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ORIGINAL RESEARCH

Should *CYP2C19* Genotyping Be Recommended as a Straight Forward Approach to Optimize Clopidogrel Utilization in Patients with Ischemic Stroke Complicated by Type 2 Diabetes Mellitus?

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Background: There have been few studies on *CYP2C19* genotypes and clopidogrel response associated with ischemic stroke (IS), especially IS complicated by type 2 diabetes mellitus (T2DM). This study aimed to investigate the possible association between *CYP2C19* polymorphisms and high on-treatment platelet reactivity (HTPR) in IS patients with T2DM in China.

Patients and Methods: A total of 426 consecutive IS patients with T2DM were enrolled in this case-control study and they were divided into HTPR group and non-HTPR group according to the ADP-induced platelet inhibition (PIADP) assessed by thromboelastography (TEG). Genotypes were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Various clinical and demographic data were also recorded. The association between *CYP2C19* genetic variants and platelet function was assessed.

Results: Carriers of *CYP2C19*2* heterozygous and mutant homozygous genotypes showed significantly lower PIADP than non-carriers (27.2% vs 38.3%, $p < 0.001$; 27.41% vs 38.3%, $p = 0.012$, respectively). Compared with the control group, the *CYP2C19*2* A allele was more frequent in the HTPR group (34.51% vs 25.82%, $p = 0.002$). The carriage of *CYP2C19*2* mutant allele was significantly associated with increased risk of HTPR (odds ratio (OR) = 1.94, 95% confidence interval (CI) = 1.32–2.85). There was no significant correlation between *CYP2C19*3* or **17* genotypes and HTPR risk.

Conclusion: *CYP2C19*2* mutant allele was associated with attenuated platelet response to clopidogrel and increased risk of HTPR in IS patients with T2DM, suggesting that *CYP2C19*2* polymorphism might be an important predictor of HTPR in this high-risk population.

Keywords: clopidogrel, ischemic stroke, type 2 diabetes mellitus, *CYP2C19*, high on-treatment platelet reactivity

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Introduction

Stroke is a globally fatal disease that can be classified into two categories: ischemic and hemorrhagic. Among them, ischemic stroke (IS) is the main type, accounting for 70% to 80% of stroke patients. It is characterized by high morbidity, mortality, and disability, as well as high relapse rate. According to the guidelines for the early management of patients with acute ischemic stroke, antiplatelet therapy is considered

an important treatment for secondary prevention of IS.[1,2] Clinical studies have shown that long-term antiplatelet therapy can reduce the incidence of vascular events (for example, myocardial infarction, stroke, and vascular death) by 15%-34%.[3] The antiplatelet drug clopidogrel is recommended as an effective treatment according to current guidelines.[1,2]

Although many studies have confirmed the clinical efficacy of clopidogrel as an antithrombotic agent, its effectiveness in preventing platelet aggregation is not consistent in all patients.[4–6] 5–44% of patients receiving this drug show high platelet reactivity, leading to a number of subsequent ischemic events.[7] Such a situation is called high on-treatment platelet reactivity (HTPR). Many studies have indicated that patients with HTPR are more likely to have adverse ischemic events,[8–11] with diabetes mellitus (DM) patients being at a higher risk, about two- to fourfolds increased risk compared with non-DM patients, and being considered to be a high-risk group.[11–13] The mechanisms leading to HTPR in DM patients have not been fully elucidated, and a variety of factors have been identified, including genetic, demographic, and clinical factors.[14–16]

As a prodrug, clopidogrel requires metabolic activation through the hepatic cytochrome P450 (CYP450) system. *CYP2C19* is the main metabolic enzyme of the CYP450 system and plays an important role in the oxidative activation of clopidogrel.[17] Its polymorphisms are thought to be closely related to the poor clopidogrel response. *CYP2C19*2* (681G > A, rs4244285) and *CYP2C19*3* (636G > A, rs4986893) are the most common loss-of-function (LOF) alleles in the Asian population (frequency of 25–35% and 5–15%, respectively), while *CYP2C19*17* (−806C > T, rs12248560) is a gain-of-function (GOF) allele.[18] In china, approximately 35% of the population are carriers of *CYP2C19* LOF alleles, higher than that of Caucasians and Africans (15%).[18,19] Studies have shown that there is a correlation between *CYP2C19* LOF genotypes and HTPR in IS patients.[20–22] However, there is no information about the impact of *CYP2C19* alleles on IS patient with type 2 diabetes mellitus (T2DM), who is a special group with particular metabolic profiles.

