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Original Article

The effects of CES1A2 and CYP2C19 polymorphisms on responsiveness to clopidogrel and clinical outcomes among Chinese patients with acute ischemic stroke

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Abstract: Little research about genotype and clopidogrel response related to acute ischemic stroke has been published. The study evaluated the gene polymorphisms involved in the metabolic process affecting clopidogrel response and acute ischemic stroke clinical outcomes. We evaluated 191 non-cardioembolic ischemic stroke (NCIS) patients treated with clopidogrel. The platelet reactivity with clopidogrel treatment for 5 days was measured using thrombelastography platelet mapping assay. CES1A2 A (-816) C and CYP2C19*2/*3 were screened by the Ligase Detection Reaction. The primary endpoint was the composite of vascular death, stroke recurrence and myocardial infract. The associations between the genotype and the primary endpoint were evaluated. The rate of platelet inhibition in patients with homozygous (CC) of CES1A2 A (-816) C was significantly lower than that in heterozygous (AC) and wild-type genotype (AA) in co-dominant model (P = 0.021); similar result was observed for CYP2C19 loss of function (LOF) alleles carriers (P = 0.015). The risk of primary endpoint increased with the presence of the CES1A2 (recessive model) and CYP2C19 LOF alleles (dominant model) during the mean follow-up of 9.5 months (P = 0.023 and P = 0.028, respectively). The two gene polymorphisms were independent prognostic factors for clinical outcomes in the final multivariate Cox regression model. Both the CES1A2 and CYP2C19 polymorphisms may be associated with platelet reactivity with clopidogrel treatment and increased the risk of vascular events in Chinese patients.

Keywords: Ischemic stroke, genetic polymorphism, clopidogrel, CYP2C19, CES1A2 A (-816) C

Introduction

Clopidogrel treatment is recommended for prevention of stroke recurrence in non-cardioembolic ischemic stroke (NCIS) patients [1]. However, clinical practice suggested that a fraction of patients still developed vascular events disregarding regular clopidogrel treatment [2]. In recent years, genetic variants of genes encoding enzymes involved in the clopidogrel metabolic process have been proposed to be related to the different individual response to clopidogrel [3]. The genetic variants involved in the pharmacokinetics of clopidogrel have been investigated extensively in coronary heart disease patients. Some studies revealed that the poor response to clopidogrel was related to CYP2C19 and CES1A2 gene polymorphisms [4-6].

The researches about gene polymorphisms in clopidogrel responsiveness among Chinese stroke patients are relatively limited [7]. Thus, our study was to investigate the effect of CES1A2 and CYP2C19 polymorphisms on platelet reactivity to clopidogrel and clinical outcomes in Chinese Han ischemic stroke patients.

Material and methods

Study population

The study protocol was approved by the Medical Ethical Review Board of Jinling hospital

(Nanjing, China). Written informed consent was obtained from each subject. Between Feb 2012 and Feb 2014, consecutive acute ischemic stroke patients were enrolled from Nanjing Stroke Registry Program (NSRP) [8]. Ischemic stroke subtypes were determined according to the TOAST criteria [9] by neurologists blinded to the platelet inhibition rate and genotype. The inclusion criteria were as follows: clinical diagnosis of acute cerebral infarction within 7 days after stroke onset; aged 35 years or older; with head magnetic resonance imaging or computerized tomography scan; Chinese Han ethnicity; treated with clopidogrel at time of enrollment. Exclusion criteria were: exposure to thienopyridine or glycoprotein IIb/IIIa inhibitor within one week; allergy to clopidogrel; atrial fibrillation; oral anticoagulation therapy; National Institutes of Health Stroke (NIHSS) score was > 15 (severe NCIS); serious kidney or liver disorders; hematological disease; malignancies; active bleeding; major bleeding or intracranial hemorrhage within 3 months; autoimmune disease; platelet count < 100×10^9/L or > 500×10^9/L; hemorrhage transformation after cerebral infarction.

