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The association of ABCC3 promoter methylation with clopidogrel response in Chinese ischemic stroke patients

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Multidrug resistance protein 3 (MRP3), encoded by ABCC3, is an ATP-dependent efflux pump mediating the transport of many drugs, implicated in clopidogrel resistance. This study enrolled 87 ischemic stroke patients with CYP2C19*1/*1 genotype, who received clopidogrel (75 mg/day) for at least 5 days before discharge. The maximum platelet aggregation (MPA) was measured by light transmittance aggregometry (LTA) to assess platelet function. Whole blood samples were obtained to evaluate the ABCC3 promoter methylation and mRNA expression of ABCC3. Pyrosequencing was carried out to investigate ABCC3 methylation and ABCC3 mRNA expression was evaluated by qPCR. The ABCC3 methylation was neither significantly different among the four MPA quartile groups ($P=0.275$) nor independently associated with MPA values ($R=0.100$, $P=0.358$). However, the ABCC3 promoter methylation status in 87 clinical samples from patients correlated inversely with the expression of ABCC3 ($R=-0.854$, $P<0.001$). In addition, the ABCC3 expression was neither significantly different among the four quartile groups ($P=0.499$) nor independently associated with MPA values ($R=0.060$, $P=0.582$). ABCC3 promoter methylation does not seem to exhibit any impact on MPA and clopidogrel response at all.

1. Introduction

Clopidogrel, an oral antiplatelet agent, is widely used for the prevention of recurrent ischemic stroke and vascular death in patients with recent stroke (Aw et al. 2012; Benavente et al. 2012; James et al. 2012). However, a substantial number of ischemic events with severe clinical consequences still occur after regularly taking clopidogrel as anti-platelet therapy (Fukuoka et al. 2011; Zhou et al. 2013). Some factors including the CYP2C19 polymorphism and clinical factors have been used to explain such a therapy failure (Mega et al. 2009; Paré et al. 2010; Simon et al. 2009); however, they may explain only a small 12% of the overall variation in response to clopidogrel (Notarangelo et al. 2013; Geisler et al. 2011; Zou et al. 2014). Up to now, the precise mechanism of clopidogrel resistance is still unknown.

It is well known that the multidrug resistance protein 3 (MRP3) encoded by ABCC3 is an ATP-dependent efflux pump mediating the transport of endogenous glucuronides and conjugated drug metabolites across cell membranes (Luchessi et al. 2012; Zou et al. 2013a; Zhao et al. 2013). A study reported that ABCC3 transporter might influence the efflux of many drugs on platelet membrane interfering with the drug availability and efficacy (Niessen et al. 2010). In addition, two clinical studies also demonstrated that ABCC3 mRNA expression is associated with the variation in response to clopidogrel (Luchessi et al. 2012, 2013) indicating a possible relationship of ABCC3 expression with the efflux of clopidogrel and its antiplatelet activity.

The expression of transporter gene is mainly regulated by its genetic factors (Fandino et al. 2013; Partanen et al. 2012) and epigenetic factors (Ding et al. 2004; Gao et al. 2009; Zolk et al. 2013), but many studies reported that the ABCC3 expressions were not influenced by ABCC3 polymorphism (Gradhand et al.

2007; Sasaki et al. 2011). In addition, the antiplatelet effect of clopidogrel was not influenced by ABCC3 polymorphism (Zou et al. 2013a). Therefore, we thought that ABCC3 epigenetic factors might be important causes for regulating the expression of ABCC3. So far, the transcriptional regulation of ABCC3 expression through epigenetic mechanisms has been reported in human squamous cell carcinoma (Zolk et al. 2013). DNA methylation is important in epigenetics (Heberlein et al. 2013; Welch et al. 2013). However, no information is available as to whether ABCC3 methylation is associated with mRNA expression of ABCC3 and the antiplatelet effect of clopidogrel in patients.

Thus, in the present prospective study we investigated such a potential association in a cohort of clopidogrel-treated Chinese ischemic stroke patients with CYP2C19*1/*1 to further explore the role of ABCC3 methylation in the mRNA expression and response to clopidogrel.

