Introduction

The adenosine diphosphate (ADP) receptor blocker clopidogrel is routinely administered for the prevention of cardiovascular events in patients suffering ST-elevation myocardial infarction (STEMI), especially in those undergoing percutaneous coronary intervention (PCI) [1,2]. Although new standard of antiplatelet agents such as prasugrel and ticagrelor are available now, clopidogrel is still one of the most frequently prescribed drugs in many countries. However, interindividual variability in pharmacodynamics response to clopidogrel is widespread in patients treated with this medication [3]. Patients with high platelet reactivity (PR) to ADP are more likely to experience ischemic events, while low PR to ADP may contribute to increased risk of bleeding events [4]. Although the mechanisms have not been fully elucidated, many factors have been reported to involve in the clopidogrel response variability. Gene polymorphisms...
play a critical role in clopidogrel metabolism, strongly affecting the prognosis of patients under clopidogrel treatment [5–7].

Clopidogrel is an inactive prodrug that requires intestinal absorption and subsequent biotransformation to active metabolites by cytochrome P450 enzymes. We have recently observed that the CYP2C19 loss-of-function (LOF) alleles responsible for clopidogrel metabolism had a gene dose effect on the pharmacodynamics and composite ischemic events of clopidogrel in Chinese people after PCI [8]. As for clopidogrel absorption, a key drug transporter involved is the P-glycoprotein at the intestinal barrier, which is encoded by the ABCB1 (ATP-binding cassette, sub-family B, member 1, also called MDR1) gene [9]. The P-glycoprotein is an ATP-dependent efflux pump that transports various molecules across extracellular and intracellular membranes. The increased expression or function of P-glycoprotein on intestinal epithelial cells can affect bioavailability of its substrate drugs, such as clopidogrel. The contribution of the ABCB1 gene to clopidogrel response continues to be of great interest. More than 50 single nucleotide polymorphisms (SNPs) reside in the coding region of ABCB1 gene which can possibly cause altered function [10]. Most studies focused on a synonymous SNP C3435T (rs1045642) in the gene. Previous research has shown that the minor T allele causes altered function of P-glycoprotein to affect the absorption of clopidogrel [11].

Antiplatelet effect can be evaluated through clinical outcomes and laboratory platelet function tests. Although accumulating data from large studies underscore the importance of high on-treatment PR to ADP as a prognostic risk factor of ischemic events, the association between on-treatment PR and bleeding events is less clear[4]. Thus, we are interested in exploring a potential link between thromboelastography (TEG) results and bleeding events. Meanwhile, epidemiological evidence on ABCB1 gene-association with clopidogrel response is largely inconsistent [12–14]. In contrast to the numerous studies linking ABCB1 polymorphisms to an increased risk of ischemic events, there is less evidence about the relation of ABCB1 gene and bleeding events. We previously found a significant association between ABCB1 C3435T and bleeding events. In order to further investigate the relation of other ABCB1 polymorphisms on the risk of bleeding and ischemic events, we analyzed tag SNPs across the ABCB1 gene in Chinese STEMI patients treated with clopidogrel, and assessed the association of the ABCB1 polymorphisms in the context of CYP2C19 status to reveal an independent relation of ABCB1 gene variants with clinical outcome. Here, we demonstrated that in STEMI patients treated with clopidogrel after PCI, the ABCB1 tag SNP rs1045642 is associated with higher risk of bleedings while rs7779562 is associated with lower bleeding risk, and ADP inhibition in TEG has predictive value of bleeding risk.

Methods

Study population

Between January 2011 and July 2012, 467 consecutive patients with STEMI were enrolled in our prospective, randomized, single-center study. The inclusion criteria were: age of >18 years, had an uneventful PCI, and could be followed up for >1 year after PCI. The major exclusion criteria were hemodynamic instability, active bleeding and bleeding diatheses, oral anticoagulation therapy, use of intensified antiplatelet agents other than standard dual antiplatelet therapy, contraindication to antiplatelet therapy, non-cardiac disease with a life expectancy of <1 year, or inability to follow the protocol. The Institutional Review Board approved the study protocol, and the patients were provided written informed consent for participation and agreed to the TEG testing and genotype determination. The study conformed to the principles outlined in the Declaration of Helsinki.

