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ABCB1 hypomethylation is associated with decreased antiplatelet effects of clopidogrel in Chinese ischemic stroke patients

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Current predictive models including the CYP2C19 polymorphism and clinical factors still explain only about 12% of variability of clopidogrel responsiveness. Up until recently, the precise mechanism of clopidogrel resistance remains unclear. P-glycoprotein (P-gp) encoded by ABCB1, a transmembrane calcium-dependent efflux pump for clopidogrel, implicated a role in clopidogrel resistance. In this present study, we investigated the methylation status of ABCB1 gene promoter in relation to ABCB1 mRNA expressions and the antiplatelet effects of clopidogrel. This study was a prospective cohort analysis of eligible stroke patients (n = 183, aged 18∼75 years) who received clopidogrel (75 mg/day) for at least 5 days before discharge. A final subcohort of 87 patients with CYP2C19*1/*1 genotype were enrolled in the study population. Patients were grouped in quartiles of maximum platelet aggregation (MPA values (Q1, Q2, Q3 and Q4, $MPA_{Q1} < 14.1\%$, $MPA_{Q4} > 35.4\%$). The methylation status of the ABCB1 promoter was 1.8 times in the Q1 MPA group ($10.1 \pm 2.4\%$) than in the Q4 MPA group ($5.5 \pm 2.1\%$) ($P < 0.001$). ABCB1 methylation correlated inversely with MPA ($R = -0.764$, $P < 0.001$) and mRNA expression ($R = -0.839$, $P < 0.001$). Results of a multivariate linear regression model demonstrated that ABCB1 methylation was independently associated with MPA ($\beta_{coefficient} = -4.71$, $P < 0.001$). ABCB1 expression was 0.62 times in the Q1 MPA group ($5.3 \pm 1.4‰$) than in the Q4 MPA ($8.5 \pm 2.5‰$), and the expression of ABCB1 correlated positively with ADP-induced MPA ($R = 0.791$, $P < 0.001$). ABCB1 promoter methylation status in whole blood appears to be inversely associated with ABCB1 mRNA expressions and MPA. In conclusions, hypomethylation of ABCB1 promoter is associated with a decreased response to clopidogrel in ischemic stroke patients *via* increased ABCB1 mRNA expression.

1. Introduction

Clopidogrel is an antagonist of the platelet P2Y12 adenosine diphosphate (ADP) receptor that prevents platelet aggregation Yang et al. 2012; Labarthe et al. 2012. Treatment with clopidogrel has become the standard protocol for the prevention of recurrent ischemic stroke Aw et al. 2012; James et al. 2012; Benavente et al. 2012. However, nearly one third of the patients are nonresponsive to clopidogrel as a result of high interindividual variability in platelet inhibition that has been associated with increased risk for ischemic events with severe clinical consequences Simon et al. 2009; Mega et al. 2009; Rideg et al. 2011. Current predictive models including the CYP2C19 polymorphism and clinical factors explain only about 12% of variability of clopidogrel responsiveness Geisler et al. 2011; Notarangelo et al. 2013; Zou et al. 2014. Up until recently, the precise mechanism of clopidogrel resistance still remains unclear.

It is well known that efflux trans-membrane P-glycoprotein (P-gp) encoded by ABCB1 is responsible for the active efflux of drugs from cells Sjostedt et al. 2014; Kis et al. 2013. Clopidogrel is absorbed in the intestine and this process is influenced by P-gp, which accelerates the efflux across cell membranes and limits the intestinal absorption of clopidogrel Taubert et al. 2006; Campo et al. 2011, indicating a possible relationship of P-gp/ABCB1 expression with the absorption of clopidogrel and its antiplatelet activity.