Therefore, this study aimed to investigate the possible association between *CYP2C19* polymorphisms and HTPR in Chinese Han patients with IS complicated by T2DM. The data obtained may help to improve individualized antiplatelet therapy opinions and reduce adverse side effects in IS patients with T2DM.

Patients and Methods

Study Protocol

We conducted a case-control study consisting of consecutive patients with IS complicated by T2DM from the Chinese Han population who were admitted to the Affiliated Hospital of Qingdao University between January 2018 and October 2019. The inclusion criteria were as follows: diagnosis of IS with computer tomography or magnetic resonance imaging; diagnosis of T2DM according to the International Diabetes Federation criteria;[23] older than 18 years; continuous clopidogrel treatment for 7 to 10 days, with platelet function testing. Exclusion criteria included: exposure to thienopyridine or glycoprotein IIb/IIIa inhibitor within 1 week; diagnosis of severe kidney or liver diseases; inability to give informed consent. Furthermore, to determine whether the results of this study were specific to patients with T2DM, pharmacodynamics evaluations were also extended to a cohort of IS patients without T2DM. We included the same sample size for each group. The study was conducted in accordance with the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. Written informed consent was obtained from each participant.

Clinical Data Collection

The demographic characteristics and baseline data were collected and evaluated, including general condition (age, gender, and body mass index (BMI)), personal history (smoking and drinking), medical history (hypertension, dyslipidemia, coronary artery disease, peripheral arterial disease, previous percutaneous coronary intervention (PCI) and previous stroke), laboratory tests, and use of other medications (insulin, oral hypoglycemic agents, aspirin, lipid-lowering drugs, β-blockers, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), calcium channel blockers (CCBs), and proton-pump inhibitors (PPIs)). These data were obtained through reviewing medical records of the hospital.

ADP-Induced Platelet Aggregation Test

ADP-induced platelet inhibition (PIADP) was measured by TEG® 5000 Thrombelastograph® Hemostasis Analyzer system (Haemoscope Co., Niles, IL, USA). Venous blood was drawn from patients on an empty stomach in the morning within 7 to 10 days of clopidogrel treatment.

The first 2 to 4 mL of blood was discarded to avoid spontaneous platelet activation. 2.7 mL of venous blood anticoagulated with 3.2% sodium citrate and 4.0 mL of venous blood anticoagulated with 14.7 U mL^{-1} lithium heparin were extracted and tested within 2 hrs.

The analyzer had three channels: 20 μL of 0.2 mol L^{-1} $CaCl_2$ and 340 μL of blood anticoagulated with sodium citrate and mixed with Kaolin were added to channel 1; 10 μL of activator F and 360 μL of heparin-anticoagulated blood were added to channel 2; and 10 μL of activator F, 10 μL of ADP, and 360 μL of heparin-anticoagulated blood were added to channel 3. PIADP was calculated by the instrument software and the results were expressed as a percentage (%). Patients with PIADP < 30% belonged to the HTPR group (case group).

CYP2C19 Genotyping

Three single nucleotide polymorphisms (SNPs) of *CYP2C19*, including *CYP2C19*2*, **3*, and **17*, were genotyped in this study by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. Genomic DNA was extracted from citrated whole blood with commercially available kits (DP348 Blood Genomic DNA Extraction Kit, Tianjian Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Genotyping was performed on a 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) using the *CYP2C19* Genotyping Assay Kit (Shandong Biomultitech Co., Ltd., Shandong, China) following the supplier's protocols. The results were analyzed by Applied Biosystems 7500 Real-Time PCR System Sequence Detection Software v1.4.1. Control DNAs supplied by the manufacturer were genotyped to ensure accuracy.

Sample Size Calculation

The sample size was calculated using PASS Software v15.0 (NCSS, LLC, Kaysville, UT, USA). It was estimated that at least 56 HTPR participants would be required to detect an odds ratio (OR) of 2.654 with an expected prevalence of *CYP2C19*2* allele of 0.288,[24,25] a power of 90% and a two-sided alpha-level of 0.05. To ensure accuracy, we included a larger and equal sample size for each group.

