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Haplotype of platelet receptor *P2RY12* gene is associated with residual clopidogrel on-treatment platelet reactivity*

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Abstract: Objective: To investigate a possible association between common variations of the *P2RY12* and the residual clopidogrel on-treatment platelet reactivity after adjusting for the influence of *CYP2C19* tested by thromboelastography (TEG). Methods: One hundred and eighty patients with acute coronary syndrome (ACS) treated with clopidogrel and aspirin were included and platelet function was assessed by TEG. Five selected *P2RY12* single nucleotide polymorphisms (SNPs; rs6798347, rs6787801, rs6801273, rs6785930, and rs2046934), which cover the common variations in the *P2RY12* gene and its regulatory regions, and three *CYP2C19* SNPs (*2,*3,*17) were genotyped and possible haplotypes were analyzed. Results: The high on-treatment platelet reactivity (HTPR) prevalence defined by a platelet inhibition rate <30% by TEG in adenosine diphosphate (ADP)-channel was 69 (38.33%). Six common haplotypes were inferred from four of the selected *P2RY12* SNPs (denoted H_0 to H_5) according to the linkage disequilibrium *R* square (except for rs2046934). Haplotype H_1 showed a significantly lower incidence of HTPR than the reference haplotype (H_0) in the total study population while haplotypes H_1 and H_2 showed significantly lower incidences of HTPR than H_0 in the nonsmoker subgroup after adjusting for *CYP2C19* effects and demographic characteristics. rs2046934 (T744C) did not show any significant association with HTPR. Conclusions: The combination of common *P2RY12* variations including regulatory regions rather than rs2046934 (T744C) that related to pharmacodynamics of clopidogrel in patients with ACS was independently associated with residual on-clopidogrel platelet reactivity. This is apart from the established association of the *CYP2C19*. This association seemed more important in the subgroup defined by smoking.

Key words: *P2RY12*; *CYP2C19*; Haplotype; Single nucleotide polymorphism (SNP); Clopidogrel; Thromboelastography
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1 Introduction

Dual antiplatelet therapy with aspirin and a platelet P2Y12 adenosine diphosphate (ADP) receptor antagonist reduces recurrent adverse cardiovascular events in acute coronary syndrome (ACS) and/or following percutaneous coronary intervention (PCI) (Yusuf *et al.*, 2001; Beinart *et al.*, 2005). It is recommended for patients with ACS and/or PCI in China (Emergency Medical Branch of Chinese Medical Doctor Association *et al.*, 2016; Section of Interventional Cardiology of Chinese Society of Cardiology of Chinese Medical Association *et al.*, 2016) and

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worldwide (Levine *et al.*, 2016; SIGN, 2016). Clopidogrel is currently one of the most commonly used platelet P2Y12 receptor inhibitors in China, although new guidelines have recommended ticagrelor as the first-line medicine in patients with ACS or following PCI.

Clopidogrel is an orally administered thienopyridine prodrug, which needs to be biotransformed into its active metabolite. After oral administration, the absorption of clopidogrel is regulated by adenosine triphosphate (ATP)-binding cassette, sub-family B, member 1 (ABCB1) transporters (Mega *et al.*, 2010a), and the hepatic biotransformation of clopidogrel prodrug to its active metabolite is mediated by CYP450 enzymes, especially CYP2C19. After activation, the active metabolite of clopidogrel selectively and irreversibly inhibits the P2Y12 receptor located on the surface of platelets and decreases platelet aggregation. Many studies have demonstrated the close relationship between *CYP2C19* genotype and high on-treatment platelet reactivity (HTPR) (Mega *et al.*, 2010b; Simon *et al.*, 2011), including some studies focusing on Chinese subjects (Chen *et al.*, 2014) and our previous study (Liu *et al.*, 2015).

