CYP2C19 polymorphisms in acute coronary syndrome patients undergoing clopidogrel therapy in Zhengzhou population

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ABSTRACT. The goal of this study was to explore the polymorphisms of CYP2C19 (CYP2C19*2, CYP2C19*3) in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI) on clopidogrel therapy in Zhengzhou city for guidance on clinical medication and reduction in the incidence of thromboembolic events. Two hundred and thirty-four ACS patients undergoing PCI were included in the study, including 171 males (average age = 64.13 ± 12 years) and 63 females (average age = 67.86 ± 10.20 years). Pyrosequencing analysis detected CYP2C19*2/*3 genotypes, which were divided into wild-type homozygous C/C, mutant heterozygous C/T, and mutant homozygous T/T. This study further explored the relationship between CYP2C19 polymorphisms and clopidogrel resistance in ACS patients. Gene frequencies of C/C, C/T, and T/T for CYP2C19*2 were 39.74, 50, and 10.26%, respectively, while the frequencies of C/C, C/T, and T/T for CYP2C19*3 were 94.02, 5.55, and 0.43%, respectively. According to platelet aggregation analysis, 203 cases normally responded to

clopidogrel (86.8%) and 31 cases were clopidogrel resistant (13.2%). There was a correlation between gender and genotype distribution but none between age and genotype. In addition, patients with clopidogrel resistance were treated with ticagrelor antiplatelet therapy instead of clopidogrel, and only 1 case in all patients suffered thrombotic events during a 3-12 month follow-up. In conclusion, CYP2C19*2/*3 polymorphisms may be associated with clopidogrel resistance. Wild-type homozygote and single mutant heterozygote of CYP2C19*2/*3 can be given a normal dose of clopidogrel, while carriers with single mutant homozygote or double mutant heterozygote require ticagrelor antiplatelet therapy as an alternative.

Key words: Clopidogrel; Polymorphism; Coronary heart disease

INTRODUCTION

As life styles change, the prevalence of coronary heart disease in China has increased year by year, and interventional therapy has become an important means to alleviate the symptoms of angina pectoris. Antiplatelet therapy is an important method for the prevention of thrombosis and in-stent restenosis before and after surgery. The combination of aspirin and clopidogrel is the standard medication of anti-platelet therapy for patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI), but a number of patients are resistant to clopidogrel. As a prodrug, clopidogrel cannot play an anti-platelet role unless it exerts activity after enzymatic metabolism. Therefore, more attention has been focused on the association between the CYP2C19*2/*3 polymorphisms that affect enzymatic metabolism and clopidogrel resistance.

Clopidogrel exerts its inhibition on platelet aggregation after transformation with the help of the cytochrome P450 (CYP450) enzyme *in vivo*, which can effectively reduce the risk of intra-arterial and postoperative in-stent thrombosis. CYP2C19 is an important member of the CYP450 isoenzyme family and is involved in the catalytic metabolism of endogenous substrates, environmental pollutants, and about 2% of clinical drugs. Chinese and international studies have confirmed that the CYP2C19 gene polymorphism is associated with clopidogrel metabolism. Moreover, due to the genetic polymorphisms, there are obvious individual and racial differences in CYP2C19 enzyme activity, which has a serious impact on the clinical efficacy and safety of clopidogrel. Therefore, it is important to understand the genetic polymorphisms of CYP2C19 for the implementation of individual anti-platelet therapy. In this study, the distribution of CYP2C19*2/*3 gene polymorphisms in patients with ACS undergoing PCI in Zhengzhou, China was examined.