Statistical Analysis

Data were analyzed by IBM SPSS Statistics Software v26.0 (IBM Co., Armonk, NY, USA). Continuous variables were presented as mean and standard deviation (SD) or median and quartile ranges (for non-normally distributed data). Categorical variables were expressed as numbers or percentages. The normality of continuous variables was evaluated using the Shapiro–Wilk test, with homogeneity verified by the Levene test. Continuous variables between groups were compared by the Student's *t*-test or the Mann–Whitney *U*-test, as appropriate. Categorical variables were compared using the χ^2 test (or Fisher exact test for expected counts less than 5). The χ^2 test was also used to evaluate differences in allele and genotype frequencies between groups, and to determine whether individual polymorphism was in Hardy–Weinberg equilibrium (HWE). Result with a two-tailed p value < 0.05 was considered statistically significant.

Results

Baseline Characteristics and Platelet Reactivity

We consecutively recruited 426 IS patients with T2DM who were all from the Chinese Han population. The median PIADP of the entire subject, the case group, and the control group was 30.05% (IQR, 21.1–58.6%), 21.1% (IQR, 12.95–25.8%), and 58.6% (IQR, 44.20–75.05%), respectively. Table 1 lists the baseline demographics, clinical characteristics, and laboratory data of the study population. No significant differences in the baseline characteristics between the HTPR and non-HTPR groups were observed.

The baseline characteristics of the IS study population without T2DM are shown in Table S1. The differences between the HTPR and non-HTPR groups were not statistically significant ($p > 0.05$).

Genotyping results

Table 2 shows the genotyping results of *CYP2C19* in the IS patients with T2DM. The genetic distribution of the assessed polymorphisms did not deviate from the HWE ($p > 0.05$). The frequencies of *CYP2C19*2*, **3*, and **17* mutant allele carriers in this population were 50.94%, 12.21%, and 4.46%, respectively. Results showed that the Chinese had a high prevalence of *CYP2C19*2* A allele (30.16%), but a relatively low prevalence of **3* A (6.46%) and **17* T (2.35%) alleles. Similar results could be observed in the cohort of IS patients without T2DM (Table S2). The genetic distribution of the evaluated polymorphisms was consistent with previously reported findings.[18,26]

The distribution of *CYP2C19*2*, **3*, and **17* genotypes of the study population is presented in Table 3. Compared