As well as *CYP2C19*, other genetic factors may contribute to the inter-individual variability of clopidogrel, among which *P2RY12*, which encodes the P2Y12 receptor on platelets, is of importance. Previous studies have implied that *P2RY12* single nucleotide polymorphism (SNP) might play a role on the large variability in clopidogrel response, but the results were inconsistent and controversial (Angiolillo *et al.*, 2007; Zee *et al.*, 2008; Jana *et al.*, 2014; Oestreich *et al.*, 2014; Li *et al.*, 2016; Zhang *et al.*, 2016). Most of those studies focused on single haplotype-tagging SNPs (ht-SNPs), such as rs2046934 (i-T744C) or rs6785930, or two common haplotypes H_1 and H_2 (constituted by single nucleotide polymorphism database (dbSNP) rs10935838, rs2046934, rs5853517, and rs6809699), especially the previous studies in the Chinese population (Chen *et al*., 2014; Zhang *et al*., 2016). Several studies focused on rs10935838, rs5853517, and some other SNPs at the same time, but mostly in the Caucasian population (Zee *et al.*, 2008; Rudež *et al.*, 2009). rs10935838, rs2046934, rs5853517, and rs6809699 are in close linkage disequilibrium (LD) but not covering the regulatory regions of the *P2RY12* gene. A comprehensive study of common variations in the *P2RY12* gene that cover common haplotypes within the *P2RY12* gene and its regulatory regions is lacking in the Chinese population with ACS. Moreover, most present studies focus on the *P2RY12* gene separately, without adjusting for the influence of the main proven gene to affect clopidogrel on-treatment residual platelet activities—the *CYP2C19**2 and *3 alleles. The aim of this study was to investigate a possible association between the common genetic variations of the *P2RY12* locus, including the promoter region after adjusting for the influence of *CYP2C19**2 and *3 alleles, and the residual on-clopidogrel platelet reactivity by thromboelastography (TEG) assay in a Chinese population with ACS.

2 Materials and methods

2.1 Study population

Consecutive patients aged from 18 to 80 years with established ACS at Peking University Second Hospital, Beijing, China, during May 2014 to April 2015 were included in the study. The diagnosis of ACS (unstable angina or myocardial infarction) was confirmed by ischemic symptoms and electrocardiograph (ECG) changes with or without increase in creatine kinase and troponin levels plus coronary angiography if needed. The exclusion criteria included contraindications to clopidogrel, aspirin, heparin, and contrast agents as well as quantitative coronary angiography drugs, and whether there had been a major operation within the last one month and the use of a glycoprotein IIb/IIIa antagonist during hospitalization.

All the eligible patients received 75 mg/d clopidogrel and 100 mg/d aspirin for at least 5 d with or without a loading dose of 300 or 600 mg clopidogrel according to institutional practice in addition to the other available medical therapy, including statins, β-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, or antidiabetic agents.

2.2 Definition

Smokers were defined as current or recent smokers (stopped smoking for <1 year). Hypertension, diabetes mellitus, and dyslipidemia were defined according to the World Health Organization criteria.

A family history of cardiovascular disease was defined as an early history of coronary artery disease in women younger than 65 years and in men younger than 55 years among the first-degree relatives.

2.3 Blood collection

On the 5th to 7th days after clopidogrel administration, blood samples were collected from peripheral veins with vacutainer tubes (BD Medical Systems) containing heparin or 32 g/L sodium citrate. TEG assay was conducted within 3 h after blood collection. The study protocol was approved by the Ethics Committee of Peking University, China (IRB 00001052-14087).

2.4 Platelet function assay

Citrated whole blood (2 ml) and heparin-anticoagulated whole blood (2 ml) were drawn at least 5 d after the administration of the first clopidogrel maintenance dose. These samples were sent to the hemostasis laboratory of the hospital and TEG was performed within 3 h after blood collection according to the manufacturer's instruction (TEG 5000, Haemoscope Corp., IL, USA) by trained technicians at the hospital. Platelet reactivity was calculated using the TEG platelet mapping system. TEG utilizes four channels to detect the effects of antiplatelet therapy activity induced by arachidonic acid or ADP. The platelet inhibition rate (PIR) in the ADP channel was recorded. A TEG PIR of less than 30% was defined as clopidogrel HTPR, while patients with non-high on-treatment platelet reactivity (nHTPR) had a PIR of at least 30% according to the instruction and previous study (Bliden *et al.*, 2007).

2.5 Selection of SNPs

Based on the latest LD data of the *P2RY12* locus (*P2RY12* gene with 2.5 kb flanking sequence) provided by SNP Annotation and Proxy Search (SNAP) for a population of Han Chinese in Beijing and Japanese in Tokyo (CHBJPT) (http://www.broadinstitute.org/mpg/snap), we chose the five most common *P2RY12* SNPs which cover common haplotypes within the *P2RY12* gene and its regulatory regions to be analyzed. LD data were calculated using Haploview 4.0, based on phased genotype data from the 1000 Genomes Project (1000 Genomes Pilot 1) (Table 1). The five selected SNPs that were genotyped were rs6798347 (c.−281−3614C>t), rs6787801 (c.−217+2739T>c), rs6801273 (c.−216−4445A>g), rs6785930 (c.18C>t, which was also identified as C34T), and rs2046934 (c.742T>c, which was also identified as T744C).

