PRELIMINARY DATA FROM A STUDY ON POLYMORPHISM RS4244285 OF P4502C19 CYTOCHROME GENE IN PATIENTS WITH ACUTE CORONARY SYNDROME, UNDERGOING TREATMENT WITH DUAL ANTIPLATELET THERAPY WITH CLOPIDOGREL AND ASPIRIN

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Summary

Administration of antiplatelet therapy Aspirin and Clopidogrel (CLP) is a corner stone in patients with Acute Coronary Syndrome (ACS) undergoing Percutaneous Coronary Intervention (PCI) with/without stent implantation. The CYP2C19*2 allele is the most important genetic variant determining response to CLP. We aim to investigate frequency of CYP2C19*2 polymorphism in patients with ACS and significance for the individual response to CLP therapy. The preliminary data of a study including a total of 120 patients with ACS undergoing PCI with stent placement and treated with dual antiplatelet therapy (CLP and Aspirin) are presented. So far 18 patients (41-81 year age) are tested for CYP2C19*1/*2 polymorphisms. The genotype CYP2C19*1/*1; CYP2C19*1/*2 and CYP2C19*2/*2 is demonstrated in 50%, 33%, 17% respectively, of the patients. The established frequency of CYP2C19*2 allele (33%) is significantly higher (χ^2=5.220; p=0.022) than in healthy Bulgarian individuals (16%). In-stent thrombosis have developed 3 (17%) of patients: 2 are CYP2C19*1/*2 carriers, and 1 – homozygous CYP2C19*2/*2. The preliminary data demonstrate high prevalence of CYP2C19*2 polymorphism in patients with ACS and point to significance of the variant for CLP therapy. Further extension of the study with larger samples and monitoring of the patients are required to determine the effects of the polymorphism on the prognosis for major adverse cardiovascular events.
Key words: CYP2C19 polymorphism, Clopidogrel, Acute Coronary Syndrome, in-stent thrombosis

Introduction

Acute coronary syndrome (ACS) is the most severe manifestation of ischemic heart disease, which remains among the leading causes of death worldwide, despite the use of modern treatment. ACS is a life threatening condition and includes the following categories of patients: diagnosed with STEMI (myocardial infarction with ST-segment elevation), NSTEMI (myocardial infarction without ST-segment elevation) and UA (unstable angina pectoris).

According to the recommendations of the European Society of Cardiology, in patients with

ACS the use of antiplatelet drugs leads to decrease of the risk of acute ischemic complications, as well as recurrent thrombotic events [1, 2]. Platelet adenosine diphosphate P2RY12 receptor inhibitors are one of the main classes of drugs used in ACS patients and performed percutaneous coronary intervention (PCI) with or without stent implantation. The main representative member of this group is Clopidogrel (CLP) and dual antiplatelet therapy (Aspirin and CLP) is a standard in clinical practice [3]. Although the combination has a proven effect, for some patients pharmaco-dynamic response of CLP is reduced, these patients show a lower level of platelet inhibition and increased risk for cardiovascular complications – death, myocardial infarction (MI) and stent-thrombosis [4].

Clopidogrel is a second generation thienopyridine, and is an adenosine diphosphate (ADP) antagonist, that inhibits direct binding of ADP to the platelets and selectively blocks P2RY12 receptors on the platelet membrane. Its double effect on platelet function is due to both the direct and irreversible inhibition of ADP-dependent platelet activation as well as the indirect influence on platelet aggregation by blocking the ADP-mediated activation of GP IIb/IIIa receptors for binding with fibrinogen [5-7].

The response during treatment with CLP shows the wide variability between individuals and has multifactorial etiology involving both the polymorphisms of genes of the cytochrome P450 enzyme system and environmental factors. Among the non-genetic factors are certain drug interactions, concomitant diseases (such as diabetes and renal failure), age and other [8, 9]. Pharmaco-genetic reasons for this variability include participation of various and complex pharmacokinetic, pharmacodynamic and metabolic pathways. In literature [9], between 4% and 30% of patients show resistance to therapy with CLP and inadequate antiplatelet response. Among the reasons for inter-individual variability in response to this drug is a cytochrome P450 enzyme system responsible for the metabolism of thienopyridine including CLP. The drug is initially a prodrug and by hepatic cytochrome (P450 CYP2B6, CYP3A4, CYP 3A5, CYP2C19) isoenzymes is converted to an active metabolite and irreversibly inhibits platelet adenosine diphosphate receptor (P2RY12) [4, 10, 11]. CYP2C19 enzyme is involved in two steps of oxidative metabolic