The expression of ABCB1 is mainly regulated by genetic factors Taubert et al. 2006; Xie et al. 2011 and epigenetic factors Oberstadt et al. 2013; Tomiyasu et al. 2014, but many studies reported that the antiplatelet effect of clopidogrel was not influenced by ABCB1 polymorphism Rideg et al. 2011; Jaitner et al. 2012. Therefore, we thought that ABCB1 epigenetic factors might be important causes to regulate the expression of ABCB1. The transcriptional regulation of ABCB1 expression through epigenetic mechanisms has been reported in expression of cancer cells Oberstadt et al. 2013; Tomiyasu et al. 2014. DNA methylation is one of the important contents of epigenetics Heberlein et al. 2013; Welch et al. 2013, and many researchers have reported that ABCB1 expression was influenced by DNA methylation in the ABCB1 promoter region Oberstadt et al. 2013; Tomiyasu et al. 2014; Reed et al. 2010; Chen et al. 2012. Moreover, the overexpression of the ABCB1 is negatively correlated with disease prognosis and reduced patient survival Baker et al. 2005. Therefore, a better understanding of ABCB1 methylation status may improve clopidogrel treatment efficacy. However, up to now, no studies have shown an association between the level of promoter methylation of ABCB1 and antiplatelet response to clopidogrel, the role of ABCB1 methylation in the antiplatelet response to clopidogrel is not known.

In the present prospective study, we investigated the promoter methylation status of ABCB1 in whole blood of patients with

Table 1: Demographic and clinical characteristics of 87 ischemic stroke patients with CYP2C19*1/*1 genotype according to the response of clopidogrel (MPA)

	Q1 (n = 23)	Q2 (n = 21)	Q3 (n = 21)	Q4 (n = 22)	p value
MPA, %	8.9 ± 3.5	18.9 ± 3.0	29.7 ± 3.8	50.9 ± 13.9	0.001
Age, yrs	59.6 ± 10.6	63.1 ± 8.1	63.5 ± 7.5	62.9 ± 8.0	0.400
Male, %	19(82.6)	16(76.2)	15(71.4)	14(63.6)	0.531
BMI, kg/m^2	24.4 ± 2.7	25.3 ± 3.2	24.2 ± 3.7	24.7 ± 2.6	0.693
Hypertension, %	15(65.2)	17(81.0)	15(71.4)	16(72.7)	0.712
Hyperlipidemia, %	11(47.8)	12(57.1)	11(52.4)	12(54.5)	0.937
Diabetes mellitus, %	7(30.4)	7(33.3)	8(38.1)	4(18.2)	0.525
Current smoking, %	8(34.8)	5(23.8)	9(42.9)	4(18.2)	0.289
Statins, %	22(95.7)	18(85.7)	21(100.0)	21(95.5)	0.236
ACE inhibitor, %	7(30.4)	7(33.3)	5(23.8)	11(50.0)	0.310
CCB, %	7(30.4)	11(52.4)	7(33.3)	8(36.4)	0.453
Platelet count, × 10^9/L	217.7 ± 57.2	206.1 ± 40.5	212.8 ± 70.7	202.0 ± 37.6	0.779
HDL, mmol/L	0.92 ± 0.21	0.93 ± 0.28	1.04 ± 0.23	0.98 ± 0.23	0.414
LDL, mmol/L	2.49 ± 0.75	2.48 ± 0.61	2.67 ± 0.76	2.61 ± 0.82	0.821

Patients were grouped in quartiles of MPA values (Q1, Q2, Q3 and Q4)
Values are n (%) or mean ± SD, MPA = maximum platelet aggregation; BMI = body mass index; Statins, including atorvastatin, lovastatin, or simvastatin; ACE = angiotensin-converting enzyme; CCB = Calcium-channel blocker; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

ischemic stroke, and evaluated the impact of ABCB1 methylation on the mRNA expressions and ADP-induced maximum platelet aggregation (MPA) in consecutive Chinese ischemic stroke patients treated with clopidogrel.