In addition to the five selected *P2RY12* genes, we also genotyped the three most common reported *CYP2C19* SNPs, *CYP2C19**2 (c.681G>A, rs4244285), *CYP2C19**3 (c.636G>A, rs4986893) and *CYP2C19**17 (c.806C>T, rs12248560), to adjust their effect on the platelet reactivity for the above selected SNPs.

2.6 Genetic and haplotype analysis

After TEG testing, the used heparin-anticoagulated whole blood was collected from the hemostasis laboratory. Blood sample genomic DNA was isolated using the DNA extractor AxyPrep-96 (AXYGEN Scientific Inc., California, USA). *CYP2C19**2 (rs4244285), *CYP2C19**3 (rs4986893), *CYP2C19**17 (rs12248560), and *P2RY12* SNPs (rs6798347, rs6787801, rs6801273, rs2046934, and rs6785930) were identified by the ligase detection reaction (LDR)-real time polymerase chain reaction (PCR) analysis system (Perkin-Elmer Gene Amp PCR Systems 9600, Perkin-Elmer Shanghai Inc., Shanghai, China). GENESCAN™ 672 and Genemapper of the system were used to analyze the *CYP2C19* alleles. In this study, 10% of the samples were randomly reanalyzed,

Table 1 Linkage disequilibrium *R*-square of the selected *P2RY12* SNPs

P2RY12 SNP	Pairwise R^2				
	rs6798347	rs6787801	rs6801273	rs2046934	rs6785930
rs6798347	rs6798347				
rs6787801	**0.354**	rs6787801			
rs6801273	**0.347**	0.144	rs6801273		
rs2046934	0.147	0.114	0.169	rs2046934	
rs6785930	**0.336**	0.148	**0.446**	0.076	rs6785930

Linkage disequilibrium data are calculated based on phased genotype data from the 1000 Genomes Project (1000 Genomes Pilot 1), and the pairwise R^2 values of >0.3 were selected for haplotype analysis (shown in bold numbers)

and the results were confirmed in 98.92%. According to the LD *R*-square of the selected *P2RY12* SNPs, SNPs with pairwise R^2 values >0.3 were selected for haplotype analysis, which turned out to be rs6798347, rs6787801, rs6801273, and rs6785930.

2.7 Statistical analysis

Statistical analysis was carried out using STATA for Windows, Version 12.0 (Stata Corp., Texas, USA). Demographic data were presented as means and standard deviations (SDs) for continuous variables and as counts and percentages for categorical variables. Hardy-Weinberg equilibrium analysis was conducted using a chi-square test. Multivariate logistic regression analysis was carried out to study the association between individual SNPs/haplotypes and HTPR. Homozygotes for the common allele of these SNPs were used as the reference for each comparison analysis. *P2RY12* haplotype analysis was done separately in nonsmokers. Analyses were adjusted for age, sex, body mass index, smoker, diabetes, hypertension, hyperlipidemia, family history of cardiovascular disease, platelet count, clopidogrel dose regimen (with or without loading dose), prior vascular disease and diagnosis (ST-segment elevation myocardial infarction (STEMI) or non-ST-segment elevation acute coronary syndrome (NSTE-ACS)). Results were expressed as odds ratio (OR) with 95% confidence interval (95% CI). A 2-sided value of $P<0.05$ was considered statistically significant.

3 Results

3.1 Platelet reactivity detection and baseline characteristics

One hundred and eighty patients were included in this study. All were from the Chinese Han population, and 120 (66.67%) patients underwent PCI. We identified 69 (38.33%) patients with HTPR. Demographic characteristics according to different clopidogrel responses are shown in Table 2. There

Table 2 Demographic characteristics of the studied population according to residual platelet reactivity

