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Association of CYP2C19 Polymorphism with Clopidogrel Resistance in Patients with Acute Coronary Syndrome in China

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Manuscript Preparation E
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Background: The relationship between clopidogrel-resistance (CR) and polymorphism located in genes encoding clopidogrel metabolism-related enzymes has not been fully explored. Thus far, few studies on CR-associated polymorphism have been conducted in the Chinese population. The purpose of this study was to identify CYP2C19 polymorphism associated with CR in patients with acute coronary syndrome in China.

Material/Methods: There were 125 patients with acute coronary syndromes (ACS) selected for this study. Of these, 27 patients (21.6%) showed CR (less than 10% reduction in platelet accumulation rate), while the remaining 98 patients (78.4%) were non-clopidogrel-resistant (NCR).

Results: There were significant differences in the allele frequencies of CYP2C19 (rs4244285) (P=0.03) and CYP2C19 (rs4986893) (P=0.005) between the 2 groups; however, there was no significant difference in allele frequencies of ABCB1 (rs1045642) (P=0.661) and PON1 (rs662) (P=0.690) between the 2 groups. The null allele in the CYP2C19 (rs4244285) [odds ratio (OR)=5.317, 95% confidence interval (CI) 1.542–26.428, P=0.001] and CYP2C19 (rs4986893) (OR=4.295, 95%CI 1.312–17.517, P=0.013) is one of the causes of CR in patients with ACS in China.

Conclusions: The CYP2C19 polymorphism (rs4244285 and rs4986893) is the correlative factor of CR in patients with ACS in China. It was found that the null allele in the CYP2C19 polymorphism was related to the higher CR risk. According to the key role of CYP2C19 in the clopidogrel activation and the evaluated role of CYP2C19 in this study, further studies should be carried out to formulate therapeutic alternative methods for CR in patients with ACS.

MeSH Keywords: Acute Coronary Syndrome • Enzymes • Metabolism

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Background

Coronary heart disease (CAD) has a high mortality and disability rate. CAD is divided into stable angina pectoris and acute coronary syndrome (ACS) [1]. According to the CDC, every 25 seconds one American is diagnosed with ACS. Furthermore, CAD incidence has increased rapidly in China in the past decades and has remained high [2]. Aspirin and clopidogrel dual anti-platelet therapy is the standard treatment in many guidelines for the treatment of type I ACS [3,4]. But some patients develop relapse myocardial infarction or even death in 30 days of dual anti-platelet therapy. Sub-acute thrombosis is reported to occur in coronary stents in 1% to 3% of patients [5]. Clopidogrel resistance (CR) is defined as the decline in platelet accumulation rate by less than 10% compared to the baseline after clopidogrel treatment [6–9]. CR is thought to be critically involved in recurrent myocardial infarction after antiplatelet therapy. Low response to clopidogrel predicts poor antiplatelet treatment effect in 4% to 30% of the patients, whereas hyper-responsiveness of platelets is the primary cause of recurrent ischemic events in ACS [10].

The impact of diabetes in causing worse prognosis in STEMI [11,12] and in NSTEMI events [13], by different mechanisms, includes atherosclerotic plaque rupture, inflammatory/oxidative stress, and loss of regenerative myocardial muscle properties. Intriguingly, also pre-diabetic status alters glucose homeostasis and insulin resistance which might cause major adverse coronary events in patients in absence of severe coronary stenosis thus leading to coronary artery endothelial dysfunction [14]. This point is remarkable also in patients with insulin resistance and normal glucose homeostasis [15], where it causes restenosis in percutaneous coronary intervention (PCI) treated patients. On other hand, multiple inflammatory/oxidative processes, such as over reactivity of thrombotic structure, can be a cause of worse prognosis in patients with acute coronary syndrome admission event [16]. Biotransformation in the liver is an important mechanism required for metabolizing exogenous chemicals and maintaining homeostasis in the human body. Clopidogrel is a precursor drug that is converted into its active form through 2 sequential oxidative steps after absorption into the bloodstream via the intestines [17]. Studies have shown that the enzymes encoded by the *ABCB1*, *CY2C19*, and *PON1* genes influence clopidogrel metabolism by affecting the absorption and activation of clopidogrel [18]. Thus far, the majority of studies that investigated the associations between *ABCB1*, *CY2C19* and *PON1* polymorphisms and CR have been conducted in western populations [19]. The relationship between CR and polymorphisms located in genes encoding clopidogrel metabolism-related enzymes has not been fully elucidated. Further, only a few studies have been conducted in the Chinese population.

