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Original Research

Effects of genetic factors to stent thrombosis due to clopidogrel resistance after coronary stent placement

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Abstract: Stent thrombosis (ST) is considered as a multifactorial problem which is mostly occurs due to clopidogrel resistance. It may be due to some CYP450 enzyme deficiencies which play role in clopidogrel metabolism. Therefore the aim of this study is to detect the mutations in CYP2C19 and CYP2C9 genes which may cause ST, and to investigate the relation between other risk factors and ST. 50 individuals who have stent thrombosis and 50 individuals who haven't got any complication were enrolled as patient and control group respectively. *2,*3,*4,*5,*17 mutations in CYP2C19 gene and *2 ve *3 mutations in CYP2C9 gene were investigated with RT-PCR. Clopidogrel and aspirin resistance were investigated with multiple electrode platelet aggregometry. Results were evaluated statistically. CYP2C19*2 mutation was found statistically higher in patients (% 18), whereas CYP2C19*17 was found statistically higher in controls (% 36)($p<0.05$). Additionally, it was found that patients who have clopidogrel and/or aspirin resistance also have CYP2C19*1/*2 or CYPC19*2/*2 genotype. These relations were also found statistically significant. (p=0,000005 for clopidogrel resistance and p=0,000059 for aspirin resistance). In conclusion, it was suggested that there is a relation between CYP2C19*2 mutations and ST due to clopidogrel resistance, and CYP2C19*17 may have a protective role in this process. The use of novel and more potent drug or high clopidogrel maintenance dosing before stent implantation may be beneficial treatment options for antiplatelet therapy in CYP2C19*2 carriers.

Key words: CAD, stent thrombosis, clopidogrel resistance, CYP2C19, CYP2C9.

Introduction

Coronary artery disease (CAD), although closely associated with environmental factors and life style, is also concerned by the complex patterns of inheritance. The relation between CAD and conventional factors has been completely investigated, but the role of genetic markers is not exactly understood. In patients scheduled for percutaneous coronary intervention (PCI), dual antiplatelet treatment with clopidogrel and aspirin is routinely administered to prevent thrombotic events after coronary stent placement (1). In spide of this treatment, significant number of thrombotic events still occur. The most serious thrombotic complication is stent thrombosis (ST). This acute re-occlusion of the artery cause acute myocardial infarction (MI) and is associated with morbidity and mortality (2).

Clopidogrel is an antiplatelet drug used by approximately 40 million patients worldwide to treat or prevent atherothrombotic events after PCI (3). Although the standard 75 mg daily maintenance dose of clopidogrel has proven to be clinically adequate for patients, in some cases different pharmacodynamic responses to clopidogrel were detected. Therefore patients with higher platelet reactivity while receiving clopidogrel are at risk of cardiovascular events (4)

Clopidogrel, an inactive prodrug, is metabolized and activated by the hepatic cytochrome P450 (CYP) system to generate its active thiol metabolite. Patients with mutations in CYP2C19 gene, have more adverse clinical events following PCI. (5) Also it has been suggested that clopidogrel may be less effective in reducing the rate of cardiovascular events in patients who are carriers of loss of function CYP2C19 like CYP2C19*2 and*3 and CYP2C9 alleles such as CYP2C9*2 and *3, are associated with reduced conversion of clopidogrel to its active metabolite (2,6,7). Additionally it has been suggested that the presence of the gain-of-function CYP2C19 allele like CYP2C19*17 may be associated with enhanced response to clopidogrel (8). Many CYP2C19 and CYP2C9 mutations were identified (9). The CYP2C19*2 (rs4244285) is a G681A nucleotide substitution at the intron 4/exon 5 junction, which introduces a splicing defect resulting in a truncated, nonfunctional protein. The CYP2C19*3 polymorphism (rs4986893) is a G636A nucleotide substitution in exon 4, which creates a premature stop codon at amino acid position 212 resulting in a truncated, metabolically inactive protein (10). The CYP2C19*4 (rs28399504) and CYP2C19*5 (rs56337013) are rare variant alleles which may also determine the poor metabolizer phenotype. CYP2C19*4 polymorphism is a A/G substitution in the initiation codon, resulting in a Met1Val substitu-