Characteristics	Overall (*n*=180)	HTPR (*n*=69)	nHTPR (*n*=111)	*P*-value
Demographics				
Age	64.09±11.08	64.91±10.47	63.59±11.46	0.434
Male	137 (76.11%)	50 (72.46%)	87 (78.38%)	0.367
Risk factors				
BMI (kg/m^2)	25.81±3.49	25.78±3.69	25.83±3.38	0.921
Smoker	54 (30.00%)	15 (21.74%)	39 (35.14%)	0.046
DM	70 (38.89%)	28 (40.58%)	42 (37.84%)	0.714
Hypertension	122 (67.78%)	48 (69.57%)	74 (66.67%)	0.686
Dyslipideamia	78 (43.33%)	28 (40.58%)	50 (45.05%)	0.557
FH of CVD	63 (35.00%)	25 (36.23%)	38 (34.23%)	0.785
Medication				
Clopidogrel loading dose	42 (23.33%)	11 (15.94%)	31 (27.93%)	0.068
β-Receptor antagonist	132 (73.33%)	46 (66.67%)	86 (77.48%)	0.113
ACEIs or ARBs	109 (60.56%)	40 (57.97%)	69 (62.16%)	0.576
CCBs	48 (26.67%)	23 (33.33%)	25 (22.52%)	0.113
Statins	165 (91.67%)	64 (92.75%)	101 (90.99%)	0.678
PPIs	28 (15.56%)	11 (15.94%)	17 (15.32%)	0.910
NSTE-ACS	124 (68.89%)	44 (63.77%)	80 (72.07%)	0.243
Prior MI	29 (16.11%)	12 (17.39%)	17 (15.32%)	0.713
Prior PCI	119 (66.11%)	47 (68.12%)	72 (64.86%)	0.654
Platelet count ($10^9\ L^{-1}$)	189.11±61.19	193.06±64.50	186.70±59.25	0.502
Haematocrit (%)	38.35±6.37	37.74±5.16	38.73±7.01	0.320
Hemoglobin (g/L)	133.50±18.52	130.40±22.43	133.50±18.52	0.319

Values are expressed as mean±SD or *n* (%). *P*-values refer to comparisons between HTPR and nHTPR groups. HTPR: high on-treatment platelet reactivity; nHTPR: non-high on-treatment platelet reactivity; BMI: body mass index; DM: diabetes mellitus; FH of CVD: family history of cardiovascular disease; ACEIs/ARBs: angiotensin-converting enzyme inhibitors/angiotensin receptor blockers; CCBs: calcium channel blockers; PPIs: proton pump inhibitors; NSTE-ACS: non-ST-segment elevation acute coronary syndrome; MI: myocardial infarction; PCI: percutaneous coronary intervention

were no significant differences among age, gender, risk factors, medications, or other general information between the two groups (P>0.05) except for smoking (P=0.046).

3.2 Genotyping and haplotype results

Allele frequencies and genotype distribution of the genetic variations studied are shown in Table 3. The distribution of all the *CYP* genetic variants did not deviate from Hardy-Weinberg equilibrium.

3.2.1 *CYP2C19*

Among the 180 patients studied, 89 (49.44%) were carriers of at least one *CYP2C19**2 loss-of-function (LOF) allele and 18 (10.00%) were carriers of at least one *CYP2C19**3 LOF allele. For *CYP2C19**17 gain of function (GOF) allele, only one heterozygous carrier was detected.

On the basis of the distribution of genotypes, the patients were divided into extensive metabolizers (EMs) without an LOF mutation allele (LOF non-carriers), which was also the *CYP2C19**1/*1 (681GG/636GG) wild type genotype, intermediate metabolizers (IMs) carrying only one LOF mutation allele (*CYP2C19**2 or *CYP2C19**3) with or without the GOF mutation allele, and poor metabolizers (PMs) carrying two LOF mutation alleles (*CYP2C19**2 and *CYP2C19**3), accounting for 42.78%, 42.78%, and 14.44% of all cases, respectively.

3.2.2 *P2RY12*

Genotype distribution and allele frequencies of the studied *P2RY12* SNPs were given in Table 3. According to the LD R-square of the selected *P2RY12* SNPs shown in Table 1, we combined the four *P2RY12* ht-SNPs (rs6798347, rs6787801, rs6801273, and rs6785930) and 12 haplotype alleles were inferred, among which 6 had an allele frequency higher than 5%: (H_0) CTAC (7.78%), (H_1) tcgt (14.44%), (H_2) tTgt (16.11%), (H_3) CcgC (18.33%), (H_4) CcAC (21.67%), and (H_5) tTAC (7.22%), as shown in Table 4. Finally, the defined haplotypes cover 85.56% of the total common selected DNA sequence variations in the *P2RY12* locus. The last *P2RY12* SNP rs2046934 was not combined as the pairwise LD R^2 values with the other four SNPs were low (<0.3).