The aim of this study was to investigate the relationship between polymorphism of *CYP2C19*2*, *CYP2C19*3*, *ABCB1*, and *PON1* and CR in patients with ACS in China.

Material and Methods

Patients

This study was approved by the Ethics Committee of Nanchong Central Hospital with the clinical trial registration No. ChiCTR-ROC-17012115 (*http://www.chictr.org/en/*). There were 125 patients who had a first diagnosis of ACS with PCI (18~75 years of age) in Cardiovascular Medicine Department of Nanchong Central Hospital, who were selected for this study from February 2014 to January 2015. The diagnosis of ACS depended on the results of electrocardiogram (ECG), myocardial enzyme test, coronary angiography, and clinical manifestations [2]. Before the PCI procedure, 300 mg clopidogrel+300 mg aspirin loading dose was given to study patients, with routine maintenance dose of 75 mg/day clopidogrel+100 mg/day aspirin. The whole study was carried out according to Helsinki Declaration in 1975 and was approved by the Institutional Review Committee of Nanchong Central Hospital. Before the study began, all study participants signed informed consents. Exclusion criteria was as follows: 1) allergy to clopidogrel; 2) taking aspirin, clopidogrel, or other anti-platelet drugs within 7 days before the study; 3) platelet count >300×10^9/L or <100×10^9/L; 4) abnormal liver and kidney functions (alanine aminotransferase [ALT] or aspartate transaminase [AST] levels at least 3-fold higher than the upper limit of normal range; glomerular filtration rate [GFR] <60 mL/min); 5) active peptic ulcer; and 6) received major surgeries, cerebrovascular accidents or recent history of hemorrhage.

Collection and treatment of blood samples

Venous blood samples (2.7 mL) were collected before clopidogrel administration at 24 hours after the loading dose of 300 mg clopidogrel+300 mg of aspirin (81 cases), and at 5 to 7 days after the maintenance dose of the drugs (75 mg/day clopidogrel+100 mg/day aspirin (44 cases). The blood samples were placed in tubes and 0.3 mL of sodium citrate was added as anticoagulant. The tubes were inverted several times to ensure proper mixing between the anticoagulant and blood. The platelet accumulation rate was measured within 2 hours. At the same time, 2 mL of blood samples were collected before medication and cryopreserved in the refrigerator at –20°C until DNA extraction. For blood sample collection, care was taken not to take the venous band, and repeated slapping and puncture was avoided.

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Detection of the platelet aggregation rate (PAR)

The platelet aggregation rate **(**PAR) was detected by turbidimetric method according to the following steps: the venous blood sample was centrifuged at 800 rpm for 10 minutes at 37°C. After the centrifugation, the light-yellow serum in the supernatant was collected and used for the preparation of platelet-rich plasma (PRP). Care was taken not to collect the blood cells in the lower layer. PRP was further centrifuged at 37°C at 4000 rpm for 10 minutes, and platelet-free plasma in the supernatant was collected for the preparation of platelet-poor plasma (PPP). Platelet count was determined in the PRP and was adjusted to 100×10^9/L to 300×10^9/L using PPP. Next, 300 µL aliquots of PRP were added separately to 2 tubes, each containing a small magnet. The tubes were preheated at 37°C for 5 minutes. After removing the magnet, the tube containing the PPP was placed in the test channel, and the system was automatically zeroed. The tube containing PRP was placed in the test channel in the same manner and loaded with 5 µL of 20 µmol/L ADP using a micro-syringe. The rate of ADP-induced platelet accumulation was then measured for 10 minutes and recorded.

Grouping

CR was defined as less than 10% reduction in the platelet accumulation relative to the baseline [6–9]. The study participants were assigned to the CR group or the NCR group based on the decrease in platelet accumulation rate.