Blood sampling and platelet function assay

Blood samples for platelet reactivity testing were obtained from ulnar vein after clopidogrel treatment for more than 5 days. The blood was collected in vacuum tubes contained lithium heparin and trisodium citrate. The tubes were then inverted three times to ensure the blood was completely mixed with the anticoagulant. The platelet reactivity was determined using a Thrombelastograph (TEG) Analyzer (Haemoscope Corp, Niles, USA) according to the manufacturer's instructions [10]. Inhibitory rate of platelet aggregation was calculated with a computer software using the following formula: % inhibition = $[(MA_{Thrombin}-MA_{ADP})/(MA_{Thrombin}-MA_{Fibrin})] \times 100$. $MA_{Thrombin}$ is the thrombin-induced clot strength, representing the platelet maximal potential reactivity. MA_{ADP} is the ADP-induced clot strength, reflecting the contribution of platelets not inhibited by clopidogrel. MA_{Fibrin} is the fibrin-induced clot strength, representing the contribution of fibrin alone to clot strength.

Genotyping

Genotyping were determined by improved Multiple Ligase Detection Reaction (iMIDR) [11], with technical support from Center for Human Genetic Research, Shanghai Genesky Biotech Co., Ltd. The technicians performing genotyping were blinded to the platelet inhibition rate and clinical outcome. About 10% samples were selected randomly for repeated assay, and the results were 100% concordant.

Primary endpoint and clinical follow-up

The primary endpoint was a composite of ischemic stroke, vascular death and myocardial infarction. Ischemic stroke was defined as focal neurologic deficit at least lasting twenty-four hours or new ischemic lesion found through CT or MRI scan. Vascular death indicated any vascular-cause mortality. Myocardial infarction (MI) was diagnosed according to the third universal definition of MI, which requires a rise cardiac troponin plus either ischemia symptoms or electrocardiographic changes [12]. Follow-up was done for each subject at three months after enrollment via outpatient visit or telephone interview, with a detailed report of drug treatment. We excluded those who changed anti-platelet regime or disruption antiplatelet agents. The subjects were further evaluated at 6, 12, 24 months after enrollment. The endpoint was adjudicated by a physician unaware of the genotype and platelet function measure of the patients. Follow-up was censored when the primary endpoint was identified, clopidogrel discontinued, or the follow-up completed. The final follow-up date was December, 2014.

Statistical analyses

The relationship between platelet inhibition rate and genotype in codominant model was analyzed using a one-way analysis of variance, followed by the least significant difference or Dunnett's T3 test for post-hoc multiple comparison. Cumulative risk of the primary endpoint according to different genotypes was presented with Kaplan-Meier survival curve, and compared with log-rank test. Multivariable Cox proportional hazards regression model was applied to explore independent risk factors for primary endpoint. The statistical analyses were performed by the SPSS 22.0 software.

Results

Characteristics of the study population

A total of 191 patients were included in the present study after excluding those who

Table 1. Characteristics of the patients (n = 191)

Characteristics	Value
Age (years)	61.5 ± 10.5
Male (n, %)	128 (67%)
Height (cm)	166.8 ± 7.3
Weight (kg)	68.2 ± 10.4
BMI (kg/m^2)	24.5 ± 3.0
NIHSS	3.9 ± 3.4
Risk factors	
Diabetes mellitus (n, %)	71 (37.2%)
Hypertension (n, %)	121 (63.4%)
Smoker (n, %)	97 (50.8%)
Dyslipidemia (n, %)	77 (40.3%)
Medications	
ACEI or ARB (n, %)	38 (19.9%)
CCB (n, %)	68 (35.6%)
Beta-blocker (n, %)	14 (7.3%)
Stains (n, %)	182 (95.8%)
PPI (n, %)	10 (5.2%)
Laboratory parameters	
Platelet count (×10^9/L)	198.9 ± 54.5
PT (S)	11.6 ± 1.1
APTT (S)	28.4 ± 4.7
Creatinine (μmol/L)	70.0 ± 19.8

BMI: body-mass index; NIHSS: National Institutes of Health Stroke Scale; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker; CCB: calcium-channel blocker; PPI: proton pump inhibitor; PT: prothrombin time; APTT: activated partial thromboplastin time.

