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Review Article

The Impact of Cytochrome P450 2C19 Polymorphism on Cardiovascular Events in Indonesian Patients with Coronary Artery Disease

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Abstract

Background: The polymorphism of cytochrome P450 2C19 (CYP2C19) has been documented as the determinant variability in the antiplatelet effect of clopidogrel. The relation between CYP2C19 polymorphism and the antiplatelet efficacy of clopidogrel in Indonesian patients with coronary artery disease (CAD) is unknown. To address this issue, we examined the distribution of CYP2C19 genotypes and platelet aggregation, and assessed the impact of CYP2C19 polymorphism on response to clopidogrel and cardiovascular events.

Methods: This observational analytic study with prospective cohort approach was conducted in Wahidin Sudirohusodo and Hasanuddin University Hospital, Makassar. We measured the CYP2C19 genotype by polymerase chain reaction-restriction fragment linked polymorphism (PCR-RFLP) method and platelet aggregation by optical platelet aggregometry with 10 µmol of adenosine diphosphate (ADP) in 69 patients with stable CAD who were treated with clopidogrel. Platelet hyperaggregation was defined as maximal platelet aggregation > 94.3%. The patients were followed up every month at the outpatient department for 6 months or at end point. The end point was acute myocardial infarction, ischemic stroke, or cardiovascular death.

Results: Distribution of CYP2C19 alleles were 89.8%, 40.6%, and 11.6%, in CYP2C19*1, CYP2C19*2, and CYP2C19*3, respectively. Distribution of CYP2C19 genotype were 50.7%, 29.0%, 8.7%, 8.7%, and 2.9% in CYP2C19*1/*1, *1/*2, *1/*3, *2/*2, and *2/*3, respectively. Platelet hyper aggregation was more in patients with polymorphism than wild type (*p* 0.034; OR 3.707) and was associated with cardiovascular events (*p* 0.030; OR 13.250). There was acute myocardial infarction in 2 patients, ischemic stroke in 1 patient, and cardiovascular death in 1 patient. All of these patients were carrying at least one variant allele of CYP2C19; details of genotype were in two patients with CYP2C19*1/*2, one patient with *2/*2, and one with *2/*3 alleles.

Conclusion: CYP2C19*2 and *3 were associated with cardiovascular events due to platelet hyper aggregation.

Keywords: Polymorphism, CYP2C19, Clopidogrel, Coronary artery disease, Indonesia.

Introduction

Cardiovascular diseases (CVDs) are the number one cause of death globally; exceeding other diseases. An estimation of 17.3 million people died from CVDs in 2008, representing 30% of all global death [1]. Of these, an estimated 7.3 million were due to coronary artery disease (CAD) [2].

Reperfusion of the coronary arteries is the first-line treatment in ischemic heart disease. The treatment methods are the administration of fibrinolytic drugs, percutaneous coronary intervention (PCI), or coronary artery bypass surgery (CABG). Indeed, PCI is the treatment of choice for acute myocardial infarction (MI), if it is done on time [3,4].

However, stent thrombosis can occur after stent placement. Many studies have been conducted on the prevention of stent thrombosis with antiplatelet therapy. The American Society of Cardiology (ASC) guideline has recommended the administration of clopidogrel, in combination with aspirin, in patients using bare metal stent (BMS) for at least one month and up to 12 months in recent studies [5] and in patients using drug-eluting stent (DES) for at least 12 months [6].

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Clopidogrel is a pro-drug agent that, after becoming an active metabolite, selectively blocks ADP dependent platelet activation and aggregation. The drug requires the enzyme cytochrome P450 2C19 (CYP2C19) function for its activation and antiplatelet effect. In some patients, clopidogrel has no antiplatelet effect or its effect is reduced. The responsiveness to clopidogrel is determined by genetic and acquired factors, and is one of the important factors in stent thrombosis and cardiac events after the stent placement in patients with CAD.[7] Single nucleotide polymorphism of CYP2C19 that reduce the activity of this enzyme are among the causes of racial differences in response to the antiplatelet effect of clopidogrel [7]. The presence of CYP2C19*2 allele decreases the response to antiplatelet effects of clopidogrel [8]. To date, the effect of the CYP2C19 polymorphism on antiplatelet effect of clopidogrel and cardiovascular events in Indonesian patients with CAD remains unknown.