2. Investigations and results

2.1. Study population and response to clopidogrel

Many studies reported that carriage of the CYP2C19*2 loss-of-function genetic variants is significantly associated with attenuated platelet response to clopidogrel (Simon et al. 2009; Mega et al. 2009; Yang et al. 2013; Zou et al. 2013b). To clarify the effect of ABCC3 methylation on the antiplatelet effect of clopidogrel, all patients carrying CYP2C19*2 variants were excluded from the study population. Therefore, of the 183 eligible ischemic stroke patients, 83 patients with CYP2C19*1/*2 genotype and 13 patients with CYP2C19 *2/*2 were excluded. Thus,

Table 1: Demographic and clinical characteristics of the patients with ischemic stroke according to the quartiles of MPA values

	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
ACEI, %	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

Values are n (%) or mean ± SD. MPA = maximum platelet aggregation; BMI = body mass index; Statins, including atorvastatin, lovastatin, or simvastatin; ACEI = angiotensin-converting enzyme inhibitor; CCB = Calcium-channel blocker; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

87 patients with CYP2C19*1/*1 (wild-type homozygotes) were enrolled in the present study, as described elsewhere (Yang et al. 2013). The antiplatelet effect of clopidogrel was categorized according to the quartiles of maximum platelet aggregation (MPA). 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. Baseline characteristics of the study population according to the quartiles of MPA are summarized in the Table. The baseline variables were well balanced among the various clopidogrel response groups ($P > 0.05$).

2.2. ABCC3 methylation and MPA

ABCC3 gene methylation status in whole blood was compared among the MPA quartile groups, as shown in Fig. 1. The median value of ABCC3 methylation in the study population of 87 ischemic stroke patients with CYP2C19*1/*1 was 5.2% (IQR, 4.2 to 6.8%). The median ABCC3 methylation values among MPA quartile groups were as follows: 6.2% (IQR, 4.3 to 7.2%) for Q1, 5.7% (IQR, 3.6 to 6.2%) for Q2, 4.7% (IQR, 4.0 to 6.8%) for Q3, and 4.7% (IQR, 4.0 to 5.9%) for Q4. The ABCC3 methylation was not significantly different among the four MPA

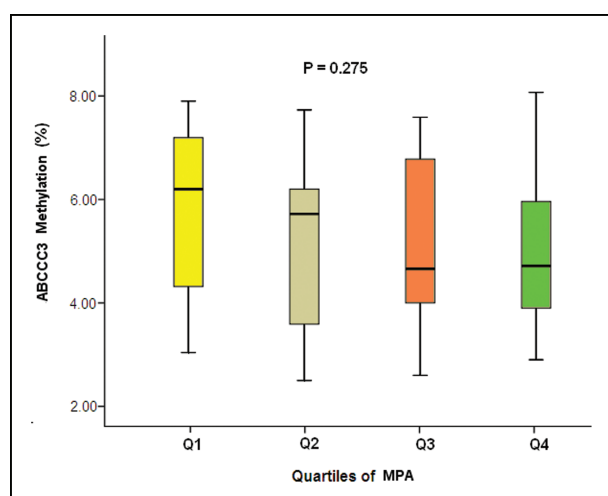


Fig. 1: ABCC3 promoter methylation status in whole blood according to the quartiles of MPA (Q1, Q2, Q3 and Q4). MPA, maximum platelet aggregation.

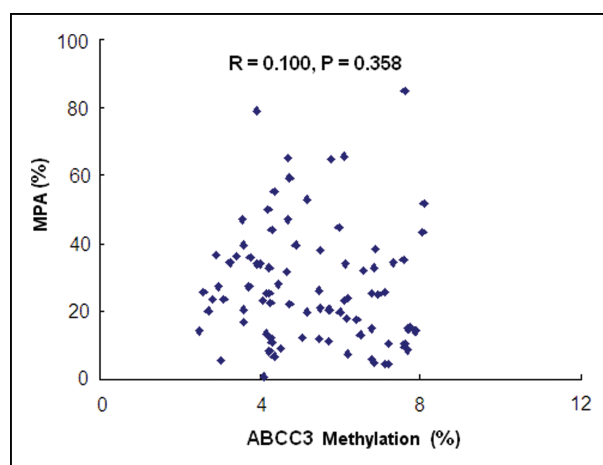


Fig. 2: The correlation of ABCC3 promoter methylation with MPA assessed by linear regression. MPA, maximum platelet aggregation.

quartile groups ($P = 0.275$). Overall, ABCC3 methylation was 1.16 times in the Q1 MPA group ($5.9 \pm 1.5\%$) than in the Q4 MPA ($5.1 \pm 1.5\%$), and no significant difference in ABCC3 gene methylation was observed in Q1 and Q4 groups ($P = 0.090$). Moreover, in a linear regression model, ABCC3 gene methylations was not independently associated with MPA values ($R = 0.100$, $P = 0.358$), suggesting that ABCC3 gene methylation might not link to MPA, as demonstrated in Fig. 2.