Study design

All patients were pre-treated with aspirin and a loading dose of 300 mg clopidogrel before PCI, followed by a maintenance dose of 100 mg/day aspirin for life and 75 mg/day clopidogrel for 1 year. The decision for PCI was based on the coronary angiography results, and all interventions were conducted according to the current standard guidelines. The stent type was chosen by the operator, and tirofiban was administered if a glycoprotein IIb/IIIa receptor inhibitor (GPI) was required. Anticoagulation with low-molecular-weight heparin (enoxaparin) or unfractionated heparin was initiated before angiography in all patients.

Selection of tag SNPs

Using the pairwise tagging approach, tag SNPs were selected from the HapMap CHB databank (HapMap Data Rel 27 Phasell + III, Feb09, on NCBI B36 assembly, dbSNP b126) with aid of tag SNPs’ online software (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36/#search). The selected tag SNPs covered the complete ABCB1 region, from 5,000 bp upstream to 5,000 bp downstream. Common variants were defined as those with a minor allele frequency (MAF) greater than 0.05, with a linkage disequilibrium (LD) measure r² threshold of 0.8. Twenty tag SNPs were identified to capture 86 percent of SNPs over the entire ABCB1 gene. Before analysis, one tag SNP rs2032582 was excluded because of significant deviation from Hardy-Weinberg equilibrium in the study population (p < 0.001). No such deviation was detected in all other enrolled tag SNPs.

Genetic analysis

Genomic DNA was extracted from peripheral whole blood samples according to a salting-out protocol. All 20 selected SNPs were genotyped using the ligase detection reaction (LDR) and a commercially available detection system (ABI3130XL DNA Analyzer System; Applied Biosystems, USA). Repeat genotyping was performed on random duplicate samples (n = 21), and sequencing techniques were used to ensure quality control. Based on the known association of CYP2C19 genetic variation with pharmacological response and adverse outcomes in clopidogrel-treated patients, we assessed the LOF alleles CYP2C19*2 (rs4244285, C.681G > A) and CYP2C19*3 (rs4986893, C.636C > A), and the gain of function (GOF) allele CYP2C19*17 (rs12248560, g. -808C > T) to show the relation of ABCB1 polymorphisms to clinical adverse outcomes.

Thromboelastograph platelet-mapping assay

Blood was collected at least 6 h after the patient had taken the clopidogrel dose in a vacutainer tube containing 3.2% trisodium citrate and lithium heparin. The vacutainer tube was filled to capacity and inverted three to five times to ensure complete mixing of the anticoagulant. Modified TEG® used four channels to detect effects of antiplatelet therapy with arachidonic acid (AA) and adenosine diphosphate (ADP) activators. A detailed description of this method is outlined previously [15]. The TEG Hemostasis Analyzer (Haemonetics Corp, Braintree, MA) and automated analytical software were used to measure the physical properties. The percentage of platelet inhibition by clopidogrel was computed as the contribution of ADP-stimulated platelets to maximal clot strength (ADP inhibition): 100–100 x |(MAADP−MAFBRIN)/(MAFBRIN−MAHROMBIN−MAFBRIN)|, where MAADP is the ADP-induced clot strength (measurement of clopidogrel effect), MAFBRIN is the activator-induced clot strength (measurement of fibrin contribution), and MAHROMBIN is the thrombin-induced clot strength (maximum clot strength).
Study endpoints and definitions