Table 3 Genotype distribution and allele frequencies of investigated genetic variations

Variation	Non-carriers, *n* (%)	Heterozygous carriers, *n* (%)	Homozygous carriers, *n* (%)	Carriers of at least one allele, *n* (%)	Allele frequency (%)
CYP2C19					
*2 (rs4244285)	91 (50.56%)	67 (37.22%)	22 (12.22%)	89 (49.44%)	30.83
*3 (rs4986893)	162 (90.00%)	18 (10.00%)	0 (0.00%)	18 (10.00%)	5.00
*17 (rs12248560)	179 (99.44%)	1 (0.56%)	0 (0.00%)	1 (0.56%)	0.28
P2RY12					
rs6798347	94 (52.22%)	67 (37.22%)	19 (10.56%)	86 (47.78%)	29.17
rs6787801	60 (33.33%)	81 (45.00%)	39 (21.67%)	120 (66.67%)	44.17
rs6801273	71 (39.44%)	83 (46.11%)	26 (14.44%)	109 (60.56%)	37.50
rs6785930	116 (64.44%)	54 (30.00%)	10 (5.56%)	64 (35.56%)	20.56
rs2046934	122 (67.78%)	54 (30.00%)	4 (2.22%)	58 (32.33%)	17.22

Table 4 Composition and frequency of *P2RY12* haplotypes

Haplotype	(1) (C>t)	(2) (T>c)	(3) (A>g)	(4) (C>t)	SNP allele composition	Frequency (%)
H_0	C	T	A	C	CTAC	7.78
H_1	t	c	g	t	tcgt	14.44
H_2	t	T	g	t	tTgt	16.11
H_3	C	c	g	C	CcgC	18.33
H_4	C	c	A	C	CcAC	21.67
H_5	t	T	A	C	tTAC	7.22

Haplotype alleles were coded H_1 to H_5 in the descending order of their effect on platelet inhibition rate by TEG where H_0 is the reference haplotype allele. (1)–(4) represent rs6798347, rs6787801, rs6801273, and rs6785930, respectively, with the minor alleles in lower case

3.3 Relationship of genotypes and haplotypes with residual platelet reactivity

The results of multivariate logistic regression for HTPR in the entire study population are presented in Table 5. After adjusting for demographic characteristics, the presence of at least one LOF allele (PMs or IMs) or *P2RY12* haplotype was independently associated with HTPR in the entire study population and in nonsmokers (Fig. 1).

3.3.1 *CYP2C19*

Carriers of at least one LOF allele (PMs and IMs) had a higher residual platelet reactivity than non-carriers (EMs), and IMs had a higher incidence of HTPR than EMs in the total study population and the nonsmoker subgroup (46 (46.46%) vs. 19 (24.68%), OR 6.00, *P*-value 0.000, 95% CI 2.27–15.82; 32 (51.61%) vs. 20 (32.26%), OR 4.69, *P*-value 0.007, 95% CI 1.52–14.46). PMs had 100% incidence of HTPR.

Table 5 Influence of all genetic variants/haplotypes on platelet reactivity

SNP/haplotype	HTPR, *n* (%)	nHTPR, *n* (%)	Total, *n* (%)	*P*-value
CYP2C19				
EM	19 (24.68)	58 (75.32)	77 (42.78)	Reference
IM	39 (50.65)	38 (49.35)	77 (42.78)	0.001
PM	11 (42.31)	15 (57.69)	26 (14.44)	0.091
P2RY12 (rs2046934)				
Non-carriers	44 (36.07)	78 (63.93)	122 (67.78)	Reference
Carriers	25 (43.10)	33 (56.90)	58 (32.33)	0.223
P2RY12 haplotype				
H_0	8 (57.14)	6 (42.86)	14 (7.78)	Reference
H_1	9 (34.62)	17 (65.38)	26 (14.44)	0.016
H_2	10 (34.48)	19 (65.52)	29 (16.11)	0.062
H_3	13 (39.39)	20 (60.61)	33 (18.33)	0.104
H_4	15 (38.46)	24 (61.54)	39 (21.67)	0.105
H_5	7 (53.85)	6 (46.15)	13 (7.22)	0.296