MATERIAL AND METHODS

Subjects

A total of 234 ACS patients undergoing PCI in Zhengzhou consecutively registered from October 2013 to August 2014 were admitted to the Zhengzhou Central Hospital Affiliated

to Zhengzhou University. They were 39-90 years old with the average age being 65.13 ± 11.64. The group included 171 males (average age $= 64.13 \pm 12$) and 63 females (average age $= 67.86 \pm 10.20$). This study was conducted in accordance with the declaration of Helsinki and with approval from the Ethics Committee of Zhengzhou Central Hospital Affiliated to Zhengzhou University. Written informed consent was obtained from all participants. The inclusion criteria were: 1) Han patients with ACS undergoing PCI; 2) born and currently lived in Zhengzhou; and 3) had not taken clopidogrel before admission. The exclusion criteria were: 1) allergy and intolerance to clopidogrel; 2) severe liver or renal dysfunction or abnormal coagulation function; 3) perioperative gastrointestinal bleeding or active bleeding history within the last 30 days; 4) platelet count $<100 \times 10^9/L$ or $>300 \times 10^9/L$ and white blood cell count $<4 \times 10^9/L$ or $>10 \times 10^9/L$ in hospital blood test; and 5) minority patients. For surgery, 1-2 rapamycin-eluting stents were implanted into all patients with release at 12-14 atm and were expanded with balloon post-dilatation at 12-16 atm to make in-stent residual stenosis less than 10%.

CYP2C19 genetic testing

Two-milliliter blood samples were collected before PCI and was placed into an EDTA anticoagulated tube. Blood was preserved at 4°C to analyze genotypes within 24 h.

Pyrosequencing reaction

PCR amplification product (15 µL) was transferred onto 96-well plates with 38 µL binding buffer (Qiagen, Dusseldorf, Germany) added to each PCR reaction system (Table 1). Next, the reaction was placed on a multifunction oscillator (Eppendorf, Hamburg, Germany) at 2000 rpm with 2 µL Streptavidin Sepharose beads (GE, Beijing, China) and incubated for 10 min. After Ultrapure water, 70% ethanol, denaturation buffer (0.2 M NaOH) and washing buffer (Qiagen) were consecutively added in 4 pools to the Vacuum Prep Workstation (Qiagen). The vacuum pump was turned on and the vacuum prep tool was rinsed in ultrapure water for 30 s and inserted onto 96-well plates to absorb Sepharose beads. The vacuum prep tool was washed in 70% ethanol for 5 s, denatured in denaturation buffer for 8 s (only biotin-labeled single-stranded DNA bound to Sepharose beads), and rinsed in washing buffer for 10 s. As the vacuum pump was turned off, the vacuum prep tool was placed onto PyroMark Q24 pyrosequencing reaction plates (Qiagen) with 25 µL annealing buffer (Qiagen) and 1.5 µL (10 µM) sequencing primer already added to each well. After gentle friction, Sepharose beads were fully released. The reaction plate was placed at 80°C for 2 min and cooled to room temperature. The test procedure was set according to the manufacturer instructions for Q24 PyroMark. After enzyme mixture (E), fluorescent substrate (S), and four kinds of dNTPs in PyroMark™ Gold Q96 Reagents kit (Qiagen) were added to the reagent chamber, the reaction plate was detected in the detection chamber. Amplification and sequencing primers used in the pyrosequencing reaction are shown in Table 1.

Detection method of platelet aggregation

After dual antiplatelet therapy of aspirin and clopidogrel for 1 week, 2 mL blood was collected from each patient into an EDTA anticoagulated tube. Patients could not ingest milk,

soymilk, or other high fat foods the day before blood sampling for the prevention of reducing the transmittance artificially. The ADP-induced platelet aggregation method was applied for the determination of platelet aggregation within 30 min. To prepare, 1 mL ADP buffer was added into ADP powder and mixed then another 1 mL ADP buffer was added and mixed. This ADP solution (10 mL) was divided into several low-dose aliquots. For dosage, each patient received 10 µL (final concentration = 5 µM). The Platelet-Poor Plasma (PPP) sample was centrifuged at 2500 rpm for 10 min, while Platelet-Rich Plasma (PRP) sample was centrifuged at 800 rpm for 10 min. For detection, 300 µL PPP plasma (without test beads) was added into the test cup and placed into the test channel for sampling in the PPP state. In addition, 300 µL PRP plasma (with test beads) was added into the test cup, transferred into the test channel, and rewarmed at 37°C for 1 min. The device was then turned on. Immediately, 10 µL inducer (ADP) was added into the cup bottom with a pipette. An automatic test was performed and the results recorded.