2. Investigations and result

2.1. Study population and response of clopidogrel

It is well known that CYP2C19 loss-of-function variants have a marked impact on the attenuated response to clopidogrel Mega et al. 2009; Rideg et al. 2011; Geisler et al. 2011; Notarangelo et al. 2013; Zou et al. 2013; Yang et al. 2013. To clarify the effect of the ABCB1 methylation on MPA, all patients carrying CYP2C19 variants were excluded from the present study. Therefore, of the 183 eligible ischemic stroke patients, 83 patients with CYP2C19*1/*2 genotype (mutant heterozygotes) and 13 patients with CYP2C19 *2/*2(mutant homozygotes) were excluded. Thus, a final subcohort of 87 patients with CYP2C19*1/*1 (wild-type homozygotes) were enrolled in the study population, as described elsewhere Yang et al. 2013. Platelet response to clopidogrel was categorized in to MPA quartiles. Individuals with MPA values within the first quartile (Q1, MPA < 14.1%) were considered good responders, while those who had MPA within the fourth quartile (Q4, MPA > 35.4%) were considered non-responders. Demographic and clinical characteristics of the study population according to the response of clopidogrel (MPA) are summarized in Table 1. Hyperlipidemia, hypertension and diabetes mellitus were the major risk factors for stroke patients, but their baseline and clinical characteristics of these patients were well balanced among the various clopidogrel response groups ($P > 0.05$).

2.2. Correlation of ABCB1 gene methylation with MPA

ABCB1 gene methylation level in whole blood was compared among MPA quartile groups, as shown in Fig. 1. The median value of ABCB1 methylation in the 87 ischemic stroke patients with CYP2C19*1/*1 (wild-type homozygotes) was 8.0% (IQR, 5.8 to 10.2%). The median ABCB1 methylation values among MPA quartile groups were as follows: 10.7 % (IQR, 8.1 to 11.6%) for Q1, 8.7% (IQR, 8.3 to 9.8%) for Q2, 6.8% (IQR, 6.0 to 9.5%) for Q3, and 4.6 % (IQR, 4.0 to 6.7%) for Q4. The ABCB1 methylation was significantly different among the four quartile groups ($P < 0.001$). Overall, ABCB1 methylation was 1.8 times in the Q1 MPA group (10.1 ± 2.4%) than in the Q4 MPA (5.5% ± 2.1%), a significant difference in ABCB1 gene methylation was observed in Q1 and Q4 groups ($P < 0.001$). In addition, ADP-induced MPA was significantly different between the 4 groups ($P < 0.001$) (Table 1). In a linear regression model, ABCB1 gene methylation was independently associated with MPA induced by ADP (20 μmol/L), the methylation status of the ABCB1 promoter correlated inversely with MPA (R = − 0.764, $P < 0.001$), suggesting that ABCB1 gene methylation might link to decreased MPA, as demonstrated in Fig. 2. On the other hand, ABCB1 hypomethylation might lead to increased MPA, resulting in a decreased response to clopidogrel.