DNA extraction of blood samples

The cryopreserved blood samples were thawed, and the tubes were inverted several times to ensure proper mixing. Then, 200 µL of each sample was placed into a 1.5 mL centrifuge tube and added with 1 mL of ddH_2O. After shaken and mixed, the samples were placed at room temperature for 3 minutes, and centrifuged with 12 000 rpm for 5 minutes. Then the supernatant was discarded. The aforementioned steps were repeated when a large amount of red precipitate remained. Afterwards, 400 µL of 1x digestion buffer and 10 µL of protease K (20 µg/uL) were added, and the tube was inverted to ensure proper mixing. The tube was incubated overnight at 56°C and vertically inverted several times. The tube containing the digestion buffer was added with 400 µL of phenol-chloroform-isoamyl alcohol and then shaken vigorously for 1 minute. Centrifugation was performed at 12 000 rpm for 5 minutes. The aqueous supernatant was carefully collected and transferred to a new tube using a yellow tip. The white material at the interface of the 2 phases was collected. Next, the sample was added with 3 M NaAc at one tenth the volume of the water phase and pre-cooled absolute alcohol at twice the volume of sample. The tube was inverted vertically several times to ensure complete mixing, placed at –20°C for 10 minutes to complete precipitation, and then centrifuged at 12 000 rpm for 10 minutes. After discarding the supernatant, the precipitate was washed with 1 mL of 70% alcohol. The tube was gently vortexed for several seconds and centrifuged. The supernatant was gently poured out while taking care not to discard the white precipitate. The residual liquid was removed using clean absorbent paper or pipette tips. The tube was dried at 58°C for 30 minutes, and the precipitate was dissolved in 50 µL of DNA buffer. After vortex, the tube was cultured at 50°C water bath for 1 hour. The tube was then removed after 30 minutes and vortexed for several seconds. Centrifugation was performed at 4000 rpm for 30 seconds, and 1 µL of the sample was analyzed by 1% agarose gel electrophoresis. The sample was normalized to 10 ng/µL for analysis. Sample quality was assessed, and the concentration was estimated. The sample was diluted to the working concentration of 5 to 10 ng/µL based on the estimated sample concentration.

Genotyping

The sequences of the *CYP2C19*, *ABCB1*, and *PON1* genes were retrieved from the NCBI database (*http://www.ncbi.nlm.nih.gov*). The PCR primers of CYP2C19 (rs4244285, rs4986893), PON1 (rs662) SNPs, and ABCB1 (rs1045642) were designed by Primer 5.0 software (Table 1) and synthesized by Shanghai Genesky Biotech Co., Ltd. The PCR reaction materials (10 µL) included 1×HotStar Taq buffer, 3.0 mM Mg^{2+}, 0.3 mM dNTP, 1 U HotStar Taq polymerase (Qiagen Inc.), 1 µL sample DNA, and 1 µL PCR primer (1 µM). Restriction endonuclease reaction system (10 µL) contained 1x buffer, 1 U of restriction endonuclease, and 4 µL of PCR product. The reaction was conducted in a water bath at an appropriate temperature. Restriction enzyme digestion using the MspI, BamHI, BfuCI, and BfuCI restriction enzymes for CYP2C19 (rs4244285, rs4986893), ABCB1 (rs1045642), and PON1 (rs662) polymorphisms, respectively. Genotypes were analyzed based on the length of the products after gel electrophoresis (Table 1).

Statistical analysis

The measurement data were expressed as x ± Standard deviation (SD). The enumeration data were expressed as percentages and frequencies. Student's *t*-test and χ^2 test (enumeration data) were used for intergroup comparison. ANOVA was performed to compare the measurements and RxC tables to compare the counts among multiple groups. Hardy-Weinberg equilibrium test was performed for the recruited population, with the significance level set at 0.05. $P<0.05$ showed there were statistical significances.

The potential effect of various independent factors on CR was evaluated by binary logistic regression analysis to calculate

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Table 1. PCR primers.

Name	Upstream and downstream primer (5'→3')	Annealing temperature	Length of the product
rs4244285	F-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTGTTTTCTCTTAGATATGCAATAATTTTCCCAC	64.27°C	312 kb
	R-AACATCCGTAGTAAACACAAAACTAGTCAATG		
rs4986893	F-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTAATGAAAACATCAGGATTGTAAGCACC	54.55°C	290 kb
	R-CCTGATCTATATTGGGATATTCATTTCCTG		
rs1045642	F-CCACATGCTCCCAGGCTGTT	65.77°C	251 kb
	R-TTCAGCTGCTTGATGGCAAAGA		
rs662	F-TCCTTCTGCCACCACTCGAA	65.57°C	284 kb
	R-GCTAGCACGAAGGCTCCATCC		

Table 2. Baseline information of CR and NCR groups.