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tion. CYP2C19*5 polymorphism is a C1297T substitution in exon 9, resulting in an Arg433Trp substitution in the heme binding region resulted in an enzyme that had negligible catalytic activity toward CYP2C19 substrates (11,12). A further polymorphism, CYP2C19*17 (rs12248560), has been discovered that results in an increased enzyme function due to a 806C/T substitution in the 5′-flanking region of the gene that causes increased transcription, resulting in ultra-rapid metabolism of CYP2C19 substrates (13). CYP2C9*2 is characterized by a nucleotide substitution C430T in exon3 that produces the amino acid change Arg144Cys, and CYP2C9*3 shows a nucleotide substitution A1075C in exon 7 that generates the amino acid change Ile-359Leu. Both of these CYP2C9 allelic variants result in decreased enzyme activity of the gene products (14)

New markers for identifying high risk populations as well as novel strategies for early detection and preventive care are needed. Therefore the aim of this study was to investigate whether there is an association between CYP2C19*2, *3, *4, *5, *17; CYP2C9*2 and *3 and ST in patients undergoing coronary stent placement after pre-treatment with clopidogrel.

Materials and Methods

Study population

Totally, 100 patients (85 males, 15 females) were enrolled from Dr. Siyami Ersek Thoracic and Cardiovascular Surgery Training and Research Hospital from September 2013-March 2015 in the trial, which was conducted in patients with known cardiovascular disease taking 75 mg of clopidogrel and 100 mg aspirin daily. Patients were only selected as cases when they were still on aspirin and clopidogrel at the time of ST. Control subjects were selected from consequtive patients who underwent PCI, with no adverse cardiovascular events, including ST, during a 1 year follow up post PCI.

Common exclusion criteria of these trials were: ST-segment elevation acute myocardial infarction, stroke within the previous 3 months, haemodynamic instability, malignancies, active bleeding or bleeding diathesis, recent trauma or major surgery in the previous month, suspected aortic dissection, patients who received a glycoprotein IIb/IIIa inhibitor within 14 days, oral anticoagulation within 7 days, known allergy to the study medication; and current or suspected pregnancy.

The present study complies with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Yeditepe University, Istanbul, Turkey. All patients gave written informed consent prior to study inclusion.

Follow-Up

Patients stayed in the hospital for at least 2 days after study inclusion and after PCI. Patients were interviewed by telephone after 180 days (±7 days) and were encouraged to contact the specialist study team personnel at any time during the follow up period. Patients with cardiac symptoms were seen in the outpatient clinic for complete clinical, ECG, and laboratory checkup. Patient data were collected and entered into a computer database, and all of the related information from referring physicians, relatives, and hospital readmissions was entered.

Platelet function testing

For platelet function testing with multiple electrode platelet aggregometry (Dynabyte), whole blood was obtained from the arterial sheath of all of the patients directly before PCI. Details of this method have been reported previously (15). Blood was placed in 4.5 mL plastic tubes containing the anticoagulant lepirudin (25 mg/mL, Refludan, Dynabyte, Munich, Germany) After 1:2 dilution of whole blood with 0.9% NaCl solution and stirring for 3 min in the test cuvettes at 37°C, 6.4 µmol/l ADP was added. Platelet aggregation was continuously recorded for 5 min. Impedance with MEA is transformed to arbitrary aggregation units (AU) that are plotted against time (AU·min). Aggregation measured with MEA is quantified as AU and area under the curve of arbitrary units (AU·min). All materials used including ADP was obtained from the manufacturer (Dynabyte).

Blood sampling and Genotyping

Whole blood for genotyping was obtained from the arterial sheath of all patients directly after diagnostic angiography and before PCI. Genomic DNA was extracted from 200 mL of blood using commercially available kits (Qiagen) according to manufacturer's instructions. DNA purity and concentration were determined by NanoDrop spectrophotometer (Thermo Scientific). Real-time PCR reactions for CYP2C19*2 (rs4244285), CYP2C19*3 (rs4986893) and CYP2C19*17 (rs12248560), were carried out on Rotor-Gene 6000 Real-Time PCR Device (Qiagen). CYP2C19*4 (rs28399504), CYP2C19*5 (rs56337013), CYP2C9*2 (rs1799853) and CYP2C9*3 (rs1057910) were carried out on 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction was performed according to the manufacturer's instructions.

Statistical Analysis

SPSS 22.0 was performed for statistical analysis. The $\chi 2$ and independent two sample t tests were used for comparing mutations between two groups. Mann Whitney U and Kruskal Wallis tests were performed to investigate the association between genotypes and risk factors of ST. p values less than 0.05 ($p<0.05$) were considered to be statistically significant. Multivariable analysis between genotypes and ST was performed using logistic regression analysis. Results were represented as odd ratios (OR) with 95% confidence intervals. (95% CI).