Methods

We conducted prospective cohort study in Wahidin Sudirohusodo and Hasanuddin University Hospital, Makassar, South Sulawesi, Indonesia from September 2013 until September 2014. Ethical approval was obtained from the ethics committee of Hasanuddin University and informed consent was obtained from all participants.

Study Population

We studied 69 patients of angiographically proven CAD taking clopidogrel. Patient with acute coronary syndrome received anticoagulant, or antiplatelet agents other than clopidogrel or aspirin were excluded.

Genotyping

Genotyping of CYP2C19 alleles (CYP2C19*1, CYP2C19*2, and CYP2C19*3 alleles) was carried out by polymerase chain reaction-restriction fragment linked polymorphism (PCR-RFLP) technique [9]. Reaction mixture for PCR with 30 µL KAPA Taq DNA Polymerase that consist of 0.6 U Taq Polymerase, dNTPs 0.1 mM, in PCR Buffer 1X (500 mM Tris/HCl pH 8.3, 100 mM KCl, 50 mM $(NH_4)_2SO_4$), and 1.5 mM $MgCl_2$, added with 0.4 µM forward and reverse primer, and 6 µL DNA template (DNA extract).

There were 2 master mix for each sample, that consist of primer set *2 and *3. The PCR amplification was performed with Thermal Cycler Verity (Applied Biosystem) (Table 1).

PCR product was detected by electrophoresis method with agarose gel 2% and Ethidium Bromide staining. Bands were detected by a short wavelength UV transluminator with Gel Doc (BioRad). The results were band 321 bp for CYP2C19*2 and 271 bp for CYP2C19*3. The example of electrophoresis gel result is shown in Figure 1.

Digestion of the CYP2C19*2 amplicon with *Sma*I resulted in products of 212 and 109 bp (homozygous wild type; c.681 G/G); 321, 212 and 109 bp (heterozygote; G/A); and a single undigested product of 321 bp (homozygous *2; A/A). Digestion of the CYP2C19*3 amplicon with *Bam*HI resulted in products of 175 and 96 bp (homozygous wild-type; c.636 G/G); products of 271, 175 and 96 bp (heterozygote *3; G/A); and a single undigested product of 271 bp (homozygous *3; A/A), as shown in figures 2 and 3.

Reaction Activity	Temperature (°C)	Time (second)	Cycle
Enzyme Activation	95	120	1
Denaturation	95	20	37
Annealing	53	10	
Extention	72	10	
Conditioning	72	300	1

Table 1: The PCR amplification for CYP2C19*2 and CY2C19*3.

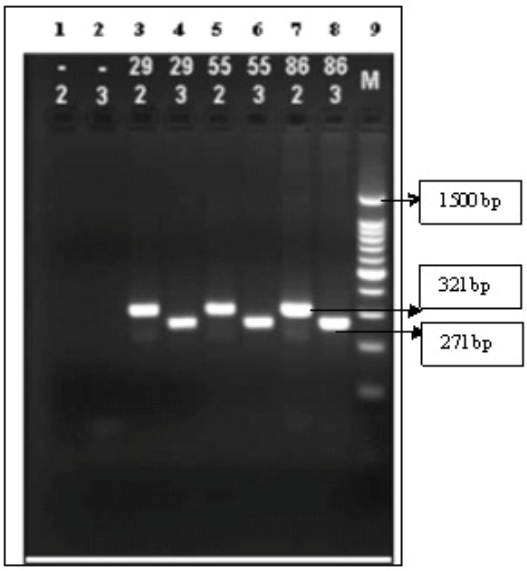

Figure 1: Electrophoresis gel shown: 1. CYP2C19*2 negative control; 2. CYP2C19*3 negative control; 3. CYP2C19*2 positive sample; 4. CYP2C19*3 positive sample; 5. CYP2C19*2 positive sample; 6. CYP2C19*3 positive sample; 7. CYP2C19*2 positive sample; 8. CYP2C19*3 positive sample; 9. Marker 100 bp (base pair).