Table 2. The inhibition rate of platelet aggregation (%) measured by TEG

Genotype	PIR (%)	*F* value	*P* value
CES1A2 A (-816) C		3.945	0.021
AA	55.0 ± 23.0		
AC	54.7 ± 26.7		
CC	30.3 ± 12.2		
CYP2C19*2/*3		4.278	0.015
EM	59.3 ± 23.2		
IM	51.8 ± 25.4		
PM	44.5 ± 22.6		

PIR: platelet inhibitor rate; EM: extensive metabolizers; IM: intermediate metabolizers; PM: poor metabolizers. (AA vs. CC, *P*=0.001; AC vs. CC, *P*=0.001; EM vs. IM, *P*=0.049; EM vs. PM, *P*=0.007).

changed anti-platelet agent and were lost to follow-up. Of the 191 patients, the mean age was 61.5 ± 10.5 years old, and the mean NIHSS score was 3.9 ± 3.4. One hundred and twenty-eight patients (67.0%) were males, 63.4% with hypertension and 37.2% with diabetic mellitus (DM) (**Table 1**).

Genotypes and platelet inhibition rate

The frequencies of the GG (wild-type homozygote), GA (heterozygote), and AA (mutant homozygote) genotypes for CYP2C19*2 were 85 (44.5%), 90 (47.1%), and 16 (8.4%), respectively. With regard to CYP2C19*3, 172 (90.1%) patients were GG (wild type), 19 (9.9%) as GA (heterozygote). The percentage of AA, AC and CC for CES1A2 A (-816) C was 56.5%, 39.3% and 4.2%, respectively. The rate of platelet inhibition in patients with homozygote (CC) of CES1A2 A (-816) C was significantly lower than that in heterozygote (AC) and wild-type genotype (AA) in co-dominant model (*P* = 0.021) (**Table 2**; **Figure 1A**). The inhibition rate decreased according to the CYP2C19 genotype (59.3 ± 23.2%, 51.8 ± 25.4%, and 44.5 ± 22.6%) in the EM (extensive-metabolizers, non-carrier), IM (intermediate-metabolizers, one *2 or *3 alleles) and PM (poor-metabolizers, two *2 or *3 alleles), *P* = 0.015) (**Table 2** and **Figure 1B**).

The gene polymorphisms and clinical endpoint

During the follow-up period, ischemic stroke was observed in 18 (9.4%) patients, vascular death was observed in 6 (3.1%) patients, and myocardial infarction was observed in 2 (1.0%) patients. A total of 26 (13.6%) patients experienced the primary endpoint.

For CYP2C19, primary endpoint was observed in 5 (6.5%) of 77 patients with EM genotype, in 15 (17.2%) of 87 patients with IM genotype, and in 6 (22.2%) of 27 patients with PM genotype. With regard to CES1A2A (-816) C, primary endpoint was observed in 23 (12.6%) of 183 patients with (AA+AC) genotype, and in 3 (37.5%) of 8 patients with CC genotype. Kaplan-Meier analysis showed that the risk of experiencing primary endpoint was increased in CES1A2A (-816) C homozygote carriers and CYP2C19 LOF (at least one LOF) allele carriers during the follow-up (*P* = 0.023 and 0.028, respectively; (**Figures 2**, **3**). Multivariable Cox regression analysis showed that the adjusted risk of primary endpoint for patients with