Results

Study Population and drug resistance

Table 1 summarizes the characteristics of the study population. There were no significant differences with regard to all characteristics between the two groups, except clopidogrel resistance.

CYP2C19 and CYP2C9 genotyping

Table 2 shows genetic analyses of cases and controls. CYP2C19*2,*3,*17 and CYP2C9*2,*3 mutations were found in various proportions. CYP2C19*4 and *5 mutations were not detected. CYP2C19*2 mutation was

Table 1. Baseline characteristics of the study population.

Characteristics	Number of participants		p values
	Controls (n=50)	Cases (n=50)	
Clopidogrel resistance (%)	3 (6%)	6 (12%)	0,0012*
Aspirin resistance (%)	0 (0%)	5 (10%)	0,056
Age (years)	58,24 ± 10,38	59,14 ± 10,66	0,67
Fasting blood glucose level	118,86 ± 37,16	126,18 ± 49,4	0,40
Hyperlipidemia (%)	24 (48%)	23 (46%)	0,84
Total cholesterol (mg/dl)	191,46 ± 47,22	184,36 ± 55,22	0,49
LDL (mg/dl)	114,74 ± 43,01	123,58 ± 57,65	0,39
HDL (mg/dl)	39,42 ± 8,09	38,82 ± 9,7	0,74
Triglyceride (mg/dl)	185,08 ± 60,95	161,36 ± 63,42	0,06
Red blood cell distribution width (RDW)	13,56 ± 1,48	14,03 ± 1,41	0,11
Mean platelet volume (MPV)	8,91 ± 1,04	9,06 ± 0,98	0,45
White blood cell (WBC) (x1000/µl)	9,15 ± 2,87	8,92 ± 2,39	0,66
Ejection fraction (EF) (%)	51,40 ± 9,9	52,58± 8,55	0,53
HbA1c	6,08 ± 0,99	6,34 ± 1,69	0,36
Serum creatinine (mg/dl)	1,11 ± 0,99	1,02± 0,90	0,63
Platelet count (x100.000/µl)	229,88 ± 56,6	253,74 ± 86,63	0,11
Hypertension (%)	28 (56%)	37 (74%)	0,06
Current smoking (%)	24 (48%)	23 (46%)	0,84
Diabetes Mellitus (%)	14 (28%)	18 (36%)	0,39
Prior myocard infarction (%)	39 (78%)	34 (68%)	0,26
Prior coronary artery bypass graft surgery (%)	7 (14%)	6 (12%)	0,77
Presence of acute coronary syndrome which presents PCI indication (%)	42 (84%)	45 (90%)	0,37
Chronic obstructive pulmonary disease (COPD) (%)	6 (12%)	4 (8%)	0,51
Familial history of CAD (%)	32 (64%)	37 (74%)	0,28
Stent length (mm)	19,63 ± 7,99	20,84 ± 8,31	0,35
Stent diameter (mm)	3,08 ± 0,4	3,04 ± 0,43	0,63
Total stent length (mm)	60,09 ± 25,65	62,61 ± 24,84	0,62

Table 2. Genetic analyses in cases and controls.

Genotype and nucleotide variation	Number of participants (%)		p values
	Controls (n=50)	Cases (n=50)	
CYP2C19			
*CYP2C219*2*			
GG	50 (100%)	41 (82 %)	
GA	0 (0%)	8 (16%)	**0,007***
AA	0 (0%)	1 (2%)	
*CYP2C219*3*			
GG	50 (100%)	48 (96%)	
GA	0 (0%)	2 (4%)	0,495
AA	0 (0%)	0 (0%)	
*CYP2C219*4*			
AA	50 (100%)	50 (100%)	
AG	0 (0%)	0 (0%)	N.c.
GG	0 (0%)	0 (0%)	
*CYP2C219*5*			
CC	50 (100%)	50 (100%)	
CT	0 (0%)	0 (0%)	N.c.
TT	0 (0%)	0 (0%)	
*CYP2C219*17*			
CC	32 (64%)	43 (86%)	
CT	11 (22%)	3 (6%)	**0,03***
TT	7 (14%)	4 (8%)	
CYP2C9			
*CYP2C9*2*			
CC	40 (80%)	35 (70%)	
CT	10 (20%)	14 (28%)	0,368
TT	0 (0%)	1 (2%)	
*CYP2C9*3*			
AA	43 (86%)	39 (78%)	
AC	7 (14%)	11 (22%)	0,298
CC	0 (0%)	0 (0%)	