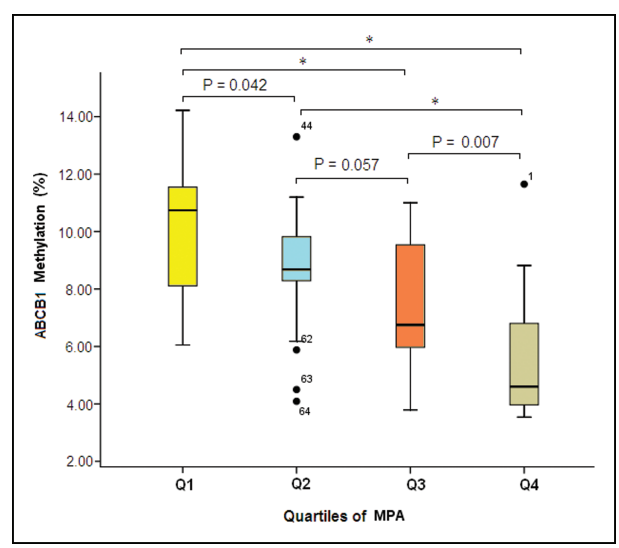

Fig. 1: Methylation status of ABCB1 promoter in whole blood according to the quartiles of antiplatelet effect of clopidogrel. Patients were grouped in quartiles of MPA values (Q1, Q2, Q3 and Q4, $MPA_{Q1} < 14.1\%$, $MPA_{Q4} > 35.4\%$). MPA was measured by light transmittance aggregometry in native platelet-rich plasma after addition of ADP at final concentrations of 20 μmol/L. Methylation status was measured by pyrosequencing. *P < 0.001; MPA, maximum platelet aggregation.

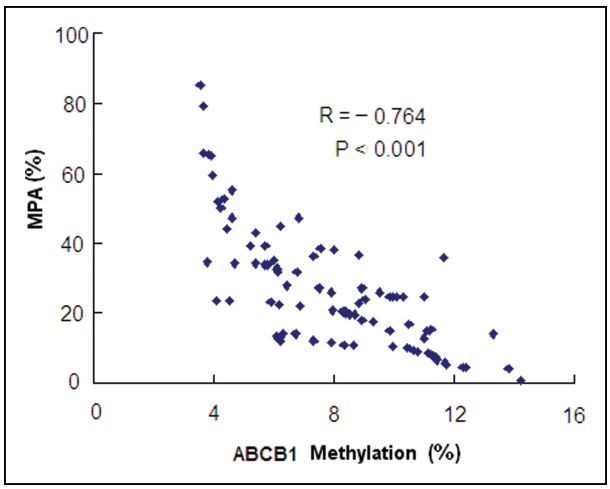

Fig. 2: The correlation of ABCB1 promoter methylation with the antiplatelet effect of clopidogrel. The effect of DNA methylation of ABCB1 on MPA was assessed by linear regression analysis. MPA, maximum platelet aggregation.

2.3. Relationship between ABCB1 methylation and ABCB1 expression

To determine the importance of DNA methylation in the expression of ABCB1, we analyzed the association between the methylation status and mRNA expression level of the ABCB1 gene in blood samples from 87 patients with ischemic stroke. In a linear regression model, we observed that the methylation status of the ABCB1 promoter correlated inversely with expression of ABCB1(R = - 0.839, $P < 0.001$), as shown in Fig. 3. That is, the up-regulation of ABCB1 mRNA had been associated with the hypomethylation of the promoter region, whereas the down-regulation of ABCB1 expression was associated with the hypermethylation of the CpG dinucleotides.

2.4. Correlation of ABCB1 gene expression with MPA

ABCB1 mRNA expressions in whole blood were compared among MPA quartile groups, as shown in Fig. 4. The median value of ABCB1 expression in the study population who were 87 patients with ischemic stroke was 5.7×10^{-3} (IQR, 4.8×10^{-3} to 7.7×10^{-3}). The median ABCB1 expression values among MPA quartile groups were as follows: 5.1×10^{-3} (IQR, 4.2×10^{-3} to 5.6×10^{-3}) for Q1, 5.3×10^{-3} (IQR, 4.6×10^{-3} to 6.2×10^{-3}) for Q2, 6.5×10^{-3} (IQR, 5.1×10^{-3} to 7.6×10^{-3}) for Q3, and 8.6×10^{-3} (IQR, 7.1×10^{-3} to 9.8×10^{-3}) for Q4. The ABCB1 expression was significantly different among the four quartile groups ($P < 0.001$). Overall, ABCB1 expression was 0.62 times in the Q1 MPA group ($5.3 \pm 1.4‰$) than in the Q4 MPA ($8.5‰ \pm 2.5‰$), a significant difference in ABCB1 gene expression was observed in Q1 and Q4 groups ($P < 0.001$).