Table 1. Amplification and sequencing primers in pyrosequencing reaction.

Primer	Primer sequence (5'→3')	Explanation
61-F	TGTGTTTCTCCCTTCTCAGGATTC	5' end labeled with biotin, PCR amplification primers
61-	TACTGGTCCCTCATTGCACTGT	PCR amplification primers
61-pyroS	ATATTCTCGACACAGCAG	Pyrosequencing primers
1213-F	ACTGGTGGAGTATTTGATAGTGTA	5' end labeled with biotin, PCR amplification primers
1213-R	CCTCTATTGTTGGATCATATTCGT	PCR amplification primers
1213-pyroS	AGCTCCAACTACCACAA	Pyrosequencing primers

Statistical analysis

All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and data are reported as means ± standard deviation. Comparison of averages was analyzed using the Student t-test between the two groups. The Pearson chi-square test was applied for correlation analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Generation information

A total of 234 ACS patients undergoing PCI in Zhengzhou were consecutively registered as they were admitted to the hospital. They were 39 to 90 years old with an average age of 65.13 ± 11.64 years (Table 2). The group comprised 171 males (average age = 64.13 ± 12 years) and 63 females (average age = 67.86 ± 10.20 years). There were 122 patients with hypertension and 89 patients with diabetes.

Table 2. General information on selected patients.

Gender	N	Age (years) (means ± SD)	BMI
Female	171	64.13 ± 12.00	26.59 ± 4.56
Male	63	67.86 ± 10.20	24.32 ± 6.23

Genotype frequencies of the polymorphic loci of CYP2C19*2 and CYP2C19*3

Statistical results showed that genotype frequencies of mutant heterozygote (50%) and wild-type homozygote (94.02%) of CYP2C19*2 were the highest, while the mutation rate of CYP2C19*3 was the lowest (0.43%; Table 3).

Table 3. Frequency of each locus of CYP2C19*2 and CYP2C19*3.

	CYP2C19*2			CYP2C19*3		
	C/C	C/T	T/T	C/C	C/T	T/T
N	93	117	24	220	13	1
Frequency (%)	39.74	50	10.26	94.02	5.55	0.43

C/C = Wild-type homozygote (fast metabolism of clopidogrel). C/T = Mutation heterozygote (intermediate metabolism of clopidogrel). T/T = Mutation homozygote (low metabolism of clopidogrel).

Genotype distribution of CYP2C19*2/*3 in patients

Statistical results showed that genotype frequencies of CYP2C19*2 C/T and CYP2C19*3 C/C were the highest, accounting for 47.01% (N = 110), while the frequency of the double mutant homozygote of CYP2C19*2/*3 was 0 (Table 4).

Table 4. Genotype distribution of CYP2C19*2/*3.

Gender	Cases						N
	CC/CC[1]	CT/CC[2]	TT/CC[3]	CC/CT[4]	CC/TT[5]	CT/CT[6]	
Female	67	83	15	1	1	4	171
Male	20	27	9	5	0	2	63
Total	87	110	24	6	1	6	234
Proportion (%)	37.18	47.01	10.26	2.56	0.43	2.56	100

[1]CC/CC = CYP2C19*2 (C/C), CYP2C19*3 (C/C), CYP2C19*2/3 wild-type homozygote. [2]CT/CC = CYP2C19*2 (C/T), CYP2C19*3 (C/C), CYP2C19*2 single mutation heterozygote. [3]TT/CC= CYP2C19*2 (T/T), CYP2C19*3 (C/C), CYP2C19*2 single mutation homozygote. [4]CC/CT = CYP2C19*2 (C/C), CYP2C19*3 (C/T), CYP2C19*3 single mutation heterozygote. [5]CC/TT = CYP2C19*2 (C/C), CYP2C19*3 (T/T), CYP2C19*3 single mutant homozygote. [6]CT/CT = CYP2C19*2 (C/T), CYP2C19*3 (C/T), CYP2C19*2/3 double mutant heterozygote.