N.c.:No statistics were computed

found statistically higher in patients (%18) (p=0,007), whereas CYP2C19*17 was found statistically higher in controls (%36) (p=0,03). Also CYP2C19*1/*2 genotype was only detected in male patients (n=8), it was not detected in male controls. This difference was found statistically significant (p=0,002). Additionally, it was found that patients who have clopidogrel and/or aspirin resistance also have CYP2C19*1/*2 or CYPC19*2/*2 genotype. These relations were also found statistically significant. (p=0,000005 for clopidogrel resistance and p=0,000059 for aspirin resistance).

Also it was found that, CYP2C19*17 may have protective effect for preventing ST. This mutation was mostly found in controls. OR:0,0226 95%CI(0,059-0,869)(p=0,042).

Discussion

Stent thrombosis is considered as a multifactorial problem related to patient, procedure, lesion, factors related to blood coagulation and response to antiplatelet therapy (16). Various clinical studies have demonstrated that patients with high residual platelet reactivity on clopidogrel were at increased risk for stent thrombosis and other cardiac complications (17,18). Multiple studies have demonstrated that both homozygotes and heterozygotes for loss-of-function CYP2C19 alleles have lower levels of the active clopidogrel metabolite (7,19), diminished platelet response to clopidogrel (8,20) and higher rates of adverse cardiovascular events when compared with noncarriers (21, 22,23). Recent studies support the significant contribution of CYP2C19 genotyping variants to the clinical efficacy of clopidogrel and cardiac outcomes in various patient populations. These studies indicated that the presence of CYP2C19 decreased-function genotype was strongly associated with a pharmacodynamic poorresponder phenotype in healthy subjects (19,24). This association has been also confirmed in patients treated with clopidogrel for coronary artery disease (8). CYP2C19 loss of function polymorphisms have been independently shown to be associated with early ST (25, 26) Similarly, the present study demonstrate that the *CYP2C19*2 polymorphism is directly related with clopidogrel and aspirin resistance, the carriage of at least one *CYP2C19*2 allele significantly increase the risk of ST ($p<0.05$).

Complementary to the associations between loss of function CYP2C19 alleles and increased cardiovascular risk, the common CYP2C19*17 gain of function allele has been associated with a better antiplatelet response to clopidogrel (27, 28). Various studies have investigated the possible linkage of CYP2C19*17 with clinical efficacy outcomes. Results might be expected to mirror the interaction with loss of function alleles, with lower risks of events in carriers of the *17 allele than in noncarriers. Similarly, the present study demonstrate that CY2C19*17 may have a protective role from ST.This mutation is mostly found in controls and the difference between groups was found statistically significant ($p<0.05$)

Studies have also shown a relationship of the CYP2C9 loss of function allelic variants with an attenuated response to clopidogrel loading (7,29) and that it may even be associated with ST (2). Despite that, some of the studies have shown that CYP2C9 genotypes have no effect in patients who were on chronic clopidogrel maintenance therapy and do not modulate platelet response to clopidogrel (30) Similarly, the present study does not demonstrate any association between CYP2C9 genotypes, ST and clopidogrel resistance.

The major result of the present study is that patients who carry at least one CYP2C19*2 allele have a significantly higher risk of ST due to aspirin and/or clopidogrel resistance following coronary stent placement compared with CYP2C19*1 wild-type homozygous patients. Also patients who carry at least one CYP2C19*17 allele have a significantly higher protective effect from ST following coronary stent placement compared with CYP2C19*1 wild-type homozygous patients.

In conclusion, genotyping of CYP2C19 gene polymorphisms may help to individualize and optimize oral antiplatelet treatment. This could be performed before PCI and helps to identify a subpopulation of patients at high risk for stent thrombosis who could then be analyzed by platelet function tests for adequate response to clopidogrel. The use of novel and more potent drug or high clopidogrel maintenance dosing may be beneficial treatment options for antiplatelet therapy in CYP2C19*2 carriers. Therefore personalized therapy targeting patients who carry CYP2C19*2 genetic variants might help to improve the clinical outcome after stent implantation.

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