Platelet Aggregation Measurement

Platelet aggregation was measured by optical platelet aggregometry with AggRam platelet aggregometer; Helena Biosciences Europe). This procedure is performed on a turbidimetric aggregometer, as first described by Born [10]. The change in absorbance is recorded as platelet rich plasma is stirred in a cuvette with aggregating reagents added. We used ADP as aggregating reagent with concentration 10 µM. The platelet aggregation reference value was 66.9 – 94.3%. Platelet hyperaggregation was defined as as maximal platelet aggregation more than 94.3%. The subject s were divided into two groups, (1) hyperaggregation group (n = 15) and (2) non-hyperaggregation group (n = 54).

Clinical Outcomes

The patients were followed up every month at the outpatient

department for 6 months or at end point. The end point was acute myocardial infarction, ischemic stroke, or cardiovascular death.

Statistical Analysis

Continuous variables are expressed as means ± SD. Categorical variables are expressed as frequencies and percentages. Differences in classifications between two or more groups were evaluated using Fisher´s exact test or likelihood ratio. Statistical significance was $p < 0.05$. All statistical analyses were performed using SPSS 18 for Windows.

Results

Patient characteristics

Clinical characteristics of each group (hyper aggregation and non-hyper aggregation) are shown in Table 2. There were no significant differences in baseline characteristics between hyper aggregation and non-hyper aggregation groups.

CYP2C19 Alleles and Genotype

Distribution of CYP2C19 alleles were 89.8%, 40.6%, and 11.6%, in CYP2C19*1, CYP2C19*2, and CYP2C19*3, respectively as shown in figure 4. Distribution of CYP2C19 genotype were 50.7%, 29.0%, 8.7%, 8.7%, and 2.9% in CYP2C19*1/*1, *1/*2, *1/*3, *2/*2, and *2/*3, respectively as shown in figure 5.

CYP2C19 Polymorphism and Platelet Aggregation

There was more platelet hyper aggregation in patients with polymorphism than wild type (p 0.034; OR 3.707) as shown in table 3.

CYP2C19 Polymorphism and Cardiovascular Events

Cardiovascular events in patient within study population were:

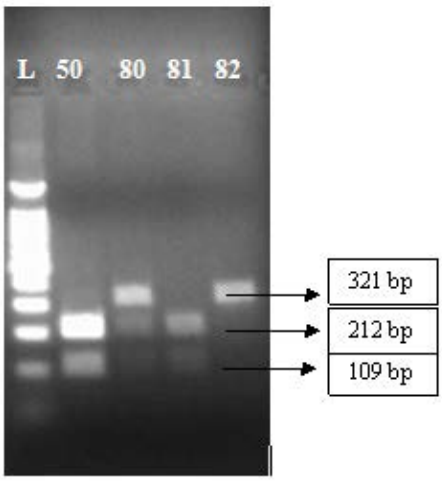

Figure 2: PCR-restriction enzyme (SmaI digestion) fragmentation patterns on the agarose gel is stained by ethidium bromide for CYP2C19*2 from subjects representing *1/*1, *1/*2, *1/*1, and *2/*2 genotypes (from 2 to 5 wells, left to right on the agarose gel); well 1 is marker 100 base pair.