These above findings were also confirmed by a linear model that showed a significant association of ABCB1 expression with platelet function after clopidogrel. The expression of ABCB1 correlated positively with ADP-induced MPA (R = 0.791, $P < 0.001$), suggesting that ABCB1 expression might lead to increased MPA, as demonstrated in Fig. 5. In addition, Results of a multivariate linear regression model demonstrated that ABCB1 methylation was independently associated with ADP-induced MPA measurements ($\beta_{coefficient}$ = - 4.71, $P < 0.001$; Table 2)

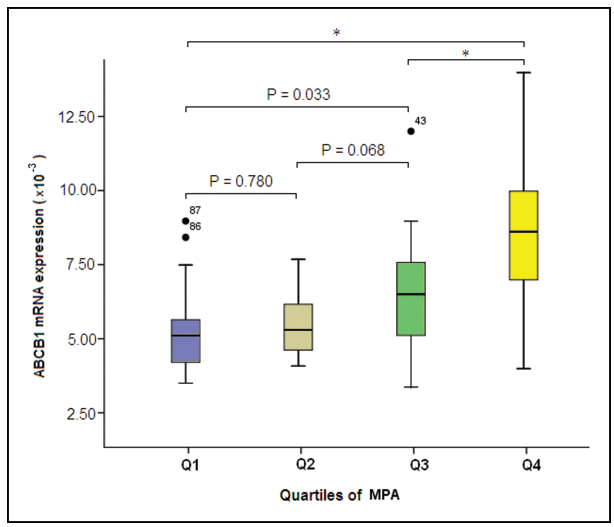

Fig. 4: ABCB1 gene expression in whole blood according to the quartiles of antiplatelet effect of clopidogrel. Patients were grouped in quartiles of MPA values (Q1, Q2, Q3 and Q4). MPA was measured by light transmittance aggregometry and ABCB1 mRNA expression was measured by qPCR. The ABCB1 methylations of four MPA quartiles (Q1 ~ Q4) were compared by Mann-Whitney test. *P < 0.001; MPA, maximum platelet aggregation.

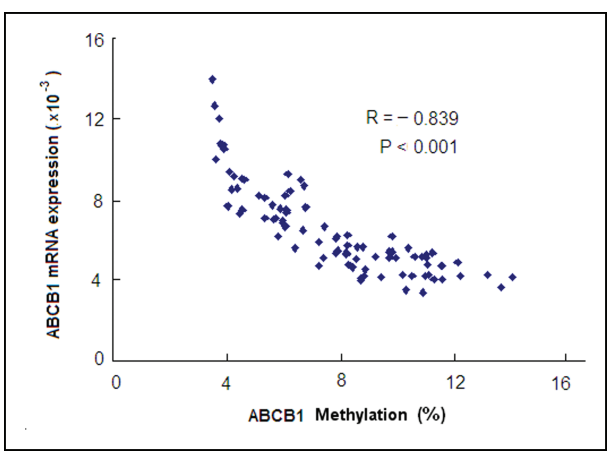

Fig. 3: The relationship between ABCB1 promoter methylation and mRNA expression in whole blood. The mRNA expression was measured by qPCR using β-actin as reference gene ($2^{-deltaCt}$ method). The effect of DNA methylation on mRNA expression of ABCB1 was assessed by linear regression analysis.

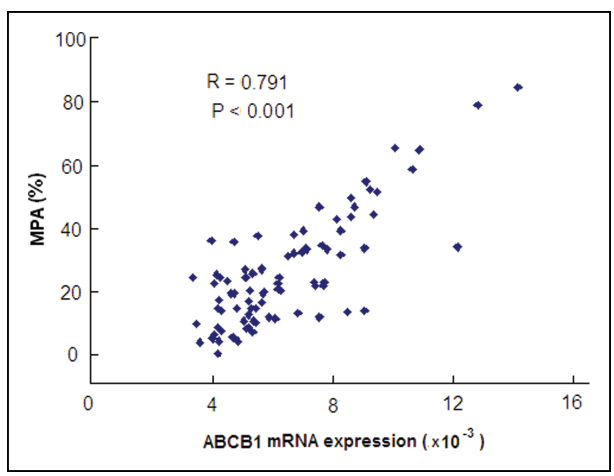

Fig. 5: The correlation of ABCB1 gene expression with the antiplatelet effect of clopidogrel. The effect of ABCB1 gene expression on MPA was assessed by linear regression analysis. MPA, maximum platelet aggregation.