Indicators	CR	NCR	P
Male (n)	17	69	0.460
Age (years)	61.7±8.34	62.3±7.95	0.824
Height (cm)	163.3±12.46	166.4±10.34	0.236
Weight (kg)	64.60±11.52	68.60±12.57	0.149
TC mmol/l	4.59±1.38	4.71±1.34	0.371
TG mmol/l	1.84±1.19	1.79±1.31	0.142
HDL mmol/l	1.35±0.97	1.33±1.03	0.786
LDL mmol/l	2.52±1.43	2.70±1.39	0.287
BMI kg/m^2	24.67±4.43	26.17±4.72	0.532
PLT (n)	(182.3±48.7)×10^9	(195.2±50.3)×10^9	0.137
Diabetes (n)	9	30	0.787
Smoking (n)	11	47	0.505
Blood pressure (n)	19	65	0.692
STEMI (n)	15	66	0.256
NSTEMI (n)	7	21	0.620
UA (n)	5	11	0.335
Statins (n)	27	88	0.897
β-receptor blockers (n)	21	89	0.091
ACEI/ARB (n)	27	85	0.892
PPI (n)	14	47	0.720
Amiodarone (n)	4	14	0.945

TC – total cholesterol; TG – triglyceride; HDL – high-density lipoprotein; LDL – low-density lipoprotein; BMI – body mass index; PLT – platelet count; STEMI – ST segment elevation myocardial infarction; NSTEMI – non-ST-elevation myocardial infarction; UA – uric acid; β-receptor blockers – beta receptor blockers; ACEI – angiotensin converting enzyme inhibitor; ARB – angiotensin II receptor antagonist; PPI – proton pump inhibitor.

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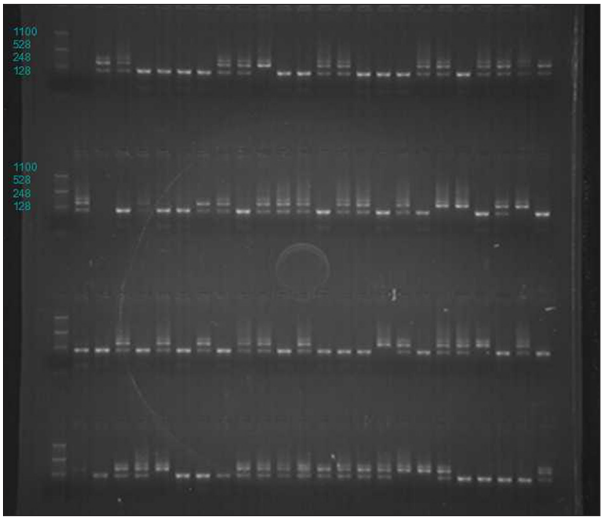

Figure 1. Genotyping of CYP2C19 (rs4244285) polymorphism by electrophoresis.

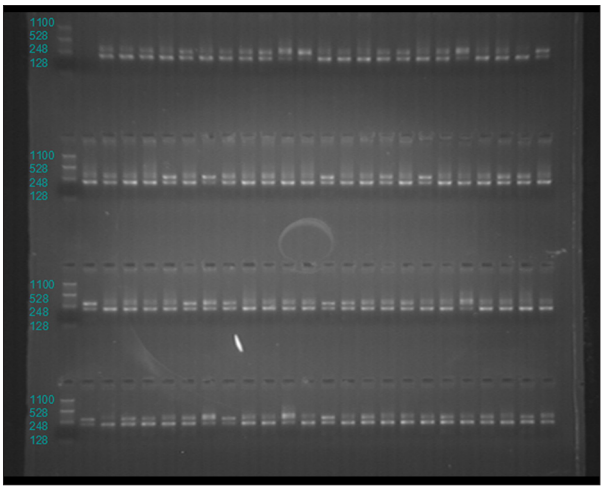

Figure 2. Genotyping of CYP2C19 (rs4986893) polymorphism by electrophoresis.

Figure 3. Genotyping of ABCB1 (rs1045642) polymorphism by electrophoresis.

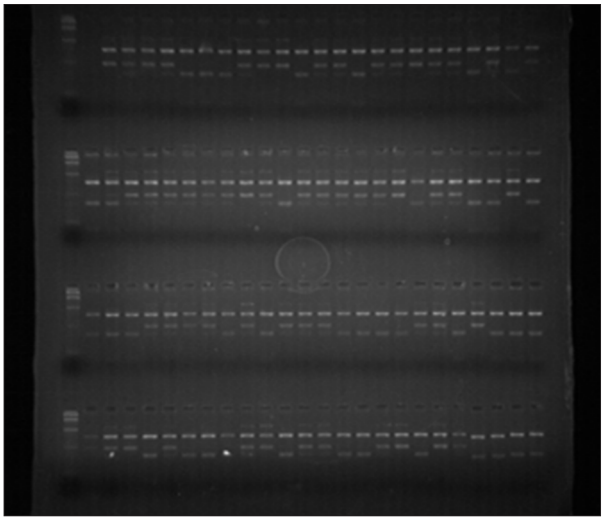

Figure 4. Genotyping of Pon1 (rs662) polymorphism by electrophoresis.

the odds ratio (OR) and 95% confidence interval (CI). The onset of CR was the dependent variable. The independent variables included age, body mass index, diabetes, hypertension, smoking, statins, β-receptor blockers, PPI, ACEI/ARB, amiodarone, CYP2C19*2 polymorphism, CYP2C19*3 polymorphism, ABCB (rs1045642) polymorphism, and PON1 (rs662) polymorphism. All statistical tests were 2-tailed.