Values are expressed as *n* (%). *P*-values refer to comparisons between HTPR and nHTPR groups by multivariate logistic regression adjusted by *CYP2C19* and covariate characteristics (including smoker). HTPR: high on-treatment platelet reactivity; nHTPR: non-high on-treatment platelet reactivity; EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer. Haplotypes H_0 to H_5 were coded by the compositions of rs6798347, rs6787801, rs6801273, and rs6785930

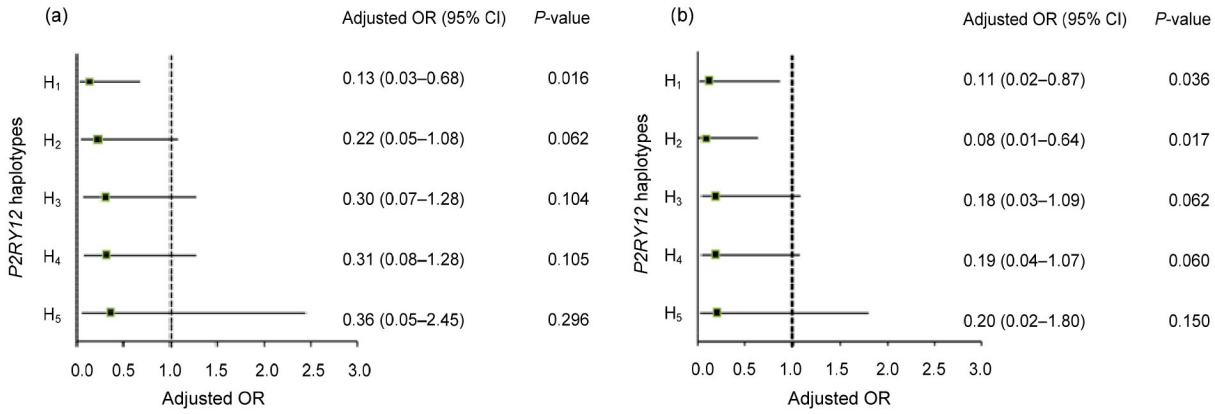

Fig. 1 Effects of *P2RY12* haplotypes on high on-clopidogrel platelet reactivity in all patients (a, *n*=154) and in nonsmokers (b, *n*=101) by multivariable logistic regression adjusted by *CYP2C19* polymorphisms and covariate characteristics
In total group smoking was also adjusted compared to nonsmoker subgroup. The subgroup of smokers was excluded from the analysis due to small sample size (*n*=53)

3.3.2 *P2RY12*

For the total study population, haplotype H_1 showed a significantly lower incidence of HTPR than the reference haplotype (H_0) after adjusting for demographic characteristics and smokers (9 (34.62%) vs. 8 (57.14%), OR 0.13, *P*-value 0.016, 95% CI 0.03–0.68; Fig. 1a). Among nonsmokers, after adjusting for demographic characteristics, haplotypes H_1 and H_2 showed a significantly lower incidence of HTPR than H_0 (8 (47.06%) vs. 7 (63.64%), OR 0.11, *P*-value 0.036, 95% CI 0.02–0.87; 7 (38.89%) vs. 7 (63.64%), OR 0.08, *P*-value 0.017, 95% CI 0.01–0.64; Fig. 1b). *P2RY12* allele rs2046934 (T744C) did not show any significant association with HTPR neither in the total population nor in nonsmokers.

4 Discussion

In our study, we found the possible association between the common genetic variations of the *P2RY12* and residual on-clopidogrel platelet reactivity using TEG in a Chinese population with ACS after adjusting the influence of *CYP2C19**2 and *3 alleles. The *P2RY12* SNPs we chose (rs6798347, rs6787801, rs6801273, rs6785930, and rs2046934) contained the promoter region of the *P2RY12* gene, which may alter its transcriptional activity instead of changing the structure of the P2Y12 receptor as other mostly analyzed SNPs may do.

In our studied population, we identified by TEG 69 (38.33%) patients with HTPR. The incidence of HTPR was similar to those of other studies on a similar population (Liu *et al.*, 2015; Tang *et al.*, 2015) and higher than those on Caucasian and African-American populations with stable coronary heart disease (Bliden *et al.*, 2007). The different incidences of HTPR might come from inadequate platelet inhibition in Chinese patients and because Chinese are more likely to carry the *CYP2C19* LOF allele compared to Caucasian and Africans (Liu *et al.*, 2015).

