C19*1/*1* homozygous who carrying the *P2RY12* rs6809699 A allele. This may, at least partially, explain that some CYP2C19 *CYP2C19*1/*1* homozygous were still resistant to clopidogrel. Therefore, construction of a comprehensive prediction model of clopidogrel responsiveness based on clinical factors and multiple gene polymorphisms, including CYP2C19 and *P2RY12* polymorphisms, has more clinical significance for guiding the precise medication of clopidogrel.

Limitations of the study include a relatively small sample size. As exemplified by only 4 patients with *P2RY12* rs6809699 mutant AA genotype in our study, further studies are warranted to verify the impact of *P2RY12* rs6809699 polymorphisms on antiplatelet effects of clopidogrel. Secondly, platelet function testing was done with only a single assessment of platelet

function, VASP-P assay, which may not be sufficient to fully reflect the response to antiplatelet therapy. Finally, follow-up data is warranted to understand the influence of the positively associated SNPs on the endpoint events and outcome of CHD patients with long-time clopidogrel therapy.

Conclusions

This study confirms the impact of *CYP2C19*2, *3* and *P2RY12* rs6809699 polymorphisms on impaired antiplatelet effects of clopidogrel in Chinese CHD patients. Moreover, the influence of *P2RY12* rs6809699 on clopidogrel response is independent of *CYP2C19* LOF alleles. But SNPs in other genes in the P2Y12 receptor pathway were not associated with antiplatelet effects of clopidogrel. A study with a larger sample size is required to confirm the association of the *P2RY12* rs6809699 with adverse ischemic events in patients receiving clopidogrel therapy. And also, the exact function of *P2RY12* rs6809699 on P2Y12 expression or function is needed.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12872-022-02988-w.

Additional file 1: Table S1. Distribution genotypes and allele frequencies and the candidate SNPs between patients with and without MACE.

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Author contributions
Xiao-Ping Chen contributed to the conception of the study; Zhongyi Li and Xiao-Lei Hu contributed significantly to analysis and manuscript preparation; Dongjie Li and Yan-Jiao Zhang performed the data analyses and wrote the manuscript; He Li and Qi-Lin Ma helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Availability of data and materials
The first author can be contacted if the raw data are needed. The email address of the first author is dongjieli@csu.edu.cn.

Declarations

Ethics approval and consent to participate
The study protocols were approved by the Ethics Committee of Central South University (No. CTXY-140002-13) and followed the Declaration of Helsinki. It was also registered on the Chinese Clinical Trial Registry (http://www.chictr.org.cn) (ChiCTR-OPN-15006260). Informed consent was obtained from all subjects involved in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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