Results

Baseline information

Before taking clopidogrel, the baseline platelet accumulation rate was 48.72±14.32, and the residual rate of platelet accumulation after clopidogrel administration was 26.34±15.37%. According to the definition of CR, there were 27 cases with CR (21.6%) and 98 cases with NCR (78.4%). The 2 groups showed no significant difference in baseline information, laboratory indicators, or medication scheme during hospitalization (Table 2).

Electrophoresis results

The ABCB1 (rs1045642), CYP2C19 (rs4244285, rs4986893), and PON1 (rs662) were digested by restriction enzymes of MspI, BamHI, and BfuCI at the target sites, respectively. The genotypes of CYP2C19, ABCB1 (C3435T, rs1045642), and PON1 (Q192R, rs662) and the lengths of the corresponding fragments are as follows. For the CYP2C19 (rs4244285) polymorphism, the samples with the GG genotype was digested into 2 fragments, with lengths of 89 and 223 bp; the samples with the AA genotype was digested into 312-bp products; the samples

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Table 3. Genotype frequency distribution of genes encoding for enzymes related to clopidogrel metabolism.

Genotype and allele	Case (N)	Proportion (%)
Genotypes of CYP2C19 (rs4244285) polymorphism		
GG	50	40.0
GA	61	48.8
AA	14	11.2
Alleles of CYP2C19 (rs4244285) polymorphism		
G	161	64.4
A	89	35.6
Genotypes of CYP2C19 (rs4986893) polymorphism		
GG	79	63.2
GA	39	31.2
AA	7	5.6
Alleles of CYP2C19 (rs4986893) polymorphism		
G	158	78.8
A	53	21.2
Genotypes of ABCB1 (rs1045642) polymorphism		
GG	45	36.0
GA	60	48.0
AA	20	16.0
Alleles of ABCB1 (rs1045642) polymorphism		
G	150	60.0
A	100	40.0
Genotypes of PON1 (rs662) polymorphism		
CC	51	40.8
CT	52	41.6
TT	22	17.6
Alleles of PON1 (rs662) polymorphism		
C	154	61.6
T	96	38.4

with the GA genotype were digested into 3 fragments with lengths of 89, 223, and 312 bp. The results of electrophoresis are shown in Figure 1. For the CYP2C19 (rs4986893) polymorphism, DNA from individuals harboring the GG genotype was digested into 2 fragments with lengths of 82 and 208 bp; individuals harboring the AA genotype produce a 290-bp product; individuals with the GA genotype produce 3 fragments with lengths of 82, 208, and 290 bp. The results of electrophoresis are shown in Figure 2. For the ABCB1 (rs1045642) polymorphism, individuals with the GG genotype produce 2 fragments with lengths of 93 and 158 bp; individuals harboring the AA genotype produce a 251-bp band; and those with the GA genotype produce 3 fragments with lengths of 93, 158, and 251 bp. The results of electrophoresis are shown in Figure 3. For the PON1 (rs662) polymorphism, digestion of samples with the CC genotype produced 3 fragments with lengths of 28 bp, 79 bp, and 177 bp; samples with the TT genotype were digested into 2 fragments with lengths of 107 bp and 177 bp; samples with the CT genotype were digested into 4 fragments with lengths of 28, 79, 107, and 177 bp. The genotypes were determined based on the results of electrophoresis (Figure 4).

Polymorphism distribution of *CYP2C19*, *ABCB1* and *PON1* genes

A total of 50 patients (40.0%), 61 patients (48.8%), and 14 patients (11.2%) harbored the GG, GA, and AA genotypes of the CYP2C19 (rs4244285) polymorphism. The frequencies of G and A alleles of CYP2C19 (rs4244285) were 0.644 and 0.356, and the minor allele frequency (MAF) was 0.356. The number of

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Table 4. H-W equilibrium test in CYP2C19 (rs4244285) polymorphism.

	G/G	G/A	A/A	P
Actual value	50	61	14	0.857
Predicted value	52	57	16	

Table 5. H-W equilibrium test in CYP2C19 (rs4986893) polymorphism.

	G/G	G/A	A/A	P
Actual value	79	39	7	0.909
Predicted value	78	42	6	

Table 6. H-W equilibrium test in ABCB (rs1045642) polymorphism.