Table 1 Baseline Characteristics of the Study Population

Variables[#]	Overall (n = 426)	HTPR (n = 213)	Non-HTPR (n = 213)	*p* value*
Age, years	69 (62–74)	69 (63–74.5)	69 (61–73)	0.359
Male, n (%)	294 (69.01)	139 (65.26)	155 (72.77)	0.094
BMI,kg/m^2	25.67 (23.88–27.68)	25.53 (23.88–28.09)	25.81 (24.22–27.51)	0.772
Active smoking, n (%)	161 (37.79)	74 (34.74)	87 (40.85)	0.250
Drinking, n (%)	121 (28.40)	54 (25.35)	67 (31.46)	0.171
Hypertension, n (%)	99 (23.24)	47 (22.07)	52 (24.41)	0.566
Dyslipidemia, n (%)	27 (6.34)	12 (5.63)	15 (7.04)	0.551
Coronary artery disease, n (%)	143 (33.57)	72 (33.80)	71 (33.33)	0.918
Peripheral arterial disease, n (%)	48 (11.27)	21 (9.86)	27 (12.68)	0.358
Previous PCI, n (%)	39 (9.15)	19 (8.92)	20 (9.39)	0.867
Previous stroke, n (%)	58 (13.62)	24 (11.27)	34 (15.96)	0.158
Platelet count, x10^9/L	203.5 (173.25–238)	202.5 (173–229.75)	204 (175–246.75)	0.542
Leucocytes, x10^9/L	6.79 (5.8–8.07)	6.78 (5.84–7.92)	6.8 (5.71–8.26)	0.903
Hemoglobin, g/L	133.23 ± 16.05	133.08 ± 16.95	133.38 ± 15.12	0.928
Fasting blood glucose, mmol/L	6.92 (5.56–8.52)	6.89 (5.56–8.62)	6.93 (5.55–8.41)	0.644
HbA1c,%	7.6 (6.7–9)	7.7 (7–9.3)	7.53 (6.5–8.3)	0.848
TC, mmol/L	3.96 (3.15–4.95)	4.01 (3.22–5)	3.9 (3.11–4.84)	0.421
LDL-C, mmol/L	2.16 (1.61–2.89)	2.29 (1.65–2.92)	2.03 (1.58–2.87)	0.085
HDL-C, mmol/L	1.07 (0.94–1.22)	1.07 (0.95–1.24)	1.07 (0.94–1.22)	0.693
TG, mmol/L	1.39 (0.95–2.08)	1.4 (0.94–2.08)	1.35 (0.97–2.09)	0.999
BUN, mmol/L	5.42 (4.41–6.74)	5.41 (4.53–7)	5.46 (4.25–6.52)	0.334
Cr, μmol/L	83.4 (66–96.88)	81.7 (63–97)	84.5 (70.15–96.5)	0.174
UA, μmol/L	309 (252.75–371)	311 (256.5–373.7)	300.9 (249.25–359)	0.270
HCY, μmol/L	11.88 (9.4–14.25)	13.1 (11.63–14.7)	10.1 (9.35–14.31)	0.301
CRP, mg/L	2.14 (1.29–4.38)	1.96 (1.28–3.46)	2.42 (1.27–5.96)	0.463
Insulin, n (%)	113 (26.53)	52 (24.41)	61 (28.64)	0.323
Oral hypoglycemic agent, n (%)	197 (46.24)	101 (47.42)	96 (45.07)	0.627
Aspirin, n (%)	179 (42.02)	84 (39.44)	95 (44.60)	0.280
Statin, n (%)	183 (42.96)	85 (39.91)	98 (46.01)	0.203
β-blocker, n (%)	153 (35.92)	75 (35.21)	78 (36.62)	0.762
ACEI, n (%)	77 (18.08)	32 (15.02)	45 (21.13)	0.102
ARB, n (%)	115 (27.00)	52 (24.41)	63 (29.58)	0.230
CCB, n (%)	140 (32.86)	70 (32.86)	70 (32.86)	1.000
PPI, n (%)	161 (37.79)	86 (40.38)	75 (35.21)	0.272

Notes: [#]Quantitative data are expressed as mean ± standard deviation or median (1st-3rd quartiles). *Variable is significantly different between HTPR and non-HTPR groups at *p* value < 0.05. Qualitative data are presented as numbers (%).

Abbreviations: BMI, body mass index; PCI, percutaneous coronary intervention; HbA1c, Hemoglobin A1c; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; BUN, blood urea nitrogen; Cr, creatinine; UA, uric acid; HCY, homocysteine; CRP, C-reactive protein; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CCB, calcium channel blocker; PPI, proton-pump inhibitor.

with the non-HTPR group, the frequency of the *CYP2C19*2* A allele was significantly higher in the HTPR group (34.51% vs 25.82%, $p = 0.002$). And the odds of HTPR among patients with a *CYP2C19*2* A allele is 1.94 times the odds of HTPR among those without this mutant allele (Figure 1). There were no significant differences in allele frequencies of the remaining two SNPs between the two groups.

Table S3 represents the distribution of *CYP2C19* genotypes of the cohort of IS patients without T2DM. No significant differences in genotype and allele frequencies were observed ($p > 0.05$).

*CYP2C19*2* Genotypes and Platelet Activity

In this study, individuals carrying the *CYP2C19*2* A allele had a lower PIADP than non-carriers (27.3% (IQR, 17.1–50.3%) vs 38.3% (IQR, 23.8–66.5%), $p < 0.001$). In addition, the PIADP was similar in GA and AA genotypes ($p = 0.897$),

Table 2 Distribution of *CYP2C19*2*, *3, and *17 Genotypes in the Patients

Gene	SNP ID	Genotype/Allele	Number	Frequency (%)	HWE *p* value*
*CYP2C19*2*	rs4244285	GG GA AA Any A allele	209 177 40 257	49.06 41.55 9.39 30.16	0.776
*CYP2C19*3*	rs4986893	GG GA AA Any A allele	374 49 3 55	87.79 11.50 0.70 6.46	0.326
*CYP2C19*17*	rs12248560	CC CT TT Any T allele	407 18 1 20	95.54 4.23 0.23 2.35	0.106

Note: *The genetic polymorphism distribution is considered to deviate from HWE at *p* value < 0.05.