activation of CLP. CYP2C19 gene is localized on the short arm of chromosome 10 and encodes the enzyme S-mephenytoin 4-hydroxylase, which is one of the cytochrome P450 enzymes involved in the formation of active metabolites of CLP. Significant number of single nucleotide polymorphisms (SNPs) in genes encoding CYP3A4, CYP 3A5, CYP2C19 are established, some of these variants decrease and others increase enzyme activity [10, 12]. CYP2C19*2 (rs4244285) is the most common allelic variant of the gene for S-mephenytoin 4-hydroxylase associated with partial or complete loss of function (LOF). CYP2C19*2 allele is a substitution of guanine by adenine at position 681 (681G>A) in exon 5, leading to the appearance of an early stop codon, disruption of splicing, and fusion of a non-functional enzyme and reduce the active metabolite of CLP [13, 14-17]. According to the CYP2C19 genotype and enzyme activity, individuals are defined as: homozygotes for allele *2 also known as slow ("bad") metabolisers (producing the less active metabolite and have a reduced platelet inhibition), compared to the homozygotes for allele *1 (normal metabolisers) and heterozygotes known as intermediate metabolizers [15, 18, 19]. Allelic frequency of the CYP2C19*2 LOF is 15% among Caucasian and African populations, and 29-35% among Asian [20]. Other polymorphic variants with reduced function are *3 (rs4986893), *4 (rs28399504), *5 (rs56337013), but they are rare with an incidence of less than 1% [13].

Aim of the study is to investigate frequency of CYP2C19*2 polymorphism in patients with ACS and significance of this SNP for the individual response during treatment with CLP.

Patients and Methods

The study is planned to include a total of 120 patients with ACS and so far 18 are tested (age from 41 to 81 years). All the patients are admitted from the Specialized Hospital for Active Treatment in Cardiology – Pleven for the period from February 2015 to April 2016 as a part of the selected patient group in a research project supported by the Medical University – Pleven "Investigation on the significance of the polymorphism rs4244285 in P4502C19 cytochrome gene in patients with acute coronary syndrome, undergoing treatment with dual antiplatelet therapy with Clopidogrel and Aspirin".

PCI is performed for all patients with drug-eluting stent implantation. All the patients have received 300-600 mg CLP during the procedure followed by 75 mg CLP daily for next 18 months, along with 100 mg Aspirin daily. Exclusion criteria are any contraindications for therapy with Aspirin or Clopidogrel, clinical conditions such as anemia (Hb<100 g/l), pulmonary edema and cardiac asthma, haemorrhagic stroke. The information about personal data, diagnosis, family history, smoking, clinical information, drug history is obtained from medical records or personally by the patient.

Informed consent is obtained by each of the patients. The study protocol is approved by the Ethics Commission of Medical University – Pleven.

Sample collection and DNA analysis:

From each of the patient 7-10 ml venous blood (in EDTA vacutainers) is collected. The DNA isolation is carried out using salt extraction method or commercial kit „AccuPrep Genomic DNA Extraction Kit" – BIONEER. Single nucleotide polymorphisms rs4244285 are detected by TaqMan® Drug Metabolism Enzyme (DME) Genotyping Assays (Life Technologies) by real-time polymerase chain reaction (qPCR) with the PicoReal 96® instrument (Thermo Scientific, United States), which is used according to instructions to optimize the sample preparation steps.

Amplification of the fragments for the rs4244285 polymorphism is performed using fluorescent VIC- and FAM- probes and set of predesigned primers. PCR amplification is carried out using 96-well Piko PCR plates (Thermo Scientific, UK), with 4.5 µL 20 ng/µL of genomic DNA, 5.0 µL 2X TaqMan Genotyping Master Mix (Life Technologies), 0.5 µL 20X TaqMan Genotyping Assay (Life Technologies) in total reaction volume of 10 µL. A two-step 40-cycle qPCR is carried out with the following conditions: initial AmpliTaq Gold – activation 95°C for 10 min denaturation of the template DNA for each cycle of 95°C for 15 s, annealing and extension of 60°C for 90 s followed by data acquisition after each annealing and extension cycle.