Platelet aggregation of each genotype

The platelet aggregation of each genotype was compared, and the results showed that there were no significant differences in CC/CC, CT/CC, CC/CT (P > 0.05) and in TT/CC, CC/TT, CT/CT (P > 0.05). Therefore, CC/TT, TT/CC, CT/CT were considered as clopidogrel resistant and theses carriers needed ticagrelor antiplatelet therapy instead of clopidogrel therapy (Table 5).

Table 5. Platelet aggregation of each genotype.

Genotype	N	Mean platelet aggregation
CC/CC	87	19
CT/CC	110	16
TT/CC	24	16
CC/CT	6	28
CC/TT	1	32
CT/CT	6	31

Analysis of the ratio of clopidogrel sensitive group and clopidogrel resistant group

According to the platelet aggregation results, 203 patients had normal metabolism of clopidogrel (86.8%) while 31 cases were clopidogrel resistant (13.2%). Timely identification of patients with clopidogrel resistance can reduce cardiovascular thrombotic events and the mortality and morbidity of coronary heart disease.

Correlation analysis of genotype with gender and age

The analysis showed that the genotype of CYP2C19*2/*3 was correlated with gender (P = 0.028). The frequencies of wild-type homozygous CYP2C19*2/*3 and mutant heterozygous CYP2C19*2 in males was higher (28.63 and 35.47%, respectively), which might be related to the high proportion of males in registered patients. Moreover, there was no correlation between genotype and age (P = 0.144).

Comparison of the number of stents in both groups

The average number of stents in the clopidogrel group was 1.34 ± 1.13, while the number in the ticagrelor group was 1.35 ± 1.18. The difference between the two groups was not statistically significant (P > 0.05).

Follow-up

A total of 203 patients had a normal response to clopidogrel while 31 patients were resistant to clopidogrel. In addition, patients with clopidogrel resistance were treated with ticagrelor antiplatelet therapy instead of clopidogrel. During a 3-12 month follow-up, the primary endpoint events were: patient was hospitalized because of acute myocardial infarction, in-stent restenosis and angina pectoris, or death. One patient who was wild-type homozygous of CYP2C19*2/*3 in the clopidogrel sensitive group suffered from acute myocardial infarction after PCI.

DISCUSSION

Activation and aggregation of platelets plays an important role in arterial thrombosis after ACS and PCI. Oral anticoagulant therapy is the key treatment of ACS patients undergoing PCI. As a prodrug, clopidogrel is metabolized into active products in two steps in the liver CYP450 system after absorption. In the second oxidation step, 44.9 and 20.6% of the roles are mediated by the proteins encoded by the CYP2C19 gene (Kazui et al., 2010). The CYP2C19 gene is located in chromosome 10 in humans and includes 9 exons and 5 introns. It encodes proteases in liver microsomes, so this gene plays an important role in the transformation of clopidogrel *in vivo*. The gene encoding CYP2C19 shows polymorphic expression in the human body and the mutants are CYP2C19*2, CYP2C19*3, CYP2C19*4 and CYP2C19*5. CYP2C19*2 is the major mutant while CYP2C19*3 is relevant within certain ethnic groups.

