European Journal of Pharmacology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Influence of CYP2C19 polymorphisms on platelet reactivity and clinical outcomes in ischemic stroke patients treated with clopidogrel

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ARTICLE INFO

Article history:
Received 27 September 2014
Received in revised form
28 November 2014
Accepted 28 November 2014

Chemical compounds studied in this article:
Clopidogrel (PubChem CID: 60606)
Adenosine diphosphate (PubChem CID: 6022)

Keywords:
CYP2C19
Genetic polymorphism
Clopidogrel
Platelet function tests
Flow cytometry
Ischemic stroke

ABSTRACT

CYP2C19 genetic polymorphisms influence clopidogrel response and clinical outcomes of cardiovascular disease. However, data on their relationship in stroke patients are scarce. We aimed to investigate the influence of CYP2C19 polymorphisms on platelet reactivity and clinical outcomes in ischemic stroke patients treated with clopidogrel. A total of 211 patients were enrolled. All patients were given clopidogrel treatment and underwent CYP2C19 genotyping and platelet function testing by flow cytometry including adenosine diphosphate-induced platelet aggregation (ADP-PAg) and platelet activation markers (PAC-1, CD62P and CD63). The modified Rankin Scale (mRS) was used and ischemic events were evaluated. A total of 129 (61.1%) of the 211 enrolled patients were carriers of CYP2C19 loss-of-function (LOF) alleles (∗2, ∗3). After clopidogrel therapy for 7 days, the levels of ADP-PAg, PAC-1, CD62P and CD63 were higher in carriers than noncarriers. CYP2C19 carriage was associated with more frequent high residual platelet reactivity. CYP2C19 polymorphisms alone could explain 12.9%, 4.3%, 8.9% and 5.5% of the inter-individual variability of ADP-PAg, PAC-1, CD62P and CD63 after clopidogrel treatment, respectively. At 6-month follow-up, 38 (19%) patients were scored poor prognosis and 15 (7.6%) ischemic events were observed. Carriers had poorer prognosis than noncarriers ($P=0.025$). No significant association of CYP2C19 carriage with ischemic events was found. Multiple regression analysis showed that CYP2C19 carriage was an independent predictor of poor prognosis (odds ratio, 3.01; 95% confidence interval, 1.23–7.38; $P=0.016$). In conclusion, carriage of the CYP2C19 LOF allele has significant influence on clopidogrel response and prognosis in patients with ischemic stroke.

1. Introduction

Platelet activation and aggregation play pivotal roles in the onset of ischemic stroke (Freynhofer et al., 2012; Tohgi et al., 1991). Elevated platelet activation and aggregation level lead to thrombin generation and vascular injury, which contribute substantially to the pathophysiology of ischemic stroke. Clopidogrel is a prodrug requiring biotransformation to form active metabolites, which act by irreversibly inhibiting the platelet $P2Y_{12}$ receptor and thus platelet aggregation. It is commonly used to regulate the activated platelet for secondary prevention of ischemic stroke (Jauch et al., 2013) and to prevent early recurrence (Kennedy et al., 2007). Clopidogrel was superior to aspirin with a relative risk reduction of 7.3% in ischemic stroke patients especially in high-risk subgroups in CAPPIE study (Committee, 1996). Although the efficacy of clopidogrel is clear, observational studies have demonstrated that there exists considerable variability in clopidogrel response and suboptimal response to clopidogrel can result in recurrent ischemic events in cardiovascular disease (Gurbel et al., 2007) and stroke (Bennett and Yan, 2013).

Numerous factors including clinical, cellular and genetic aspects contribute to poor response of clopidogrel (Campo et al., 2011b). Currently, the genetic polymorphism has been considered as the most important determinant (Campo et al., 2010b). The cytochrome P450 (CYP) 2C19 enzyme system, which contributes both of two oxidative metabolic steps of clopidogrel activation, had been reported to be a certain factor of the wide inter-individual variability

Abbreviations: ADP-PAg, adenosine diphosphate-induced platelet aggregation; LOF, loss-of-function; mRS, the modified Rankin Scale; RPR, residual platelet reactivity; HRPR, high residual platelet reactivity

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http://dx.doi.org/10.1016/j.ejphar.2014.11.037
0014-2999/

of clopidogrel response (Mega et al., 2009). Carriers of at least one loss-of-function (LOF) allele of CYP2C19 (known as CYP2C19 *2 and CYP2C19 *3 alleles) have been reported to have lower levels of the active clopidogrel metabolism, reduced platelet inhibition and a higher prevalence of clinical outcomes (Brandt et al., 2007; Frere et al., 2008; Simon et al., 2009). While most studies were conducted in patients with cardiovascular disease, data on their relationship in stroke patients are scarce. In consideration of the poor clinical prognosis of stroke and high stroke recurrence (Antithrombotic Trialists' Collaboration, 2002), it merits considerable attention to clopidogrel response in stroke patients.

In the present study, we aimed to evaluate the association of CYP2C19 polymorphisms with clopidogrel response and clinical sequelae in clopidogrel-treated Chinese patients who suffered ischemic stroke.

2. Materials and methods

2.1. Study population

Between April 2012 and December 2013, a total of 211 patients were recruited from the Second Hospital of Tianjin Medical University, if they were admitted to hospital within a week after the symptom onset and were diagnosed as acute ischemic stroke by a neurologist. Exclusion criteria included: (1) treatment of anticoagulants, thrombolytic agents and other antiplatelet drugs within 2 weeks; (2) the presence of cranial bleeding or active hemorrhage; trauma, surgery; deep vein or arterial thrombosis within the preceding 3 months; (3) severe hepatic or renal dysfunction; malignant diseases; chronic inflammatory diseases or infectious conditions at study entry.