Table 2: Association of the ADP-induced MPA as the dependent variable with other independent variables as determined by the multivariable linear regression model

Independent variable	β Coefficient		
	Value	SE	P
ABCB1 methylation	−4.71*	0.55*	< 0.001
Age	0.015β	0.18	0.936
Gender	3.52	4.00	0.382
Current smoking	1.79	3.37	0.596
BMI	−0.42	0.51	0.415
Hypertension	−1.22	3.43	0.723
Diabetes mellitus	−2.69	3.26	0.412
Hyperlipidemia	−1.97	3.14	0.533
HDL	1.58	7.06	0.823
LDL	0.92	2.05	0.654
Use of ACEI	1.23	3.33	0.713
Use of Statins	−4.16	5.92	0.485
Use of CCB	2.52	3.25	0.134

*Unadjusted β coefficient for ABCB1 methylation was −4.83 (SE = 0.44, $P < 0.001$). MPA = maximum platelet aggregation; BMI = body mass index; ACEI = angiotensin-converting enzyme inhibitor; CCB = Calcium-channel blocker; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

3. Discussion

Methylation is one of the most widely studied types of epigenetic modification of DNA and is known to play a significant role in the control of gene expression (Oberstadt et al. 2013; Tomiyasu et al. 2014). Although many studies have addressed that the methylation levels of the ABCB1 promoter were inversely correlated with ABCB1 expression in cancers Reed et al. 2010; Chen et al. 2012; Baker et al. 2005, there are no studies evaluating the correlation among the ABCB1 methylation status, ABCB1 expression and the antiplatelet effects of clopidogrel in the Chinese patients with ischemic stroke.

In the present study, we analyzed the correlation between the methylation status of CpG sites at ABCB1 promoter region and mRNA expression of ABCB1 gene in whole blood obtained from 87 Chinese patients with ischemic stroke. We found that ABCB1 methylation had a significant impact on ABCB1 expression and a statistically significant inverse correlation between the methylation status and expression of ABCB1 in blood samples. Our results suggested that ABCB1 hypomethylation status was associated with higher levels of ABCB1 expression, these results were consistent with the previous reports which proposed that the promoter methylation was a dominant epigenetic regulator of ABCB1 gene expression Reed et al. 2010; Chen et al. 2012; Baker et al. 2005.

In addition, we have also investigated the association between ABCB1 methylation status and MPA induced by ADP. We observed that the lower methylation status was significantly associated with higher MPA, which might be correlated with the occurrence of clopidogrel resistance. The hypomethylation status of the ABCB1 promoter region might be a necessary condition for ABCB1 gene overexpression, which might decrease the absorption of clopidogrel, and thus might lead to decreased antiplatelet effect and clopidogrel resistance in Chinese ischemic stroke patients.

To the best of our knowledge, this is the first study to evaluate the impact of the ABCB1 methylation on ABCB1 expression and ADP-induced MPA in consecutive Chinese ischemic stroke patients treated with clopidogrel. The association of ABCB1 methylation and the antiplatelet effects of clopidogrel has not been explored before. However, some limitations should also be noted. First, we assessed the impact of only one important region of methylation status in ABCB1 gene promoter on ADP-induced MPA. The interaction of other regions of ABCB1 promoter and their combined impact on gene expression and MPA were not studied. Second, the number of stroke patients was relatively small, which underscores the need for further studies to corroborate the present results.