2.3. ABCC3 methylation and mRNA expression

In a linear regression model, we found that the ABCC3 promoter methylation status in 87 clinical samples from patients correlated inversely with the expression of ABCC3 ($R = -0.854$, $P < 0.001$), as shown in Fig. 3. This means that the down-regulation of ABCC3 mRNA has been associated with the ABCC3 promoter methylation, whereas the up-regulation of ABCC3 expression is associated with the hypomethylation of ABCC3.

2.4. ABCC3 mRNA expression and MPA

ABCC3 mRNA expression in whole blood was compared among the MPA quartile groups, as shown in Fig. 4. The median value of ABCC3 expression in the 87 ischemic stroke patients was 7.1×10^{-3} (IQR, 5.8×10^{-3} to 9.0×10^{-3}). The median

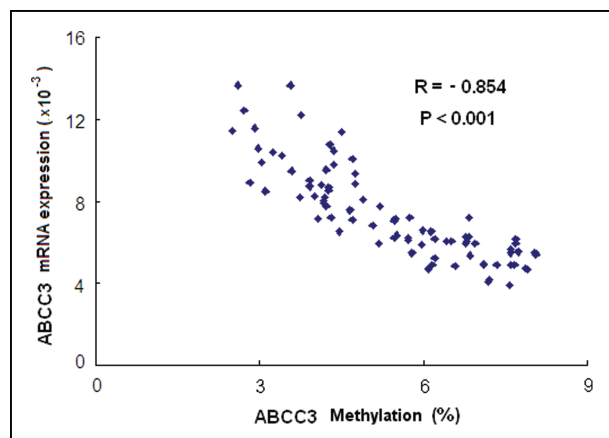


Fig. 3: The relationship between ABCC3 promoter methylation and mRNA expression in whole blood evaluated by linear regression analysis.

ABCC3 expression values among MPA quartile groups were as follows: 6.2×10^{-3} (IQR, 5.3×10^{-3} to 8.7×10^{-3}) for Q1, 7.2×10^{-3} (IQR, 6.0×10^{-3} to 8.6×10^{-3}) for Q2, 7.5×10^{-3} (IQR, 5.9×10^{-3} to 8.8×10^{-3}) for Q3, and 7.7×10^{-3} (IQR, 5.6×10^{-3} to 9.9×10^{-3}) for Q4. The ABCC3 expression was not significantly different among the four MPA quartile groups ($P=0.499$). Overall, ABCC3 expression was 0.88 times in the Q1 MPA group ($7.0 \pm 2.2\%$) than in the Q4 MPA ($8.0 \pm 2.6\%$), and no significant difference in ABCC3 gene expression was observed in Q1 and Q4 groups ($P=0.132$). These findings were confirmed by a linear model that showed no association of ABCC3 expression with MPA ($R=0.060$, $P=0.582$), as demonstrated in Fig. 5.

3. Discussion

This study is the first to investigate the impact of the ABCC3 methylation on its mRNA expression and ADP-induced MPA and was done in consecutive 87 Chinese ischemic stroke patients treated with clopidogrel. We observed that ABCC3 methylation has a significant impact on ABCC3 expression and a statistically significant inverse association between ABCC3 methylation status and mRNA expression in the whole blood samples, which suggested that ABCC3 promoter hypomethylation might be a necessary condition for the over-expression of ABCC3. However, the methylation of ABCC3 was not significantly associated with the response to clopidogrel.

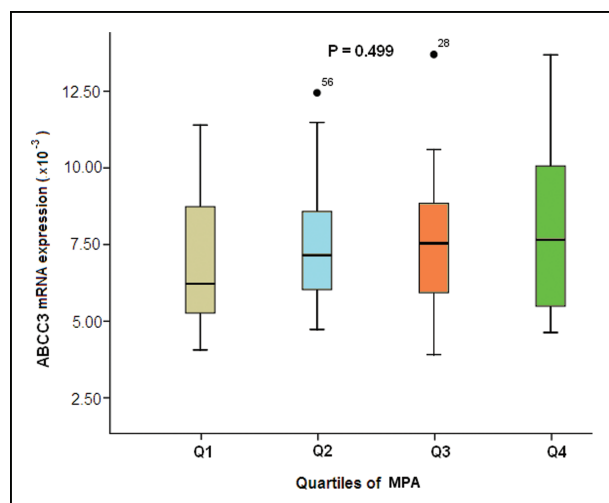


Fig. 4: ABCC3 gene expression in whole blood according to the quartiles of MPA (Q1, Q2, Q3 and Q4). MPA, maximum platelet aggregation.