	G/G	G/A	A/A	P
Actual value	45	60	20	1.00
Predicted value	45	60	20	

Table 7. H-W equilibrium test in PON1 (rs662) polymorphism.

	G/G	G/A	A/A	P
Actual value	51	52	22	0.23
Predicted value	47	59	18	

subjects carrying the GG, GA, and AA genotypes of CYP2C19 (rs4986893) polymorphism were 79 (63.2%), 39 (31.2%), and 7 (5.6%), respectively. The frequencies of G and A alleles of CYP2C19 (rs4986893) polymorphism were 0.788 and 0.212, respectively; the MAF was 0.212. The number of patients carrying the GG, GA, and GG genotypes of ABCB1 (rs1045642) polymorphism was 45 (36.0%), 60 (48.0%), and 20 (16.0%), respectively. The frequencies of the G and A alleles of the ABCB1 (rs1045642) polymorphism were 0.60 and 0.40, respectively; MAF was 0.40. The number of patients carrying CC, CT, and TT genotypes of PON (rs662) polymorphism was 51 (40.8%), 52 (41.6%) and 22 (17.6%), respectively. The frequencies of the C and T alleles of PON1 (Q192R) were 0.616 and 0.384, and MAF was 0.204 (Table 3).

Hardy-Weinberg equilibrium

The predicted and actual genotypic values of CYP2C19 (rs4244285, rs4986893), ABCB1 (rs1045642), and PON1 (rs662) polymorphisms were consistent with Hardy-Weinberg equilibrium (P>0.05) (Tables 4–7).

Association analysis

From the CR group, 8, 12, and 7 patients harbored the GG, GA, and AA genotypes of the CYP2C19 (rs4244285) polymorphism, respectively; the frequency of A allele was 0.481. For the NCR group, 42, 49, and 7 patients were carriers of the GG, GA, and AA genotypes of CYP2C19 rs4244285, respectively; the frequency of A allele was 0.321. The results showed there were differences in the frequencies of the A allele of CYP2C19 (rs4244285) between CR and NCR groups (P=0.03). For the CYP2C19 (rs4986893) polymorphism, 12, 11, and 4 patients from the CR group harbored the GG, GA, and AA genotypes, respectively; the frequency of A allele was 0.352. For NCR group, 67, 28, and 3 patients harbored the GG, GA, and AA genotypes of the CYP2C19 rs4986893 polymorphism, respectively; the frequency of A allele was 0.173. It was found that there was a difference in the frequency of A allele between CR and NCR groups (P=0.005). For the ABCB1 (rs1045642) polymorphism, 9, 13, and 5 patients from the CR group harbored the GG, GA, and AA genotypes, respectively; the frequency of the G allele was 0.426. For the NCR group, 36, 47, and 15 patients harbored the GG, GA, and AA genotypes of the ABCB1 rs1045642 polymorphism, respectively. The frequency of the T allele of ABCB1 (rs1045642) was 0.369. It was suggested that there was no difference in the frequencies of G allele in ABCB1 (rs1045642)

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Table 8. Comparison of allele and genotype frequencies in different polymorphisms between CR group and NCR group.

Genotype and allele	Cases in CR group (n)	Cases in NCR group (n)	Cases (N)	P
Genotypes of CYP2C19 (rs4244285) polymorphism				0.037
GG	9	41	50	
GA	12	49	61	
AA	6	8	14	
Alleles of CYP2C19 (rs4244285) polymorphism				0.030
G	30	131	161	
A	24	65	89	
Genotypes of CYP2C19 (rs4986893) polymorphism				0.028
GG	12	67	79	
GA	11	28	39	
AA	4	3	7	
Alleles of CYP2C19 (rs4986893) polymorphism				0.005
G	35	162	158	
A	19	34	53	
Genotypes of ABCB1 (rs1045642) polymorphism				0.904
GG	9	36	45	
GA	13	47	60	
AA	5	15	20	
Alleles of ABCB1 (rs1045642) polymorphism				0.661
G	31	119	150	
A	23	77	100	
Genotypes of PON1 (rs662) polymorphism				0.903
CC	10	41	51	
CT	12	40	52	
TT	5	17	22	
Alleles of PON1 (rs662) polymorphism				0.690
C	32	122	154	
T	22	74	96	

between the 2 groups (P=0.661). For the PON1 (rs662) polymorphism, 10, 12, and 5 patients from CR group harbored the CC, CT, and TT genotypes, respectively; the frequency of Q allele was 0.407. For the NCR group, 41, 40, and 17 patients were carriers of CC, CT, and TT genotypes of the PON1 rs662 polymorphism, respectively; the frequency of T allele was 0.378. No difference was found in the frequency of the T allele in the PON1 rs662 between CR and NCR groups (P=0.707) (Table 8).