Table 3 Distribution of *CYP2C19*2*, *3, and *17 Genotypes in Clopidogrel Responders and Non-Responders

Gene	SNP ID	Genotype/Allele	HTPR	Non- HTPR	*p* value*
			(n = 213)	(n = 213)	
*CYP2C19*2*	rs4244285	GG, n (%) GA, n (%) AA, n (%)	87 (40.85) 105 (49.3) 21 (9.86)	122 (57.28) 72 (33.8) 19 (8.92)	0.002*
*CYP2C19*3*	rs4986893	GG, n (%) GA, n (%) AA, n (%)	186 (87.32) 25 (11.74) 2 (0.94)	188 (88.26) 24 (11.27) 1 (0.47)	0.616
*CYP2C19*17*	rs12248560	CC, n (%) CT, n (%) TT, n (%)	206 (96.71) 7 (3.29) 0 (0)	201 (94.37) 11 (5.16) 1 (0.47)	0.348

Note: *Variable is significantly different between HTPR and non-HTPR groups at *p* value < 0.05.

but differed between GG and each of the other two genotypes (GA, $p < 0.001$; AA, $p = 0.012$; Figure 2A).

The PIADP of the three *CYP2C19*2* genotypes in the case and control groups was also compared, and the results are shown in Figure 2B and C, respectively. Compared with wild-type patients, the *CYP2C19*2* mutant allele carriers of both groups had lower PIADP, but the difference was only significant in the HTPR group ($p = 0.024$). Comparison between different genotypes showed that the PIADP difference between *CYP2C19*2* GG and AA genotypes in the HTPR group was significant ($p = 0.016$), while there was no significant difference between other genotypes ($p > 0.05$).

Discussion

Many studies have shown that compared with non-T2DM patients, T2DM patients have a higher incidence of HTPR, which will lead to a higher risk of adverse vascular events.[11,12,14–16] Therefore, it is of great significance to take strategies to predict and identify patients with HTPR, as they may benefit from other antiplatelet drugs to prevent ischemic events and improve clinical outcomes.

Although, some convincing genetic studies have demonstrated that *CYP2C19*2* mutant allele is associated with diminished metabolic activation of clopidogrel, decreased drug responsiveness, and increased risk of adverse vascular events in clopidogrel-treated patients, the clinical application of *CYP2C19* genotyping remains controversial.[22,27–29] Importantly, these early studies focused on patients with coronary heart disease rather than stroke, especially those with IS and T2DM. To the best of our knowledge, this was the first study to investigate patients with IS complicated by T2DM. We aimed to

Study population	Allele	HTPR No. of patients (%)	Non−HTPR No. of patients (%)	OR (95% CI)	*p* value
IS					
	*CYP2C19*2* A carriers	119 (55.87)	111 (52.11)	1.16 (0.79−1.7)	0.437
	*CYP2C19*3* A carriers	25 (11.74)	22 (10.33)	1.15 (0.63−2.12)	0.643
	*CYP2C19*17* T carriers	7 (3.29)	10 (4.69)	0.69 (0.26−1.85)	0.458
IS complicated by T2DM					
	*CYP2C19*2* A carriers	126 (59.15)	91 (42.72)	1.94 (1.32−2.85)	0.001
	*CYP2C19*3* A carriers	27 (12.68)	25 (11.74)	1.09 (0.61−1.95)	0.767
	*CYP2C19*17* T carriers	7 (3.29)	12 (5.63)	0.57 (0.22−1.48)	0.241