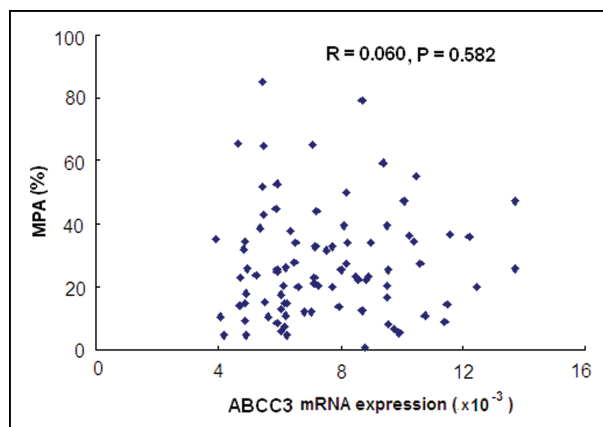


Fig. 5: The correlation of ABCC3 gene expression with MPA assessed by linear regression analysis. MPA, maximum platelet aggregation.

In addition, some limitations need to be discussed in our study. First, only 1 important region methylation status in ABCC3 gene promoter was determined to assess the impact on the antiplatelet effects of clopidogrel. Second, the number of patients with ischemic stroke was relatively small, so further studies are needed to corroborate the present results.

In conclusion, ABCC3 promoter methylation status in whole blood appears to be associated closely with ABCC3 gene mRNA expressions, which does not seem to exhibit any impact on MPA or clopidogrel response at all.

4. Experimental

4.1. Subjects

The present study was performed in ischemic stroke patients in the Department of Neurology of Nanjing First hospital in China. A total of 183 consecutive ischemic stroke patients (aged 18~75 years) were eligible for the inclusion criteria and enrolled in this study, the inclusion period lasted 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, body mass index (BMI) < 18.5 or $> 40 \text{ kg/m}^2$, use of hormone replacement therapy or contraceptives, under thienopyridine, glycoprotein IIb/IIIa inhibitor or warfarin therapy, allergic reaction and also clopidogrel contraindication. The study protocol was approved by the ethics committee of Nanjing First Hospital, Nanjing Medical University and performed in accordance with the ethical principles of the Declaration of Helsinki. All patients signed their written informed consent prior to participation.

4.2. Study protocol

All patients were under clopidogrel therapy (75 mg/day) at least for 5 days. Other medications such as angiotensin-converting enzyme inhibitors (ACEI), statins or calcium-channel blockers (CCB) were also administered. Blood samples were drawn in the morning of 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, smoking status, family history of disease and medications in use were recorded or derived from hospital admission records.

4.3. DNA extraction and bisulfite treatment

The position (-8363 bp) in human ABCC3 promoter contains G-rich sequences, which is an important region affecting ABCC3 promoter activity. Therefore, the methylation status of the position (-8363 bp) was determined in the present study. To evaluate the methylation status of ABCC3 promoter by pyrosequencing, genomic DNA was extracted from the whole blood using commercially available QIAamp DNATM 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). Modified DNA was then used for methylation analysis of the ABCC3 promoter by pyrosequencing as described below.

4.4. Pyrosequencing for promoter methylation analysis

ABCC3 promoter methylation analysis of the bisulphite 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 ABCC3 promoter, 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™ 96MA System (Biotage, Uppsala, Sweden).

4.5. Quantitative PCR (qPCR) for ABCC3 mRNA expressions

Total RNA was extracted from the whole blood samples using the TRIzol reagent (Invitrogen). The quantity of RNA was measured using a Nanodrop ND-1000 Spectrophotometer. Total RNA (2 µg) was subjected to genomic DNA digestion with DNase (Qiagen, Valencia, CA, USA), amplification 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. 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 1X Rotor-Gene SYBR Green PCR Kit (Qiagen) and carried out in a Rotor Gene Q thermocycler (Qiagen). ABCC3 mRNA expressions were measured by qPCR using β-actin as a reference for normalization. The relative quantification for ABCC3 was measured using a comparative Ct method using the $2^{-\Delta\Delta Ct}$ formula.

4.6. Determination of MPA

The antiplatelet response to clopidogrel was evaluated by detection of platelet aggregation (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 $200 \sim 250 \times 10^9$ 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). 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. Categorical variables were analyzed with Chi-square test. 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 ABCC3 methylation, mRNA levels and MPA was assessed by linear regression analysis. All statistical analyses were performed with SPSS 16.0 (SPSS Inc, Chicago, Ill, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

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