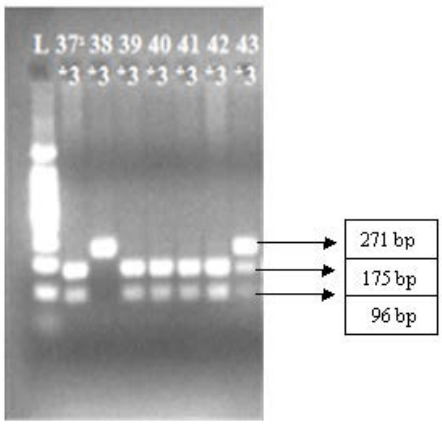

Figure 3: PCR-restriction enzyme (*Bam*HI digestion) fragmentation patterns on the agarose gel is stained by ethidium bromide for CYP2C19*3 from subjects representing *1/*1, *3/*3, *1/*1, *1/*1, *1/*1, *1/*1 and *1/*3 genotypes (from 2 to 8 wells, left to right on the agarose gel); well 1 is marker 100 base pair.

stroke, 1 patient; acute myocardial infarction, 2; ischemic stroke, 1 patient (Table 4). All of these patients were carrying at least one variant allele of CYP2C19; details of genotype were in two patients with CYP2C19*1/*2, one patient with *2/*2, and one patient with *2/*3 alleles (Table 5).

Platelet Aggregation and Cardiovascular Events

Hyperaggregation patients were associated with cardiovascular events than non-hyper aggregation group (p 0.030; OR 13.250) as shown in table 6.

Discussion

Our study found that CYP2C19*1 was most common than CYP2C19*2 and CYP2C19*3. Scott et al [11] have reported about frequencies of CYP2C19 alleles in African, American, European, East Asian, and South/Central Asiasn population; they found the similar result that CYP2C19*1 allele was most common than other alleles.

The other study in CAD patients also showed that CYP2C19*1 allele was most common than other allele. Tiong et al [12] showed that in 237 clopidogrel-treated patients among Malaysian multiethnic population, 63.0% were CYP2C19 *1, 29.0% were CYP2C19*2, 6.0% were CYP2C19*3, and 2% were CYP2C19*17. Study of Yamamoto et al in 246 CAD patients showed the frequency of CYP2C19*1, *2, and *3 were 58.9%, 30.9%, and 10.2% respectively.

There are several factors that can influence platelet aggregation. Age, smoking, diabetes, dyslipidemia, and ASA use are known as factors that can influence the platelet aggregation. Previous studies have reported that platelet activation increases with age. For example, Bastyr and colleagues demonstrated increased platelet phospholipid content, suggesting increases in transmembrane signalling with age [13]. It has also been shown that age is associated with an increase in platelet aggregability [14].

		Hyperaggregation (n)	Non-hyperaggregation (n)	p value*
Total: 69 patients		15	54	
Gender	Male	12	44	0.897
	Female	3	10	
Age (years)	< 50	5	10	0.35
	≥ 50	10	44	
BMI (kg/m²)	< 25	14	45	0.295
	≥ 25	1	9	
Ethnic	Buginese	9	26	0.415
	Non-Buginese	6	28	
Smoking status	Yes	9	37	0.540
	No	6	17	
Diabetes	Yes	7	14	0.132
	No	8	40	
Hypertension	Yes	7	27	0.891
	No	8	27	
Dyslipidemia	Yes	12	36	0.306
	No	3	18	
Coronary artery	1	7	31	0.460
with stenosis	> 1	8	23	
Statin use	Yes	5	20	0.791
	No	10	34	
ASA use	Yes	1	6	0.598
	No	14	48	
PPI use	Yes	2	4	0.492
	No	13	50	

BMI, body mass index; ASA, acetyl salicylic acid; PPI, proton pump inhibitor; *likelihood ratio

Table 2: Baseline Characteristics of Study Patients.

Kalliakmanis et al [15] reported that nicotine can inhibit vascular prostacyclin (PGI2). Prostacyclin is a prostaglandin member that can inhibit platelet aggregation. In diabetes patients, synthesis of PGI2 is decreased while prostaglandin E-like material is increased. Furthermore it will increase the synthesis f thromboxane and stimulate the platelet aggregation [16]. Hypercholesterolemia can enhance the ability of platelets to aggregate. Acetyl salicylic acid (ASA) can inhibit the formation of thromboxane A_2 in platelets, producing an inhibitory effect on platelet aggregation [17]. Likelihood ratio was performed to analyzed relationship between hyperaggregation and non-hyperaggregation group with some variables that can influence the platelet aggregation. The result was no significant differences between two groups as shown in table 2.