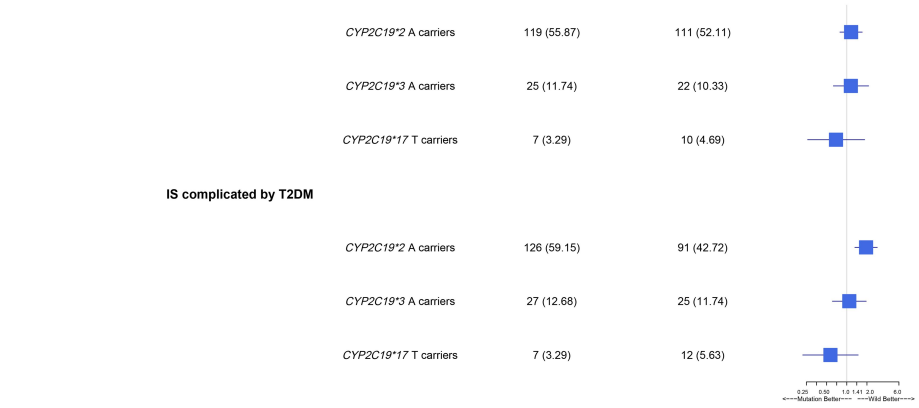

Figure 1 Association between *CYP2C19* genotypes and the risk of HTPR in IS patients with or without T2DM. Carriers were defined as patients with at least one mutant allele.