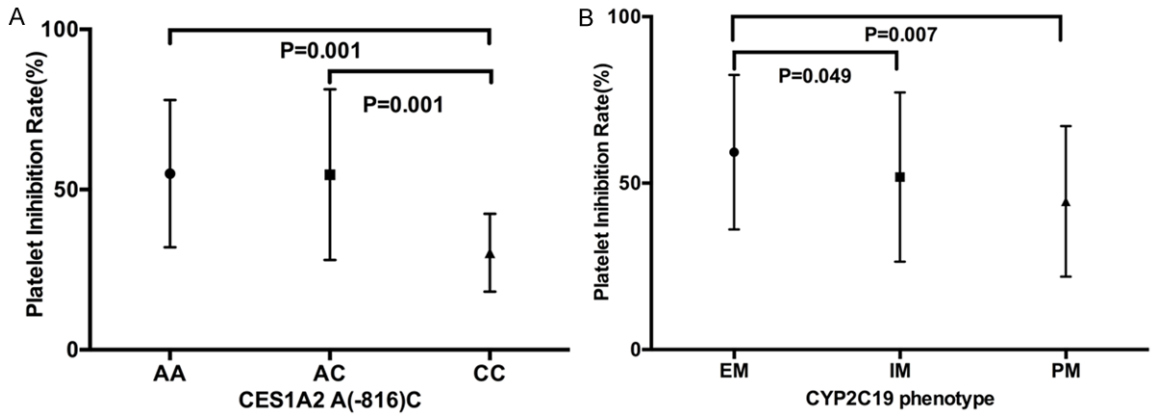

Figure 1. Platelet inhibition rate measured by thrombelastograph and grouped according to the CES1A2 A (-816) C polymorphism (A) and CYP2C19 genotype (B). Differences in the mean PIR were tested for significance using one-way ANOVA, followed by the least significant difference post-hoc test (CYP2C19 genotype) or Dunnett's T3 Test (CES1A2 A (-816) C polymorphism). EM: extensive-metabolizers; IM: intermediate-metabolizers; PM: poor-metabolizers; PIR: platelet inhibition rate.

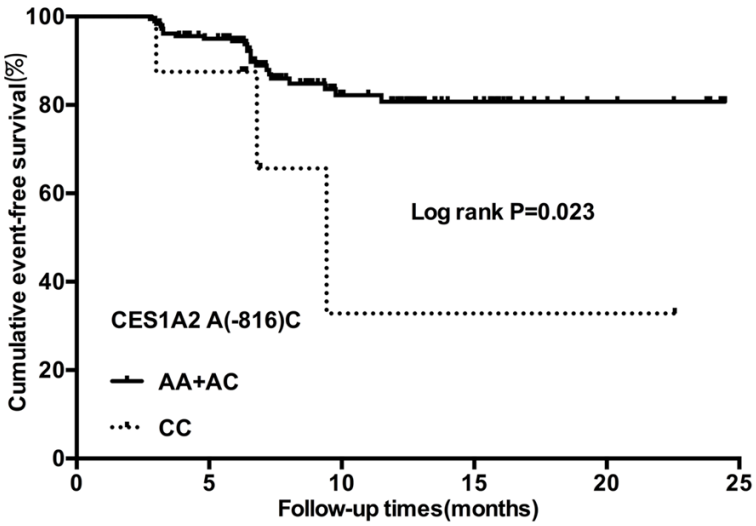

Figure 2. Kaplan-Meier survival curves for the primary endpoint according to the CES1A2 A (-816) C genotypes (AA+AC vs. CC).

CES1A2A (-816) C homozygote was 3.536-fold higher than those with the (AA+AC) genotypes (95% CI: 1.049-11.915, P = 0.042), while patients with CYP2C19 LOF allele had a 2.907-fold higher risk than non-carriers (95% CI: 1.095-7.718, P = 0.032; **Table 3**).

Discussion

Our results revealed that the CES1A2A-816C allele was associated with a higher degree of on-treatment platelet reactivity. Meanwhile, the CYP2C19 LOF alleles decreased the inhibitor rate of platelet aggregation.

CES1 acted on the primary metabolic steps of clopidogrel inactivation by catalyzing the formation of a pharmacologically inactive metabolite. Lewis et al. [13] reported that the CES1A1 (G143E) polymorphism significantly increased plasma concentrations of the active metabolite of clopidogrel. Zhu et al. [14] also reported that the defects in CES1 catalytic activity resulting from CES1 gene mutation might be associated with higher plasma contents of the active clopidogrel metabolite. Xie et al. [6] reported that the SNP in the promoter of CES1A2 attenuated the platelet reactivity on clopidogrel treatment among Chinese patients with coronary heart disease. The present study also demonstrated that the gene mutation of CES1A2 A (-816) C was associated with decreased platelet inhabitation rate on clopidogrel response among Chinese patients with non-cardioembolic ischemic stroke. It may increase the metabolic capacity of the esterase-mediated pathway, thereby reducing the content of the active metabolite and resulting in decreased platelet inhibition ratio to clopidogrel treatment. It is also suggested that the association between CES1A2 gene polymorphism and clop-