The primary clinical safety endpoint of the present study was the incidence of major bleeding events. Major bleeding was quantified according to bleeding academic research consortium definition (BARC) criteria, including type 3 and 5 in the analysis. BARC 3 bleedings include 3a, 3b and 3c. BARC 3a was defined as overt bleeding plus hemoglobin drop of 3 to < 5 g/dL or any transfusion with overt bleeding. BARC 3b was defined as overt bleeding plus hemoglobin drop ≥ 5 g/dL, cardiac tamponade, bleeding requiring surgical intervention for control (excluding dental/nasal/skin/hemorrhoid) or bleeding requiring intravenous vasoactive agents. BARC 3c was defined as intracranial hemorrhage, subcategories confirmed by autopsy or imaging or lumbar puncture, or intraocular bleed compromising vision. BARC 5 bleeding was defined as probable or definite fatal bleeding [16]. The primary clinical efficacy end point of this study was a composite of cardiovascular death, nonfatal myocardial infarction (MI), unplanned target vessel revascularization (TVR), and stent thrombosis (ST). MI was defined according to the universal definition [1]. Unplanned TVR was defined as any intervention required (surgical or percutaneous) to treat luminal stenosis (>75% on angiography) in the same coronary vessel that was treated at the index procedure, within and beyond the target lesion during the 12-month follow-up period [17]. ST was defined as definite ST according to the Academic Research Consortium [18]. The composite ischemic events included the composite of cardiovascular death, nonfatal MI, unplanned TVR, and ST. Two independent physicians blinded to the laboratory data adjudicated events after reviewing the source documents.

Statistical analysis

Sample size calculation was based on our previous study, which included a cohort with the same selection criteria and treatment strategy. The previous study showed a significant association between the rs1045642 allele carriage and BARC ≥ 3 bleedings (p = 0.023). Therefore, we assumed the number of rs1045642 allele carriers was twice more than that of wild-type homozygotes. The study was designed on the basis of the superiority principle to achieve 80% power to observe an incidence of BARC ≥ 3 bleedings in the rs1045642 allele carriers of 10.5% and 3% in wild-type homozygotes. Thus, a total of 416 patients were needed. To compensate for loss to follow-up, we recruited a population of about 450 (Statistical software: PASS 11. NCSS, LLC. Kaysville, Utah, USA).

Continuous variables were presented as the mean ± standard deviation (SD) and compared using the Student’s t test, Mann–Whitney U test, or one-way analysis of variance (ANOVA) test, as appropriate. Categorical variables were expressed as frequencies and percentages, which were compared with a chi-square test (χ2) or Fisher exact test. After significant differences among variables were demonstrated by the ANOVA test, post hoc comparisons between the groups were performed with the Student-Newman-Keuls test for multiple comparisons. Under three models (codominant, dominant and recessive), the relationships between all enrolled SNPs and the adverse events were analyzed. Receiver operator curve (ROC) analysis was performed to identify the best discriminatory level of the TEG parameters associated with ischemic or bleeding events (MedCalc software, Mariakerke, Belgium). Clinical follow-up was censored on the day of the first cardiovascular event, which corresponded to the clinical endpoints. For subjects without a clinical event, clinical follow-up was censored either at the last clinic visit after 12 months of taking clopidogrel or on the day of clopidogrel discontinuation. A multivariate logistic regression model was used to test for an independent association of ABCB1 tag SNPs carriage and the value of ADP inhibition > 92.5% with BARC ≥ 3 bleeding events. Adjustment was made for the following risk factors: the GOF CYP2C19*17 allele status, age, sex, body mass index (BMI), renal function (serum creatinine), hypertension, hypercholesterolemia, diabetes mellitus, use of proton pump inhibitors, use of tirofiban. The odds ratio (OR) and the corresponding 95% CI were estimated for each variable included in the multivariate model. We used the SHEsis software platform (http://analysis.bio-x.cn/myAnalysis.php) to perform a haplotype reconstruction analysis for ABCB1 gene polymorphisms [19]. All statistical analyses were performed using SPSS ver. 18.0 (SPSS Chicago, IL), and a two-tailed probability value of < 0.05 was considered to be significant.

To validate the genetic associations and the predictive value of TEG in the study, we enrolled another cohort of 504 patients with STEMI in a second study from July 2012 to April 2013, based on the same inclusion and exclusion criteria. The second study protocol was basically the same as the first study. The logistic regression model was replicated in the second cohort.

Results

Study population

Samples available for genetic analysis were available from 452 STEMI patients (Fig. 1). All patients were from the Chinese Han population. The average age was 59 ± 12 years, 360 (79.6%) were men. PCIs were all performed with drug-eluting stents. Baseline demographics, clinical presentations and treatment were well balanced between the rs1045642 genotype groups (Table 1). Baseline characteristics were also balanced between the rs2235047, rs7799562 and rs7802783 genotype groups.