Recent research has shown that changes in the activity of drug-metabolizing enzymes resulting from genetic mutations is one of the root causes of different metabolic ability of the same drug in individuals (Severino and Del Zompo, 2004). CYP2C19 gene polymorphisms

lead to individual differences in CYP2C19 enzyme activity, thereby causing inter-individual differences in metabolic capacity of clopidogrel (Liboredo and Pena, 2014; Sun et al., 2015). Foreign studies have indicated that the polymorphisms of CYP2C19 have obvious racial differences, as the incidence of CYP2C19*2 is 17, 15, and 30% and the incidence of CYP2C19*3 is 0.4, 4, and 5% in black Americans, Caucasians and Chinese, respectively (Zhou et al., 2009). The incidence of chronic metabolism is 3, 15-17, and 10-25% in Caucasians, Chinese, and Asians, respectively (Zhou et al., 2009). Moreover, the incidence of CYP2C19*2, CYP2C19*3, and chronic metabolism in Chinese (12-16%) is much higher than in Caucasians (2-3%) and Africans (4%) (Wedlund, 2000). At present, there are at least 25 loci for CYP2C19 polymorphisms, and it has been observed that clopidogrel therapy for carriers of CYP2C19*17 can significantly reduce the incidence of cardiovascular events when compared to carriers of CYP2C19*1. However, most current studies often focus on CYP2C19*2 and CYP2C19*3, which can diminish the efficacy of clopidogrel. These two alleles are the most common in Asians and their incidences are 29-35 and 2-9%, respectively (Scott et al., 2013). However, their incidence in Chinese patients can be as high as 52.2 and 9.6%, respectively (Zhang et al., 2013). Carriers of CYP2C19*2 and *3, referred to as having CYP2C19 chronic metabolism, present the slow activation rate of clopidogrel, resulting in decreased anti-platelet activity due to less active metabolites. The risk of in-stent thrombosis in patients with CYP2C19*2 was increased, which results in a high rate of disability and mortality; therefore, ticagrelor antiplatelet therapy is suggested for patients with CYP2C19*2 and *3. Collet et al. (2009) showed that the CYP2C19*2 polymorphism was an independent predictor of cardiovascular events and was a major determinant of prognosis in the study of 259 young patients (aged <45 years) having suffered from myocardial infarction and currently undergoing clopidogrel therapy. Hou et al. (2014) showed that CYP2C19*2 might be closely related to clopidogrel resistance in a meta-analysis of 2331 patients. However, current research on these polymorphisms is mainly conducted in Europeans and Americans, and the mechanism of their action needs to be further studied. In addition, there is no direct evidence that CYP2C19 is causally related with clopidogrel biotransformation, so some studies do not show the association between CYP2C19 gene polymorphisms and clopidogrel resistance. At present, CYP2C19 has been found to have at least 35 alleles (Zhang and Zhu, 2011). The mutant alleles affect the activity and expression of the enzyme. For example, CYP2C19*1 (wild-type or strong metabolism) encodes the normal enzyme and CYP2C19*2 (weak metabolism) is the most common enzyme-deficient allele. The CYP2C19*2 polymorphism reduces the active metabolic conversion of clopidogrel so the pharmacological metabolism of the body is weakened, which results in a low response to clopidogrel. Jeong et al. (2011) showed that 60.9% of patients had at least one locus mutation and the risk of high platelet reactivity and cardiovascular events were related to the number of variable loci in an East Asian population. There was no significant difference between carriers with one loss-of-function (LOT) gene and carriers with no loss-of-function gene, while the risks of platelet reactivity and cardiovascular events in carriers with 2 LOT genes was significantly increased when compared with carriers with no LOT gene. Tang et al. (2011) showed that urgent revascularization of CYP2C19*2 gene carriers was higher than in patients without the CYP2C19*2 gene by observing the prognosis of 267 patients undergoing 12-month clopidogrel therapy after PCI. Therefore, CYP2C19 polymorphisms have important clinical significance.

At present, there are a few of studies on the specific genotype of CYP2C19*3 and CYP2C19*2 in China. Zhang et al. (2014) showed that the frequencies of heterozygous and