Logistic regression analysis of CR potential risk factors

The occurrence of CR was the dependent variable in logistic regression analysis. The independent variables included age, body mass index, diabetes, hypertension, smoking, statins, β-receptor blockers, PPI, ACEI/ARB, amiodarone, CYP2C19*2 polymorphism, CYP2C19*3 polymorphism, ABCB (rs1045642) polymorphism, and PON1 (rs662) polymorphism. Binary logistic

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Table 9. Logistic regression analysis on risk factors of CR.

	B	P	OR	95% CI
Age	0.932	0.231	1.945	0.723–15.471
BMI	1.473	0.122	2.123	0.491–28.37
Diabetes	1.502	0.083	3.428	0.571–21.72
Hypertension	0.568	0.451	0.984	0.341–7.172
Smoking	0.031	0.732	0.841	0.652–2.131
Statins	0.882	0.541	0.907	0.472–2.793
β-receptor blockers	0.592	0.819	1.215	0.821–4.567
PPI	1.237	0.142	2.719	0.854–11.926
Amiodarone	0.013	0.678	1.047	0.884–1.237
ACEI/ARB	0.031	0.213	0.971	0.791–1.352
*2 (GA/AA)	2.479	0.001	5.317	1.542–26.428
*3 (GA/AA)	1.986	0.013	4.295	1.312–17.517
ABCB1(AG/GG)	0.272	0.357	2.154	0.872–5.818
PON1(CT/TT)	0.653	0.421	0.921	0.782–8.317

BMI – body mass index; β-receptor – beta receptor blockers; PPI – proton pump inhibitor; ACEI – angiotensin converting enzyme inhibitor; ARB – angiotensin II receptor antagonist.

regression analysis was performed, and the strength of the correlation was evaluated by OR and 95%CI. All tests were 2-tailed. It was suggested that the polymorphisms of CYP2C19*2 and CYP2C19*3 were important risk factors for CR (Table 9).

Discussion

Clopidogrel resistance (CR) is defined by a less than 10% decline in platelet accumulation rate compared to the baseline following clopidogrel treatment [6–9]. CR is thought to be critically involved in recurrent myocardial infarction after antiplatelet therapy [20]. Weak clopidogrel response predicts poor antiplatelet treatment effect in 4% to 30% of the patients, whereas hyperresponsiveness of platelets is the primary cause of recurrent ischemic events and even death of patients with ACS.

A recent study of a healthy population by Hulot et al. [21] showed a dramatic decline in the platelet accumulation rate after clopidogrel administration among normal patients harboring the wild-type CYP2C19 allele (CYP2C19*1/*1), which was absent among carriers of the null allele (CYP2C19*1/*2). CYP2C19 polymorphism is related to the bioavailability of the clopidogrel active metabolites. Compared to non-carriers, the carriers with at least one null allele had 32.4% reduction in the active metabolite of clopidogrel and a 9% increase in the platelet accumulation rate [22]. The allele of CYP2C19*2 is related to high platelet activation. For patients receiving double antiplatelet therapy after stent implantation, CYP2C19 genotyping and HPR provide complementary information for risk assessment and stratification [23].

In this study, the frequency of the A allele at the CYP2C19*2 site (681G>Ars4244285) was 35.6%, and that at the CYP2C19*3 site (626G>Ars4986893) was 21.2% of 125 patients with ACS. CR is defined as a less than 10% decline in baseline platelet accumulation rate after clopidogrel administration. Based on this criterion, CR was found in 27 out of 125 patients (21.6%) with ACS, and NCR was found in 98 out of 125 patients (78.4%.). The frequency of A allele at the CYP2C19*2 site in CR group was higher than that in NCR group (0.481 versus 0.321, *P*=0.03). The frequency of A allele at the CYP2C19*3 site in CR group was higher than that in NCR group (0.352 versus 0.173, *P*=0.005). These results suggested a potential association between CR and polymorphisms in the CYP2C19*2 and *3 sites in Chinese patients with ACS.