Clopidogrel is converted to an active thiol by the cytochrome P450 CYP 3A4 and 2C19 enzymes. Statins that metabolized by CYP3A4 suggested can attenuate the anti-aggregatory effect of clopidogrel. Analysis of relationship between concomitant use of statin and clopidogrel in this study showed that no significant differences between patient with- and without statin (p 0.791, table 2). This result showed that concomitant use of statin and clopidogrel did not influence the platelet antiaggregation effect of clopidogrel. Similar result have been found by Polena et al [18] that showed concomitant statins with clopidogrel therapy did not influence the effect of clopidogrel in platelet aggregation inhibition.

We also found that there were no significant differences between patient with- and without PPI use (Table 2). Competition with PPI with shared metabolization by CYP2C19 may diminish antiplatelet function of clopidogrel. Attenuating effects on platelet response

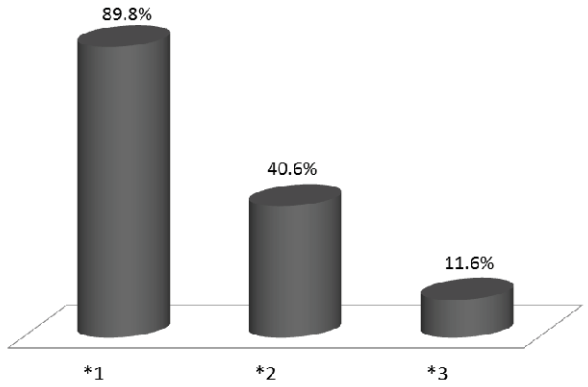

Figure 4: Distribution of CYP2C19 Alleles.

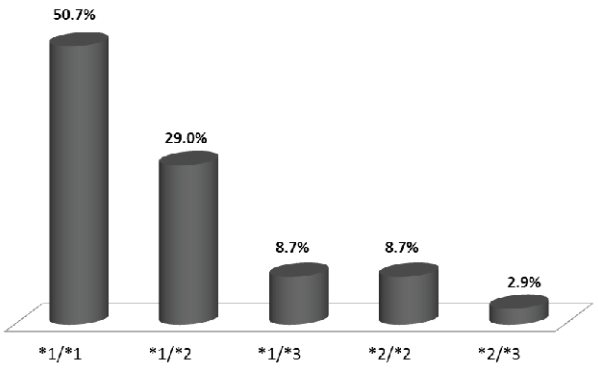

Figure 5: Distribution of CYP2C19 Genotype.

to clopidogrel have been reported solely for the PPI omeprazole. Sibbing et al [19] showed that concomitant use of clopidogrel and omeprazole associated with higher platelet aggregation compared than patient without omeprazole. But a meta-analysis showed that there were conflicting and inconsistent data on the interaction between clopidogrel and proton-pump inhibitors. The data were pooled and analysed by study design, but the substantial statistical, clinical, and methodological heterogeneity mean that it might not have been appropriate to pool the data [20].

The present study showed that patients which carrying at least one variant allele of CYP2C19 associated with 3-fold increased risk for platelet hyperaggregation (Table 3). Mega et al [21] studied 162 healthy subjects that included from six studies involving clopidogrel treatment. Polymorphic CYP enzymes tested –2C19, 2C9, 2B6, 3A5, and 1A2. The result showed that carriers of at least one CYP2C19 reduced-function allele had a relative reduction of 32.4% in plasma exposure to the active metabolite as compared to noncarriers ($p < 0.001$). In addition, carriers of at least one CYP2C19 reduced-function allele had an absolute difference in reduction of maximal platelet aggregation (ΔMPA) in response to clopidogrel that was 9% less than noncarriers ($p < 0.001$), relative risk reduction of 25%. Trenk et al [22] investigated whether the loss of function CYP2C19 681G>A *2 polymorphism is associated with high (> 14%) residual platelet aggregation (RPA) on clopidogrel and whether high on-clopidogrel RPA impacts clinical outcome after elective coronary stent placement. They found that between *2 carriers and wild-type homozygotes, there was significant ($p < 0.001$) differences in the proportion of patients with RPA > 14%, both after loading (62.4% vs. 43.4%) and at pre-discharge (41.3% vs. 22.5%).