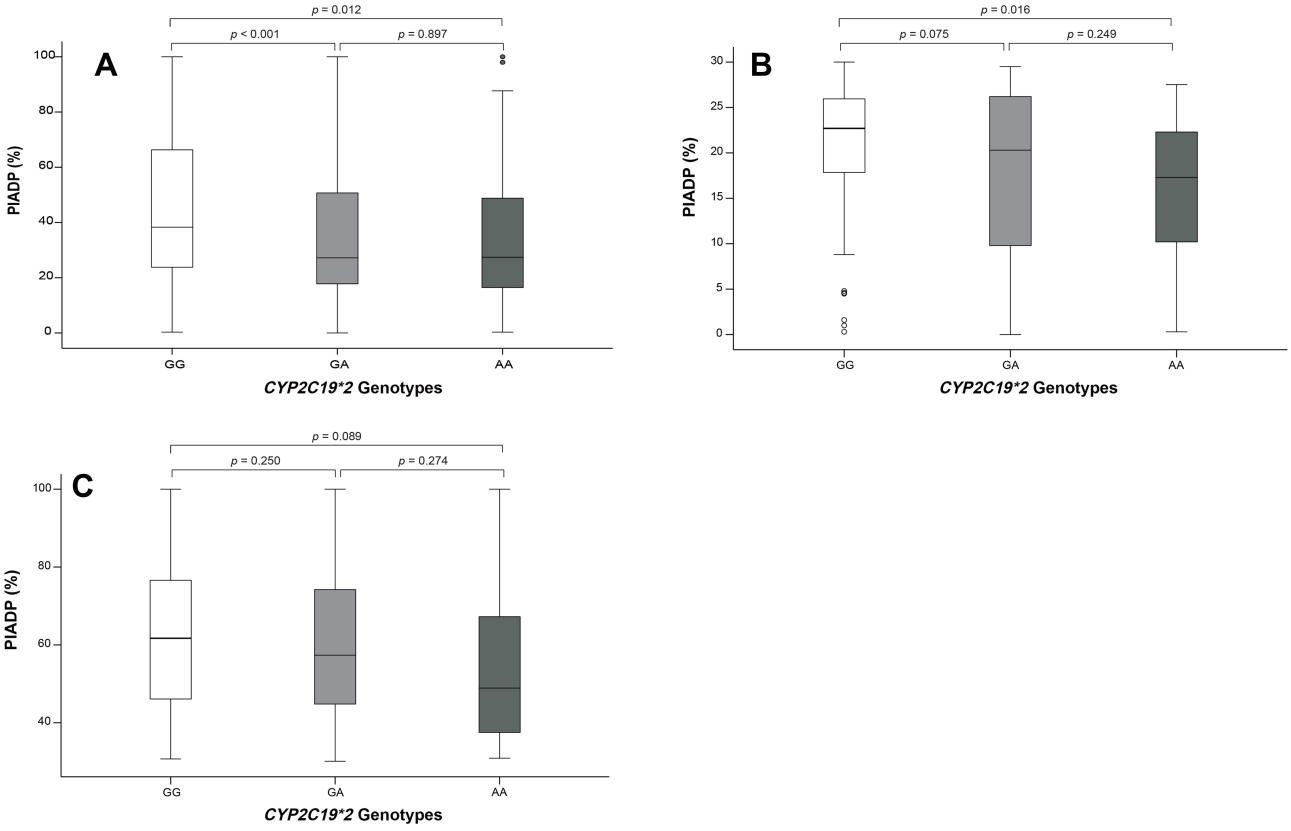

Figure 2 Box plots of ADP-induced platelet inhibition for each *CYP2C19**2 genotype in the entire subject (**A**, n = 426), HTPR group (**B**, n = 213), and non-HTPR group (**C**, n = 213).

clarify the association between *CYP2C19* gene polymorphisms and the antiplatelet efficacy of clopidogrel in this population.

In the present study, we observed that the frequencies of *CYP2C19*2* and **3* mutant alleles in the Chinese Han population were consistent with those reported by Yan et al (30.38% and 7.08%) and were higher than those reported in Swedish Caucasians and Ethiopians.[18,26] The frequency of *CYP2C19*17* T allele in our cohort was lower than that in other races, confirming that allelic

variants exhibit ethnic and geographic diversity.[18] The genetic distribution of *CYP2C19* polymorphisms was similar in T2DM and non-T2DM patients, indicating that there might be no direct correlation between *CYP2C19* genetic variants and the occurrence of T2DM. This was also confirmed by Semiz et al.[30]

Correlation analysis showed that the carriage of *CYP2C19*2* mutant allele was a related factor for HTPR in T2DM patients, similar to the results of previous studies, but was not statistically significant in non-T2DM patients.[31,32] Therefore, routine *CYP2C19*2* genotyping could be recommended as a straightforward approach for optimizing clopidogrel utilization in patients with IS, especially those complicated by T2DM. Non-responsiveness prediction through genetic profiling will help these high-risk patients to propose strategies to modify clopidogrel doses or use alternative antiplatelet drugs such as ticagrelor to prevent recurrent ischemic events and improve clinical outcomes.

Chan et al revealed that the carriage of *CYP2C19*3* and **17* mutant alleles was associated with HTPR in different directions.[33] *CYP2C19*3* reduced clopidogrel activation and was a positive predictor of HTPR. In contrast, *CYP2C19*17* enhanced clopidogrel activation and was inversely related to HTPR. However, in this study, no significant correlation was found between *CYP2C19*3* or **17* and the occurrence of HTPR. The difference may be related to their low gene distribution frequencies.

In this study, TEG was applied to test platelet function. Compared with the gold standard light transmittance aggregometry (LTA) method, TEG has the advantages of convenient operation and good reproducibility, and can provide comprehensive information about the coagulation cascade reaction. In addition, we used PCR-RFLP method to detect *CYP2C19* polymorphisms. Compared with the gold standard Sanger sequencing method, it has the advantages of high throughput and simple operation.

This study had several limitations. First, in this study, the bias of other genetic polymorphisms involved in the oxidative activation of clopidogrel, such as *CYP1A2*, *2B6*, *3A*, and *2C9*, was not excluded. Secondly, all participants in this study were patients over 18 years old, so the findings of this study might not be applicable to patients under 18 years old. Again, in a recently published article, TEG was reported to be not quite suitable for clopidogrel effect detection.[34] However, our platelet function was detected by TEG, but not confirmed by other methods. Finally, we did not collect follow-up data on MACE in the enrolled patients, so we could not provide direct evidence of the impact of *CYP2C19* polymorphisms on clinical outcomes.

Conclusion

In this study, we investigated the association between *CYP2C19* polymorphisms and HTPR in clopidogrel-treated Chinese Han patients. Results indicated that *CYP2C19*2* A allele was a related factor of HTPR events in IS patients with T2DM, but not in patients without T2DM. To validate our findings, future studies with larger sample sizes, more genetic variants, more comprehensive subgroup design, better reliability confirmation of results, and appropriate follow-up are necessary. The current results may help predict the situation of HTPR in different patient populations, guide the rational use of drugs, and reduce the incidence of adverse vascular events.

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Disclosure

The authors report no conflicts of interest in this work.

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