Results

We present the preliminary data of the study. The demographic, clinical characteristics, common risk factors, family history and CYP2C19 genotype of the studied 18 patients are presented in Table 1. The sex distribution of the patients (67% men, 33% women) shows that ACS is 2 times more frequent in men than women. In age group <64 ys men prevail while in age group above 64 ys there is no significant difference by sex. In most (89%) of the patients the main indication for PCI is MI (only two women are diagnosed with UA).

Concerning some risk factors for ACS: 33% of patients (mainly men) are tobacco smokers and 39% have obesity. Dyslipidaemia have 44% of all patients (50% of the studied female and 42% of the studied male). Hypertension have 83% of patients and there is no difference by sex. About 22% of the patients (25% of women and 17% of men) have history for Diabetes mellitus and/or previous event of MI.

Family histories for affected first-degree relatives with MI have 17% of all patients (all of them men).

Concerning rs4244285 polymorphism of the P4502C19 cytochrome gene, the genotype distribution of the patients is as follows: 9 patients (50%) are homozygous for allele 1*, 6 (33%) - heterozygous carrier 1*/2* and 3 (17%) - homozygous for allele 2*.

To compare the allele and genotype frequencies of our patients with healthy controls we used the data of a survey conducted on 142 healthy Bulgarians. The genotype distribution of the healthy individual is as follows: 102/142 (71.8%) are homozygous for allele 1*, 34/142 (23.9%) – heterozygous carrier 1*/2* and 6/142 (4.23%) – homozygous for allele 2*. The reported frequency of CYP2C19*2 allele is 16.2% [21].

Our data present significantly higher frequency of the CYP2C19*2 allele among the patients with ACS, in comparison with the healthy controls (χ^2=5.220; p=0.022). The frequency of CYP2C19*2/*2 genotype is 4 time higher (χ^2=2.609; p=0.106) in patient group than in controls.

During the treatment with CLP, 3 (17%) of patients developed early (one month after initiation of therapy) in-stent thromboses. All of them are carriers of at least one CYP2C19*2 allele (2 patients are heterozygous CYP2C19*1/*2 and one – homozygous CYP2C19*2/*2).

Table 1. Demographic and clinical characteristics, common risk factors, family history and CYP2C19 genotype of the studied patients

	Overall n=18	Males n=12 (67%)	Females n=6 (33%)
Age <64 ys	9 (50%)	8 (67%)	1 (17%)
Age >64 ys	9 (50%)	4 (33%)	5 (83%)
Smoking	6 (33%)	5 (42%)	1 (17%)
BMI*>30	7 (39%)	6 (50%)	1 (17%)
Diabetes mellitus	4 (22%)	3 (25%)	1 (17%)
Hypertension (BP>140/90 mmHg)	15 (83%)	10 (83%)	5 (83%)
Patients with MI†	16 (89%)	12 (100%)	4 (67%)
PCI‡	18 (100%)	12 (100%)	6 (100%)
DES§	18 (100%)	12 (100%)	6 (100%)
Prior MI†	4 (22%)	3 (25%)	1 (17%)
Clopidogrel and Aspirin	18 (100%)	12 (100%)	6 (100%)
In-stent thrombosis	3 (17%)	2 (17%)	1 (17%)
Dyslipidaemia	8 (44%)	5 (42%)	3 (50%)
Family history positive for MI†	3 (17%)	3 (25%)	-
CYP2C19*2			
Non-carriers	9 (50%)	6 (50%)	3 (50%)
Heterozygous carriers	6 (33%)	3 (25%)	3 (50%)
Homozygous carriers	3 (17%)	3 (25%)	-

*Body mass index; §Drug-eluting stent; †Myocardial infarction; ‡Percutaneous intervention

Discussion

Multiple genetic and non-genetic factors are implicated in individual response to Clopidogrel therapy in patients with coronary artery diseases. The CYP2C19*2 polymorphism is the most important allele responsible for resistance to antiplatelet therapy with CLP [22] and the most frequent variant allele (95%) among the reduced-function group polymorphisms [4]. The CYP2C19*2 genotype accounts for approximately 12% of variation in Clopidogrel response [18].

The distribution of CYP2C19*2 allele frequencies varied in different racial groups: significantly higher in Asian (~30%) than in Caucasian (~13%) and Africo American (~18%) populations [23]. In Bulgarian survey, the reported frequency of CYP2C19*2 allele and CYP2C19*2 homozygotes is 16.2% and 4.23% respectively [21].