Our study also showed that patients with platelet hyperaggregation associated with 13-fold increased risk for cardiovascular events (Table 6). Sofi et al [23] studied 4564 patients with stable angina, chronic CAD, or ACS (meta-analysis) and showed that clopidogrel non-responsiveness associated with an increased risk of recurrent cardiovascular events (OR 3.58; 95% CI, 2.54-5.05 ($p < 0.00001$) after adjustment for heterogeneity.

	Hyperaggregation (n)	Non-hyperaggregation (n)	OR (95% CI)	p value*
Carrier	11	23	3.707 (1.046 – 13.134)	0.034
Wild type	4	31		

OR, odd ratio; CI, confidence interval, *Fisher's Exact Test (1-sided)

Table 3: Relation between CYP2C19 Polymorphism and Platelet Aggregation.

	Stroke		AMI		CV death		Total	
	n	%	n	%	n	%	n	%
Wild type	0	0	0	0	0	0	0	0
Carrier	1	2.9	2	5.9	1	2.9	4	11.8

AMI, acute myocardial infarction; CV, cardiovascular

Table 4: Cardiovascular Events in Study Population.

CYP2C19 Genotype	Events
*1/*2	AMI
*1/*2	Stroke
*2/*2	CV death
*2/*3	AMI

AMI, acute myocardial infarction; CV, cardiovascular

Table 5: Details of Cardiovascular Events in Study Population.

	Hyperaggregation (n)	Non-hyperaggregation (n)	OR (95% CI)	p value*
CV events (+)	3	1	13. 250 (1.266 – 138.699)	0.030
CV events (-)	12	53		

CV, cardiovascular, OR, odd ratio; CI, confidence interval *Fisher's Exact Test (1-sided)

Table 6: Relationship between Platelet Aggregation and Cardiovascular Events.

In this study we found that CYP2C19 polymorphism is associated with cardiovascular events. All of cardiovascular events occurred in patients which carrying at least one variant allele of CYP2C19 (Tables 4 and 5). Singh et al [24] showed that CYP2C19*2 polymorphism was associated with higher risk of major adverse cardiovascular events [RR: 1.28, CI: 1.06-1.54; p = 0.009], cardiovascular death [RR: 3.21, CI: 1.65-6.23; p = 0.001], myocardial infarction [RR: 1.36, CI: 1.12-1.65; p = 0.002], stent thrombosis [RR: 2.41, CI: 1.69-3.41; p < 0.001]. Mega et al[21] showed that carriers of a reduced-function CYP2C19 allele have significantly lower levels of the active metabolite of clopidogrel, diminished platelet inhibition, and a higher rate of major adverse cardiovascular events, including stent thrombosis. They found that 395 subjects carrying at least one CYP2C19 reduced-function allele were at higher risk for the primary endpoint (composite death from cardiovascular causes, myocardial infarction, or stroke) 12.1% vs. 8.0%; HR for carriers 1.53; 95% CI, 1.07 to 2.19 ($p = 0.01$).

Limitations

In the present study, we do not measure plasma concentrations of the active metabolite of clopidogrel, thus, we cannot provide direct evidence of reduced antiplatelet efficacy of clopidogrel in patients carrying at least one CYP2C19*2 or *3 variant allele. In addition, we cannot exclude the effect of other drug metabolism enzymes, such as CYP1A2, 2B6, 3A, and 2C9, on clopidogrel response, besides CYP2C19. Thus, further study using larger samples are needed in the future.

Conclusion

CYP2C19*2 and *3 were associated with cardiovascular events due to platelet hyper aggregation.

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