During one-year follow-up, 26 ischemic events occurred (5.8%), including 11 (2.4%) cardiovascular deaths, 3 (0.7%) nonfatal MIs, 7 (1.5%) TVR and 5 (1.1%) ST. A total of 43 BARC ≥ 3 bleeding events (9.5%) occurred, which included 11 (2.4%) cases of BARC 3b bleedings and 32 (7.1%) cases of BARC 3a bleedings.

Patients admitted due to STEMI between January 2011 and July 2012 (n=467)

CABG (n=7)

Patients with PCI without serious clinical events (n=460)

Exclusion criteria (n=4)

Bleeding history, hemodynamic instability, etc.

Incomplete tests (n=2)

Platelet measures or genotype

Loss from long-term follow-up (n=2)

Study population (n=452)

Long-term follow-up patients receiving aspirin and clopidogrel with both platelet measures and genotype

Fig. 1. Title: Flow chart describing the study population. Caption: STEMI: ST-elevation myocardial infarction; CABG: coronary artery bypass grafting; PCI: percutaneous coronary intervention.
The four-marker combination resulted in a multivariable logistic regression model demonstrated that carriage of rs1045642 (OR 2.943, 95%CI 1.195-7.247, P = 0.019) and rs7779562 (OR 0.453, 95%CI 0.219-0.936, P = 0.032) were independent predictors of BARC ≥ 3 bleedings. The TEG value of ADP inhibition > 92.5% also predicted the risk of bleeding events (OR 2.247, 95%CI 1.082-4.665, P = 0.03) (Table 3). The renal function variation [20]. Clinically, Bliden et al. [21] demonstrated that ROC analysis

The association of TEG parameters and bleeding events was assessed with ROC analysis. In comparison with MAADP and MATHERMOMIN, ADP inhibition had the best predictive value of BARC ≥ 3 bleedings yielding an area under the curve (AUC) of 0.707 (95% CI 0.662-0.749, p = 0.009; cut-off value >93.4%). ADP inhibition can also predict BARC ≥ 3 bleedings with bleeding events after PCI.

rs7779562 and rs7802783 showed significant associations with BARC ≥ 3 bleedings. Minor allele (T) carriers at rs1045642 ≥ 3 bleedings were carriage of rs1045642 and rs7779562 (OR 1.016, 95%CI 1.004-1.029, P = 0.011; OR 1.02, 95%CI 1.008-1.032, P = 0.001), use of proton pump inhibitors (OR 0.22, 95%CI 0.064-0.752, P = 0.016; 0.2957, 95%CI 1.368-6.392, P = 0.006; respectively). We then carried out second study (Table S2), and similar results were obtained in the repeat study. In the second cohort, the independent predictors of BARC ≥ 3 bleedings were carriage of rs1045642 (OR 2.708, 95%CI 1.14-6.437, P = 0.024) and rs7779562 (OR 0.416, 95%CI 0.206-0.841, P = 0.015), TEG value of ADP inhibition > 92.5% (OR 2.447, 95%CI 1.199-4.995, P = 0.014), female gender (OR 3.226, 95%CI 1.398-7.445, P = 0.006), the renal function (OR 1.02, 95%CI 1.008-1.032, P = 0.001), use of proton pump inhibitors (OR 0.186, 95%CI 0.055-0.633, P = 0.007) and use of tirofiban (OR 3.87, 95%CI 1.817-8.243, P < 0.001).

ABC1 tag SNPs and clinical endpoints

The clinical endpoints and their association with ABC1 tag SNPs were listed in Supplementary Material Table S1. The SNP genotypes of rs1045642, rs2235047, rs7779562 and rs7802783 showed significant associations with BARC ≥ 3 bleedings. Minor allele (T) carriers at rs1045642 ≥ 3 bleedings were carriage of rs1045642 and rs7779562 (OR 1.86, 95%CI 1.16-2.98, P = 0.009) while haplotype C-C-C-C was significantly with lower bleeding risk (OR 0.46, 95%CI 0.23-0.92, P = 0.02). There was no significant difference in the composite ischemic events between any genotype groups.