homozygous CYP2C19*2 was 44.1 and 10.4%, respectively. Similarly, our study found that the frequencies of heterozygous and homozygous CYP2C19*2 was 50 and 10.26%, respectively, in Zhengzhou city. There is no uniform standard for the definition of clopidogrel resistance and the results were empirically obtained by different detection methods. Gurbel et al. (2003) compared platelet aggregation after administration of clopidogrel, using base platelet aggregation based on ADP as an inducer, and proposed that the absolute difference ≤ 10% was regarded clopidogrel resistance. Müller et al. (2003) detected the inhibition rate of platelet aggregation (IPA) to define clopidogrel responsiveness, and 10%, 10-30%, and ≥ 30% IPA was defined as non-responsive, low response, and normal response, respectively. In this study, after pairwise comparisons of the platelet aggregation of each genotype of CYP2C19*3/*2, the results showed no significant differences in CC/CC, CT/CC, CC/CT (P > 0.05) and in TT/CC, CC/TT, CT/CT (P > 0.05). Therefore, CC/CC, CT/CC, CC/CT were regarded as normal metabolism of clopidogrel, while CC/TT, TT/CC, CT/CT were considered as clopidogrel resistant, requiring ticagrelor antiplatelet therapy instead of clopidogrel therapy. In this study, the frequency of wild-type homozygous CYP2C19*2/3 was 37.2% and the normal dose of clopidogrel could be used for these carriers. However, the frequency of the mutant was 62.8%, of which the frequency of the single mutant heterozygote was 49.6%. Combined with platelet aggregation, carriers of the wild-type homozygote and single mutant heterozygote of CYP2C19*2/3 should be given a routine dose of clopidogrel by oral administration. At present, only 1 patient suffered from thrombosis and there was no increase in the risk of in-stent thrombosis during the 3-12 month follow-up. Mega et al. (2011) showed that carriers of mutant heterozygote after administration of 225 mg/day clopidogrel could obtain the equivalent inhibition level of patients with wild-type homozygote after taking 75 mg/day, but carriers with mutant homozygote, even if taking the maintenance dose of 300 mg/day, could not achieve ideal platelet inhibition. This is based on the observation of vasodilator-stimulated phosphoprotein (VASP) - plateletreactivity index (PRI) and P2Y(12) reaction units (PRU)levels in mutant heterozygote and mutant homozygote carriers of CYP2C19 after administration of the maintenance dose of 75, 150, 225, or 300 mg clopidogrel. It was safe to give a conventional dose of clopidogrel for wild-type homozygote and single mutant heterozygote of CYP2C19*2/*3, but clopidogrel resistance should be monitored in patients with coronary heart disease to reduce the incidence of thrombosis. In this study, the frequency of double mutant heterozygote or single mutant homozygote of CYP2C19*2/*3 was 13.2% and no thrombotic events occurred after ticagrelor anti-platelet therapy. In addition, this study showed correlation between gender and genotype distribution (P > 0.05) but no significant correlation between age and genotype (P < 0.05). Li et al. (2014) showed no significant correlation between age and CYP1C19 genotype based on the observation of 252 male and 119 female cases. In that study, the sample size was small and the proportion of men in the selected patients was high; however, the incidence of coronary heart disease in males is significantly higher than in females. The study also showed that the mutation rate of CYP2C19*2 in a Zhengzhou population (59.9%) was significantly higher than in Chinese (30%) based on foreign statistics, while the mutation rate of CYP2C19*3 in a Zhengzhou population (5.6%) was consistent with that in a Chinese population (5%). Fontana et al. (2008) concluded that there was a correlation between CYP2C19*2 allele and clopidogrel resistance in patients visiting cardiovascular outpatient clinics.

Currently, there appears to be a causal association between genetic variant and

clopidogrel resistance, and CYP2C19 gene polymorphisms might be only a manifestation type of the gene-decided loci. Therefore, it is unknown whether the polymorphisms of CYP2C19 directly affect the risk of cardiovascular events so further in-depth and genome-related studies are needed to solve these issues. Due to the small number of cases and short follow-up in this study, a long-term follow-up would be needed to study the outcome of carriers with mutant heterozygote and single mutant homozygote of CYP2C19*2 and CYP2C19*3 after oral administration of clopidogrel to guide clinical medication, improve prognosis, and reduce the economic burden to patients and society.

Conflicts of interest

The authors declare no conflict of interest.

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