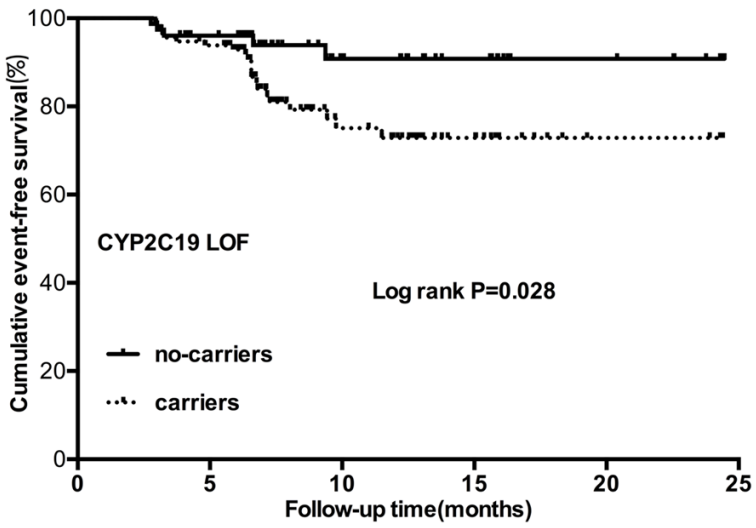

Figure 3. Kaplan-Meier survival curves for the primary endpoint according to the CYP2C19 LOF genotypes.

Table 3. Multivariable Cox regression analysis of factors associated with the primary endpoint

Variable	Beta	Hazard ratio	95% *CI*	*P* value
CES1A2 A (-816) C	1.263	3.536	1.049-11.915	0.042
CYP2C19 LOF	1.067	2.907	1.095-7.718	0.032
Age	0.048	1.050	1.007-1.094	0.021
Gender	0.103	1.108	0.469-2.620	0.815
Hypertension	-0.297	0.743	0.324-1.703	0.482
Diabetes mellitus	0.367	1.443	0.580-3.590	0.431
BMI (> 26 kg/m^2)	0.194	1.214	0.447-3.298	0.704

BMI: body mass index; CI: confidence interval.

idogrel response is common for the patients with different vascular beds diseases. Our results were consistent with previously published data that revealed CYP2C19 LOF alleles decreased the pharmacodynamics response to clopidogrel [4]. The effect of the CYP2C19 LOF alleles has been proved by many other studies [15].

There are strong links between CYP2C19 genetic mutation and adverse clinical outcomes in coronary heart disease [17-20]. With regard to ischemic stroke, Jia et al. [21] reported that the CYP2C19 genotypes significantly impacted the prognosis of patients with stroke. Sun et al. reported that the CYP2C19 LOF carriers could increase the recurrent risk among ischemic stroke patients treated with clopidogrel [22]. Similar to previous studies, the present study provided new evidences that CYP2C19 LOF allele may increase risk of vascular events in ischemic stroke patients treated with clopidogrel. Our results may be indicative for changing antithrombotic ways to prevent recurrent stroke in Chinese populations. Meanwhile, we also found that the presence of the CES1A2 A (-816) C homozygous mutation was associated with an increased risk of vascular events. Xie et al. [6] also observed that the frequency of the CES1A2 A (-816) C allele was higher in the stent thrombosis patients compared with the control group. However, the difference was not statistically significant. The inconsistent results may be due to the different diseases (non-cardioembolic ischemic stroke vs. CHD) and primary endpoints (vascular events vs. stent thrombosis). Studies with larger sample size are warranted to further investigate this relation and to better clarify whether this polymorphism is correlated with the vascular events in ischemic stroke patients.

In addition to genetic factors, advanced age was also found to be an independent risk factor for vascular events. Cao et al. [23] also observed that advanced age was a risk factor for vascular events in ischemic stroke patients taking aspirin. The prevalence of ischemic stroke in our present study seemed to be higher than the previous clinical results. The possible explanation is that patients in our study had higher percentage of advanced age, hypertension, and large-artery atherosclerosis stroke [9].

There are several limitations in our study. First, this was a small-scale, short follow-up and single-center study. Second, the plasma levels of clopidogrel and its active metabolite in each patient were not detected. Finally, the patients in this study came from southeast China. This may limit the generalization of the conclusions to other ethnic populations. However, given the

significant difference in platelet inhibition rate and the primary endpoint, our results still have a high credibility and are worthy for validation by large trials.

Conclusion

The CES1A2 A (-816) C and CYP2C19 LOF alleles were associated with attenuated platelet inhibition rate to clopidogrel in non-cardioembolic ischemic stroke patients. Carriers of CYP2C19 LOF and CES1A2 A (-816) C may increase the vascular events risk in Chinese Han ischemic stroke patients treated with clopidogrel.

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Disclosure of conflict of interest

None.

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