As well as the established association of the *CYP2C19**2 and *3 LOF alleles with HTPR, several SNPs of the *P2RY12* gene have been studied. Most studies focused on rs2046934 (T744C) or rs6809699 (G52T) representing dbSNP rs10935838, rs2046934, rs5853517 and rs6809699, and rs6785930 (C34T). Some found that it can modulate platelet response to clopidogrel or affect patients' outcomes (Fontana *et al.*, 2003; Zee *et al.*, 2008; Staritz *et al.*, 2009; Shalia *et al.*, 2013; Zoheir *et al.*, 2013; Li *et al.*, 2016), but most results were negative (Angiolillo *et al.*, 2005; Smith *et al.*, 2006; Cuisset *et al.*, 2007; Lev *et al.*, 2007; Bierend *et al.*, 2008; Bonello *et al.*, 2010; Jang *et al.*, 2012; Namazi *et al.*, 2012; Kar *et al.*, 2013; Kim *et al.*, 2013; Jana *et al.*, 2014). Our results show that the *P2RY12* allele rs2046934 (T744C) was not significantly associated with HTPR neither in the total study population nor in nonsmokers.

Instead of any single polymorphisms of the analyzed genes, we found that the coexisting of the four SNPs (rs6798347, rs6787801, rs6801273, and rs6785930) was related to on-treatment platelet reactivity tested by TEG in the total population and in the nonsmoker subgroup. Haplotype H_1, which carried four mutation alleles of the four selected SNPs at the same time, showed a significantly lower incidence of HTPR compared with the reference wild haplotype (H_0). Similarly, Rudež *et al*. (2009) composited six P2Y12 SNPs (rs6798347, rs6787801, rs9859552, rs6801273, rs9848789, and rs2046934) and found that one haplotype was associated with significantly lower residual on-clopidogrel platelet reactivity compared with the reference haplotype tested by VerifyNow and light transmission aggregometry (LTA). Rudež *et al*. (2008) also composed five *P2RY12* SNPs (rs6798347, rs10935842, rs6787801, rs6801273, and rs2046934) and found that they were associated with a higher risk of adverse cardiovascular events than the reference *P2RY12* haplotype, which contains the common alleles of all the five *P2RY12* SNPs.

Furthermore, we analyzed the co-contribution of *P2RY12* and *CYP2C19* together, after adjusting for the influence of *CYP2C19* LOG alleles and GOF alleles, and we found the extra coexisting impact of *P2RY12* on the residual platelet reactivities besides *CYP2C19*. This was partly examined in Malek *et al.* (2008)'s research with different alleles. Our study, in a similar way, found a new impact of *P2RY12* genes on platelet reactivity but by a different combination of SNPs, which helps gain a better understanding of the effect of *P2RY12* genes on clopidogrel on-treatment platelet reactivity.

In our studied population we found that smokers had a lower percentage of HTPR (OR 0.4945, *P*-value

0.046, 95% CI 0.25–0.99). This might be explained by Shanker *et al.* (2006)'s study stating that smokers might have higher platelet P2Y12 activity. Similarly, Cavallari *et al.* (2007)'s study stated that rs2046934 (T744C) was associated with a higher presence of coronary artery disease, particularly in nonsmokers, which in some way demonstrated that smoking affected P2Y12's contribution to coronary artery disease although they showed the relationship with a different *P2RY12* SNP. However, we are not the first to report the association of clopidogrel on-treatment platelet reactivity with smoking (Tantcheva-Poór *et al.*, 1999; Bliden *et al.*, 2008; Gremmel *et al.*, 2009; Price *et al.*, 2009; Jeong *et al.*, 2010; Ueno *et al.*, 2012; Gurbel *et al.*, 2013; Peng *et al.*, 2015). It was early reported that smoking increases the enzymatic activity of CYP1A2, one of the predominant pathways for the first oxidative step of the pro-drug clopidogrel, through polycyclic aromatic hydrocarbons and plasma nicotine, thereby enhancing the generation of the active metabolite of clopidogrel (Tantcheva-Poór *et al.*, 1999). One hypothesis by Bliden *et al.* (2008)'s group was that the significant cigarette smoking might affect platelet inhibition in clopidogrel-treated patients via CYP1A2. However, a study conducted by Yousef *et al.* (2008) showed that the elimination half-life of clopidogrel was shorter in current smokers than in nonsmokers, which confused the established hypothesis. Meanwhile, there are several studies which reported a negative association with smokers in larger observation studies (Hochholzer *et al.*, 2011; Sibbing *et al.*, 2012; Kim *et al.*, 2016). Kim *et al.* (2016) deduced that hemoglobin levels might be the key driver for the observed ex vivo phenomenon of lower on-treatment platelet reactivity levels and/or an enhanced response to ADP inhibitors like clopidogrel in active smokers in most of the studies published so far. More studies are needed. Recently, the study of Patti *et al.* (2016) found that cigarette smoking weakly influences antiplatelet effects of oral P2Y12 inhibition. The results on the association of smoking and platelet responsiveness to ADP-receptor antagonist are inconsistent and controversial within studies. There was one study of Cicko *et al.* (2010) reporting that smoke exposure led to an up-regulation of the P2Y2R subtypes in blood neutrophils and in bronchoalveolar lavage fluid neutrophils by in vitro mouse model. This might explain the observed relationship between HTPR and smoking on ACS patients in smokers. Further studies are required to compare the different on-treatment platelet activities between nonsmokers and smokers.