In our study, among the patients with ACS, we found significant higher frequency 33% (χ^2=5.220; p=0.022) of the CYP2C19*2 allele and 4 time higher (17%; χ^2=2.609; p=0.106) frequency of CYP2C19*2 homozygotes, in comparison with healthy individuals from the above mentioned Bulgarian study.

Currently, in the literature there are a significant number of studies that evaluate the effect of CYP2C19 genotype on the pharmacokinetics of the active metabolite of

CLP. They have shown that carriage of CYP2C19*2 and CYP2C19*3 alleles associate with a reduced function, leading to lower levels of the active metabolite, decreased platelet inhibition and increased residual platelet activity, and a higher incidence of major adverse cardiovascular events including stent thrombosis [4, 20, 24-29].

In support of that statement is data from two meta-analysis studies by Mega et al and Hulot et al [30-32], as well as by the Genome-wide association study [18]. Data from a meta-analysis establish that 84 patients have stent thrombosis among the 5894 evaluated for such and there is a significant increase risk of stent thrombosis for carriers of one (hazard ratio, HR=2.67; 95% confidence interval, CI: 1.69-4.22; p<0.0001) and two (HR=3.97; 95% CI: 1.75-9.02; p=0.001) CYP2C19 reduced-function alleles [30]. The meta-analysis by Mega et al [30], concluded that CYP2C19 genotype is an important predictor of adverse cardiovascular outcome in patients with implanted coronary stent treated with CLP.

The summarized results on this issue, however are generally controversial - some investigators confirm the specific association between LOF alleles and clinical outcome regarding cardio-vascular events, while other authors not establish strong association [33-35].

The preliminary results of our study show that 17% of investigated ACS patients treated with CLP have developed early in-stent thrombosis and CYP2C19 genotype test indicate that all of them are carriers of at least one CYP2C19*2 loss of function allele. Although the data is not conclusive, they demonstrate the significance of this SNP variant for individual response to CLP therapy. To investigate the association between CYP2C19*2 allele and risk for subsequent adverse cardiovascular events the study should continue with inclusion of a large number of patients and follow-up one year after initiation of therapy.

The treatment response to CLP depends on not only of genetic characteristics. Different non-genetic factors such as age, diabetes, obesity, smoking [20, 36], drug interactions, such as proton pump inhibitors [37, 38] and others may influence the effectiveness of therapy with CLP. Increased age, BMI, and triglyceride levels and decreased levels of high-density lipoprotein cholesterol are predictors of poorer Clopidogrel response and can explain less than 10% of variation in Clopidogrel response [18].

The preliminary data of our study also emphasized the importance of some factors for ACS that could influence on the therapy with CLP: male is the most affected sex, especially in group under the age of 64 ys; MI is the main indication for PCI; hypertension is the most common associated disease in patients with ACS. Our results about significance of risk factors such as tobacco smoking, obesity and diabetes are inconclusive may be due to the small number of the studied patients. The impact of the risk factors would be assessed more precisely after the end of the planned study, based on the final results.

Genetic testing to initiate CLP therapy is not recommended from the Food and Drug Administration and if a patient's genotype is unknown the decision to perform CYP2C19 testing is up to the individual clinician. The Clinical Pharmacogenetics Implementation Consortium (CPIC) create an algorithm for clinical actions based on CYP2C19 genotype and recommends alternative antiplatelet agents (e.g., prasugrel or ticagrelol) for ACS patients undergoing PCI who carry one or two copies of LOF allele [20].

Conclusions

The preliminary results of this study on patients with ACS undergoing PCI with stent implantation show that the frequency of CYP2C19*2 allele is high. These results, mostly resembles studies for other races such as Asians and shows significant higher prevalence of the LOF allele in our patients' group than in overall white population. Further studies with inclusion of a larger number of patients and healthy controls are required to determine the significance of CYP2C19*2 polymorphism on the prognosis of ACS patients in Bulgarian population.

The main limitations of the study: at first, this is preliminary data and the number of patients is still relatively limited. In a larger population, the results will be more reliable. Secondly, we investigate only CYP2C19*1 and CYP2C19*2 polymorphisms, considering the CYP2C19*2 is the most important allele responsible for Clopidogrel resistance. Also, we have not yet completely assessed the influence of genetic variations on final clinical outcome in CLP treated patients, so in the study there is a need of further monitoring of the patients and follow-up within one year after initiation of therapy.

69

Acknowledgments

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