All patients enrolled were given clopidogrel (75 mg once daily) therapy, underwent CYP2C19 genotyping once and platelet function testing before and 7 days after the clopidogrel regimen. The genotyping and platelet function testing were performed by two independent groups, who were unaware of patient individual information and testing results of each other. The study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University and written informed consent was obtained from every patient before enrollment.

2.2. Platelet function testing

Blood samples for platelet function tests with flow cytometry were drawn using a standardized technique as previously described (Schmitz et al., 1998). Blood was anticoagulated with 3.8% sodium citrate (0.129 M/L in dilution 1:10). Platelets aggregation was assessed on a flow cytometer (FCL500MPL, Beckman Coulter, America) by stimulating with 20 μmol/L ADP (Sigma Biosciences, America). The peridinin chlorophyll protein (PerCP)-conjugated anti-CD61 (Becton Dickinson Biosciences, America) was applied as a general platelet marker to identify the platelets. After platelets in whole blood were stimulated with ADP for 5 min, the blood mixture was compounded with saturating concentration of anti-CD61 PerCP and incubated at room temperature in the dark for 15 min. Then the sample was fixed with 1% paraformaldehyde in phosphate buffered saline for 30 min. After immunolabeling and fixation, the samples were analyzed on the flow cytometer. Side light scatter and expression of CD61 were used to discriminate platelets from other blood cells. Then 5000 platelets were gated. The result was expressed as the percentage of aggregates in the CD61-identified platelets.

Platelet activation markers were determined using antibodies including fluorescein isothiocyanate (FITC)-labeled PAC-1 (activated glycoprotein IIb/IIIa receptors), phycoethrin (PE)-labeled antibodies agonist CD62P (P-selectin) or CD63 (lysosome-associated membrane protein) (Becton-Dickinson Biosciences, America). Arg-Gly-Asp-Ser (RGDS) tetrapeptide (Sigma Biosciences, America) and mouse-IgG1 (Becton-Dickinson Biosciences, America) were used as isotype-negative controls to define nonspecific binding. The anti-CD61 PerCP was used to identify the platelets. The process of incubation and fixation was as described above. Then the samples were analyzed on FCL500MPL. Platelets were discriminate from other blood cells by using side light scatter and expression of CD61. Results were expressed as percentage of antibody-positive platelets which exceeded 99% of the control platelets.

2.3. CYP2C19 genotyping

Genomic DNA was isolated using a commercially available DNA extractor kit (Cwbiotech, Beijing, China) according to the manufacturer's instructions. Polymerase chain reaction–restriction fragment length polymorphisms (PCR–RELPs) for the CYP2C19 LOF alleles on 2 polymorphic positions, *2 (681G > A, rs4244285) and *3 (636G > A, rs4986893), were performed as previously described (de Morais et al., 1994). The primers of CYP2C19 *2 and *3 were designed and synthesized by BGI Beijing Corporation (Table 1). Fast-digest restriction enzyme SmaI and BamHI (Takara biotechnology, Dalian, China) for CYP2C19 *2 and *3 was used. Random sampling from each genotype of CYP2C19 for direct DNA sequencing confirmed the genotyping results, and the results were 100% concordant. The CYP2C19 genotypes were classified into three subgroups: an extensive metabolizer (EM) carrying normal function alleles (CYP2C19 *1/*1), an intermediate metabolizer (IM) carrying one LOF allele (*1/*2 or *1/*3) and a poor metabolizer (PM) carrying two LOF alleles (*2/*2, *2/*3 or *3/*3).

2.4. Data collection and follow-up

Data collection and follow-up were completed by another independent group and were unaware of the genotypic and platelet function information. Clinical data were collected from all patients including demographic characteristics and stroke risk factors on admission. Follow-up was performed at the end of 6 months after enrollment by clinical visits or telephone interviews. The primary end point was defined as a composite of recurrence of ischemic stroke, nonfatal myocardial infarction and death. Ischemic stroke recurrence was diagnosed as a focal neurological deficit lasting more than 24 h with ischemic cerebral lesions confirmed by computed tomography (CT) or magnetic resonance imaging (MRI). Myocardial infarction was diagnosed when two of the following three criteria were met: typical symptoms; increased cardiac-enzyme levels; and diagnostic electrocardiographic changes. Cardiovascular death was defined as deaths occurring within 24 h after symptoms onset without other causes evidence (Lonn et al., 2006). Clinical follow-up was censored when any above endpoint was defined or 6-month follow-up finished. At the same time, the modified Rankin Scale (mRS) was used. A good prognosis was defined as mRS ≤ 2, while mRS > 2 was considered as poor prognosis.

Table 1
The primer sequences of CYP2C19 *2 (G681A) and *3 (G636A).

Alleles	Primer sequences
CYP2C19 G681A	F 5′-ACCAGAGCTTGGCATATTGTATCT-3′ R 5′-GATTCTTGGTGTTCTTTTACTTTCT-3′
CYP2C19 G636A	F 5′-TTTCATCCTGGGCTGTGCTC-3′ R 5′-TGTACTTCAGGGCTTGGTCAAT-3′

Abbreviations: F, forward; R, reverse.

Please cite this article as: Qiu, L.-N., et al., Influence of CYP2C19 polymorphisms on platelet reactivity and clinical outcomes in ischemic stroke patients treated with clopidogrel. Eur J Pharmacol (2014), http://dx.doi.org/10.1016/j.ejphar.2014.11.037