5 Conclusions

Our study showed that apart from the established association of the *CYP2C19**2 and *3 LOF alleles with HTPR, haplotypes of *P2RY12* rs6798347, rs6787801, rs6801273, and rs6785930 rather than rs2046934 (T744C) that related to pharmacodynamics of clopidogrel in patients with ACS were independently associated with HTPR. This association seems to be more important in the subgroup defined by smoking. This finding suggests that *P2RY12* genetic variation and common allele composition may be important in determining platelet reactivity for patients treated with clopidogrel, especially in nonsmokers.

6 Study limitations

The first limitation of our study is the small sample size, especially the small sample of the population smoking. Secondly we cannot completely eliminate the possible bias by clinical risk factors despite multivariate analysis. Thirdly, we did not observe the association between genetic variations and clinical outcome in this study. Finally, we did not perform pharmacokinetics analysis, which might be useful for a better explanation related to the *ABCB1* and *CYP1A2* polymorphisms.

Compliance with ethics guidelines

Xiao-yan NIE, Jun-lei LI, Yong ZHANG, Yang XU, Xue-li YANG, Yu FU, Guang-kai LIANG, Yun LU, Jian LIU, and Lu-wen SHI declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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中文概要

题　目：*P2RY12* 基因单倍体分型对急性冠脉综合征患者氯吡格雷用药后血小板反应性的影响

目　的：评估血小板受体基因 *P2RY12* 常见突变位点单倍体分型对急性冠脉综合征患者氯吡格雷用药后血小板反应性的影响。

创新点：首次在中国人群中对包含调控基因在内的五个常见 *P2RY12* 突变位点进行单倍体分析，并且校正了 *CYP2C19* 基因多态性的影响，发现 *P2RY12* 常见基因位点联合突变可降低氯吡格雷用药后血小板高反应性发生率，且作用在不吸烟人群中更明显。

方　法：连续入选 180 例接受氯吡格雷药物治疗的急性冠脉综合征患者，利用血栓弹力图法检测患者用药后血小板反应性，将用药后血小板抑制率<30%定义为血小板高反应性（HTPR）。用多重连接酶检测反应（LDR）技术，对覆盖 *P2RY12* 调控基因在内的五个基因位点（rs6798347、rs6787801、rs6801273、rs6785930 和 rs2046934）以及 *CYP2C19* 常见的三个等位基因（*2、*3 和 *17）进行基因分型。根据连锁不平衡系数和基因分布频率进行单倍体分析，观察在校正 *CYP2C19* 基因多态性影响后，不同 *P2RY12* 单倍体分型对氯吡格雷用药后 HTPR 的影响。

结　论：将 *P2RY12* 基因 rs6798347、rs6787801、rs6801273 和 rs6785930 四个紧密连锁的单核苷酸多态性（SNP）分为六个常见单倍体分析（H_0~H_5，表 4）。单倍体分型与氯吡格雷用药后 HTPR 发生率显著相关，与 H_0 型相比，H_1 型 HTPR 发生率显著降低，而 rs2046934 对 HTPR 发生率无显著影响（表 5），且在不吸烟组中单倍体分型对 HTPR 影响更明显（图 1）。综上所述，*P2RY12* 基因 rs6798347、rs6787801、rs6801273 和 rs6785930 单倍体分型与氯吡格雷用药后 HTPR 显著相关。

关键词：*P2RY12*；*CYP2C19*；单倍体分型；单核苷酸多态性；氯吡格雷；血栓弹力图