In conclusion, ABCB1 promoter methylation status in the whole blood appears to be closely associated with ABCB1 mRNA expressions and the antiplatelet effects of clopidogrel. That is, CpG hypomethylation of ABCB1 promoter is associated with the decreased response to clopidogrel in ischemic stroke patients *via* increased ABCB1 expression and activity. These data suggest that routine evaluation of ABCB1 methylation in whole blood might serve as a biomarker of patients who are at higher risk of clopidogrel resistance and developing ischemic events when clopidogrel is used after ischemic stroke, which might assist in the selection of therapeutic protocols and provide further opportunities for subsequent clinical intervention in these patients.

4. Experimental

4.1. Subjects

The present study was conducted in Han Chinese patients with ischemic stroke. A total of 183 consecutive ischemic stroke patients (aged 18∼75 years) in the Nanjing First hospital (Nanjing, China) were enrolled in this study from July 2010 until July 2011. The exclusion criteria were active bleeding and bleeding diathesis, platelet count $< 100 \times 10^9$/L, severe renal or hepatic disorder, hematologic disorder, active malignancy, use of hormone replacement therapy or contraceptives, under thienopyridine, glycoprotein IIb/IIIa inhibitor or warfarin therapy, allergic reaction and also clopidogrel contraindication. The study was performed in accordance with the ethical principles of the Declaration of Helsinki and was approved by the ethics committee of Nanjing First Hospital, Nanjing Medical University. All patients gave written informed consent to study participation and blood sampling for genomic assays.

4.2. Study protocol

All patients were under clopidogrel therapy (75 mg/day) at least for 5 days and received other medication such as angiotensin-converting enzyme inhibitor (ACEI), statin and calcium-channel blockers (CCB). The predischarge samples were drawn in the morning on day 7 but before intake of clopidogrel using tubes containing 3.8 % sodium-citrate (NanGeer Biomedical Co., Ltd, Sichuan, China). Blood samples for aggregation testing were processed within 2 h after blood collecting. Information on age, gender, body mass index (BMI), systolic and diastolic pressure, diabetes, dyslipidemia, tobacco smoking, family history of disease and medications in use were recorded or derived from hospital admission records.

4.3. DNA extraction and bisulfite treatment

The -50G -box (-61 to -43) in human ABCB1 promoter contains G-rich sequences, which is an important region affecting ABCB1 promoter activity. Therefore, the methylation status of -50G -box was measured in present study. To evaluate the methylation status of ABCB1 promoter by pyrosequencing, genomic DNA was extracted from the whole blood using commercially available QIAamp DNA^{TM} Blood Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. Bisulfite treatment was performed on 1 μg of genomic DNA for methylated DNA detection by pyrosequencing using an EpiTect Bisulfite Kit (Qiagen, USA). Reactions were performed in a 140-μL volume with 85 μL bisulfite mix and 35 μL DNA protection buffer. The thermal cycler conditions were: 5 min at 95 °C, 25 min at 60 °C, 5 min at 95 °C, 85 min at 60 °C, 5 min at 95 °C, 75 min at 60 °C, and a final hold at 20 °C. The modified DNA was then used for methylation analysis of the ABCB1 promoter by pyrosequencing as described below.

4.4. Pyrosequencing for promoter methylation analysis

In the present study, quantitative ABCB1 DNA methylation analysis of the bisulfite treated DNA was performed by pyrosequencing, which is a highly accurate, sensitive and reproducible method to quantify methylation status

in a specific DNA sequence Mikeska et al. 2007; Karayan et al. 2010. We first identified the CpG islands in the DNA promoter of the ABCB1 gene, then we determined parameters to accurately quantify methylation status in this region by pyrosequencing. Methylation of target CpGs was assessed by determining the ratio of cytosine to thymine incorporated during pyrosequencing. Cytosine incorporation indicated a methylated CpG and thymine incorporation an unmethylated CpG. Pyrosequencing analysis and quantification of the methylation status were performed using the provided software from PSQ^{TM} 96MA System (Biotage, Uppsala, Sweden).