A multivariable logistic regression model demonstrated that carriage of rs1045642 (OR 2.943, 95%CI 1.195-7.247, P = 0.019) and
ADP inhibition < 30% could predict the combined ischemic outcome in patients receiving clopidogrel after PCI. In this study, ADP inhibition > 92.5% was shown to have a predictive value for BARC ≥ 3 bleeding events. Kwak et al. [22] reported that ADP inhibition > 76.5% was the only independent predictor of postoperative transfusion requirements (OR 11.44, 95%CI 2.77-47.3, P = 0.001) in patients who received clopidogrel within 5 days of off-pump CABG. This is concordant with our study.

Accumulating evidence shows genetic polymorphisms are important determinants of the large inter-individual variability in PR, aside from environmental and clinical factors [23]. ABCB1 encodes P-glycoprotein, an efflux transporter, which affects clopidogrel absorption [11]. Although several studies evaluated the relationship of ABCB1 polymorphisms with clopidogrel response or clinical outcomes, the results were inconclusive. In the current study, we found four tag SNPs were associated with bleeding events by single SNP and haplotype analysis. The logistic regression model identified two of them, ABCB1 rs1045642 and rs7779562, were independent predictors of BARC ≥ 3 bleedings.

However, we didn’t show a significant association between the GOF allele CYP2C19*17 with bleedings in our study. The frequency of CYP2C19*17 in our study population (1.6%) is much lower than that in Western populations [5]. Further studies with larger sample size might demonstrate a significant association between the CYP2C19*17 polymorphism and bleedings, which was not the aim of this study. Other studies with Chinese population reported similar results on CYP2C19*17 frequency [8,24]. Prior to our current study, only rs1045642 in ABCB1 was reported in other studies before, and this study also looked at other tag SNPs. The SNP rs1045642 (C3435T) is one of the most important markers of ABCB1 gene that were reported in many studies, but the results are inconclusive [13,14,25]. Although the rs1045642 is a synonymous SNP in exon 26, it was found to be associated with altered P-glycoprotein activity or reduced functionality by decreasing levels of mRNA expression [26], or changing the conformation of P-glycoprotein to alter the substrate specificity [27]. The decreased expression or function of P-glycoprotein efflux transporter may increase the systemic exposure to clopidogrel, its active metabolite and the clinical effects. As a result, the mutant T carriers of rs1045642 may have lower risk of ischemic events but higher risk of bleedings according to the biological plausibility. Data from PLATO trials showed that patients with the CC genotype of rs1045642 (wild-type homozygotes) had a higher rate of ischemic events than those with TC or TT genotype [14]. This result of PLATO trial suggested that the mutant T allele carriers may have higher clopidogrel absorption and clinical effect. Simon et al. [28] found that AMI patients with the TT and CT genotypes had a higher rate of subsequent ischemic events at 1 year than those with a CC wild-type genotype (15.5% vs. 10.7%; adjusted HR, 1.72; 95%CI 1.20-2.47). However, the rs1045642 polymorphism was not an independent predictor of the outcome in the subgroup of patients undergoing PCI in their study. The higher risk associated with the TT homozygous state was later confirmed in patients with ACS receiving clopidogrel in the TRITON TIMI 38 trial [13]. Discrepancies between their studies and our study could attribute to differences in ABCB1 SNP frequencies among ethnic groups, differences in study population and clopidogrel doses, or confounding by environmental factors. Although our data indicated that the ABCB1 polymorphisms were associated with bleedings after PCI in patients with STEMl, future prospective randomized clinical trials will be needed to test specific personalized antiplatelet algorithms to provide the evidence base necessary for widespread adoption into clinical practice.

**Study limitations**

First, because data from other platelet functional tests was not available in our hospital at that time, our study was limited by the use of only one method of platelet function test, the TEG platelet mapping assay. Second, this study was a monocentric rather than a multicenter
investigation. The sample size should be sufficient to draw a conclusion that may guide clinical practice but it was still not very large. Further studies in diverse populations with multiple platelet function tests are required.

Conclusions

In STEMI patients with clopidogrel administration after PCI, SNPs in ABCB1 genetic might have influence on bleedings, and ADP inhibition of TEG is predictive of bleeding risks.

Conflicts of interest

The authors declared no conflict of interest.

Acknowledgement

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Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2014.08.017.

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