Simon et al. [24] reported a link between adverse events and ABCB1 (C3435T, rs1045642) polymorphisms in patients administered clopidogrel. Carriers of the GG and AA genotypes showed considerable increased in the incidence of adverse events. ABCB1 (C3435T, rs1045642) polymorphisms were found to influence ADP-induced platelet accumulation, and carriers of the G allele were more likely to exhibit hypo-reactivity to antiplatelet therapy [7]. The TRITON-TIMI 38 clinical trial by Michalak et al. [25] showed that the maximum rate of platelet accumulation in carriers of the GG genotype in ABCB1 (C3435T, rs1045642) polymorphisms were 7.3% lower after

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taking clopidogrel compared to those in carriers of the GA or AA genotype (*P*=0.0127). Compared with patients with GA or AA genotypes, patients with GG genotype were more prone to have cardiac adverse events (CAE) after taking clopidogrel. However, the results showed that the ABCB1 (C3435T, rs1045642) polymorphisms were not related to CAE in patients taking clopidogrel and aspirin after drug-eluting stent implantation. In addition, about 72% of patients with CYP2C19*2 allele also carried ABCB1 C3435T mutation, which greatly increased the relapsed risk of CAE after stent implantation. Spiewak et al. [26] reported there was no correlation between ABCB1 (C3435T, rs1045642) polymorphisms and platelet activation to clopidogrel. In the present study, 9, 13, and 5 patients in the CR group were carriers of the AA, AG, and GG genotypes of ABCB1 (C3435T) polymorphism, respectively; the frequency of the G allele was 0.426, which is not different from the 0.393 (*P*>0.05) in the NCR group, in which 36, 47, and 15 patients were carriers of the AA, AG, and GG genotypes of the ABCB1 (C3435T) polymorphism, respectively. Therefore, the ABCB1 (C3435T) polymorphism was not associated with CR (*P*=0.661). However, this study included only 125 patients with ACS, and the sample size was relatively small. In addition, variations in geographical and ethnic background, as well as differences in the methods of the measuring platelet accumulation rate across different studies, could mask potentially significant associations between ABCB1 (C3435T) polymorphism and CR. Moreover, a large sample study is required in subsequent studies.

In vitro experiments conducted by Bouman et al. showed that PON1 (paraoxonase-1) was a rate-limiting enzyme in the second step of clopidogrel activation [27]. Patients with CAD who carried the QQ homozygous mutant genotype and took clopidogrel after PCI showed marked reduction in plasma activity of PON1 compared to carriers of the QR genotype or wild-type genotype (RR) [28]. The former also showed dramatically increased risk of recurrent stent thrombosis [29]. In a study related to *PON1* polymorphisms in the Chinese population, PON1 was found to be correlated with CR after excluding the risk factors for CAD. Compared to non-carriers, carriers of the Q allele (PON1 QQ/QR192) showed significantly higher ADP-induced platelet accumulation (48.1±25.2% versus 42.5±23.8%, *P*=0.027); however, no significant differences in platelet reactivity were observed between patients harboring the QQ192 and QR192 genotypes [9]. The study of Collet et al. [30] revealed that the *CYP2C19* gene (not the *PON1* gene) was associated with changes in pharmacokinetics and pharmacodynamics of clopidogrel and therapeutic results of myocardial infarction. In the study, the patients with CAD were divided into 2 groups with different loading doses (300 mg and 900 mg of clopidogrel), and the levels of the H4 isomer of clopidogrel metabolite and platelet function were analyzed. Results indicated no significant correlation between *PON1* polymorphism and platelet function. Dirk et al. [31] also reported no significant relationship between *PON1* (Q192R rs662) polymorphism and CR, whereas polymorphisms in the CYP2C19*2 locus was identified as the only factor associated with platelet hyperreactivity and post-PCI thrombotic events.

The current findings do not confirm a significant relationship between *PON1* (Q192R rs662) polymorphism and CR. Of the 125 patients, 51, 52, and 22 were carriers of the CC, CT, and TT genotypes of the *PON1* (Q192R rs662) polymorphism, respectively. In the CR group, 10, 12, and 5 patients were carriers of the CC, CT, and TT genotypes of the *PON1* (Q192R rs662) polymorphism, respectively. The frequency of T allele was 0.407, which is not different from that of the NCR group (41, 40, and 17 carriers of the CC, CT, and TT genotypes, respectively; the frequency of T allele was 0.378 (*P*=0.69).

Conclusions

Our results indicated a significant association between the polymorphisms in the CYP2C19*2 and CYP2C19*3 *loci* and CR in Chinese patients with ACS. The ABCB1 (C3435T, rs1045642) polymorphisms were found to be weakly associated with CR; however, the PON1 (Q192R rs662) polymorphism was not associated with CR. These results provide important clues for improved treatment strategies for patients with ACS who are at high risk of recurrence of cardiovascular events due to CR.

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