4.5. Quantitative PCR (qPCR) for ABCB1 mRNA expressions

ABCB1 mRNA levels were measured by qPCR. Total RNA was isolated and obtained from the whole blood samples using the TRIzol reagent (Invitrogen) following the manufacturer's protocols. The quantity of RNA was measured using a Nanodrop ND-1000 Spectrophotometer. Two μg of total RNA were subjected to genomic DNA digestion with DNAse (Qiagen, Valencia, CA, USA), amplifcation grade I (Invitrogen) to remove genomic DNA contamination. Isolated RNA samples were reverse transcribed to complementary DNA (cDNA) with the Superscript II Reverse Transcriptase (Invitrogen) and Oligo-dT18 (Invitrogen) kits under conditions described by the supplier. cDNA samples were kept at -20 °C for further quantitative TaqMan® real-time PCR (qPCR) analysis. Reactions were performed in 10 μL with a final concentration of 1 × Rotor-Gene SYBR Green PCR Kit (Qiagen). Forward and reverse primers were used at a concentration of 0.5 μM and were mixed with 2.5 μL 5-fold diluted cDNA. Reactions were carried out in a Rotor Gene Q thermocycler (Qiagen) with a hot-start stage step of 10 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. ABCB1 mRNA levels were measured by qPCR using β-actin as a reference for normalization. Sequences of the primers used are as follows: ABCB1 forward: 5'-TCGTGCCCTTGTTAGACAG-3', ABCB1 reverse: 5'-CATTCTGGATGGTGGACAGG-3'. The relative quantification for ABCB1 was measured using a comparative Ct method using the $2^{-deltaCt}$ formula, where delta Ct is the difference between the target gene Ct and the β-actin Ct.

4.6. Determination of MPA

The antiplatelet response to clopidogrel was evaluated by detection of platelet aggregation using a 4-channel LBY-NJ aggregometer (PuLiSheng, Beijing, China) according to manufacturer's instructions Bouman et al. 2010. MPA was the maximal amplitude of light transmission observed while residual platelet aggregation (RPA) was measured by light transmittance aggregometry (LTA) in native platelet-rich plasma (PRP) after addition of ADP (Sigma-Aldrich, Munich, Germany) at final concentrations of 20 μmol/L. Briefly, the PRP was prepared by centrifugation of citrated venous blood at 150 g for 15 min, and platelet-poor plasma (PPP) by centrifugation at 1,500 g for 20 min. PRP was adjusted to 200 $\sim$ 250 × 10^9 platelets/L by dilution with autologous PPP. Aggregation results were expressed as percentage of maximal light transmission using PPP from the same patient as reference (100% transmission). The coefficient of variation of our optical aggregometry assay was less than 10%. MPA was measured by the same laboratory staff unaware of patient's outcomes and methylation status results. All blood samples used for platelet aggregation testing were processed within 2 h of collection. *Ex vivo* platelet function testing was performed as described previously Zou et al. 2013; Yang et al. 2013.

4.7. Statistical analysis

Frequencies of categorical variables were given as counts (percentages) and continuous variables either as mean-standard deviation or as median with interquartile range as well as minimum and maximum values. Categorical variables were analyzed with Chi-square. Continuous variables with a Gaussian distribution were compared by means of the unpaired 2-tailed *t* test or ANOVA for > 2 groups, whereas continuous variables with a non-Gaussian distribution were compared by Kruskal-Wallis test or Mann-Whitney test. Correlation analysis between ABCB1 methylation, mRNA levels and MPA was assessed by linear regression analysis. Association of the MPA as the dependent variable with other independent variables as determined were performed using multivariate linear regression analysis. All statistical analyses were performed with SPSS 16.0 (SPSS Inc, Chicago, III, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

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