Association of ABCB1 promoter methylation with aspirin exposure, platelet function, and clinical outcomes in Chinese intracranial artery stenosis patients

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Abstract
Purpose DNA methylation typically acts to repress gene transcription. ABCB1 is involved in the intestinal absorption of aspirin. We aimed to investigate the impact of methylation status of ABCB1 promoter on aspirin exposure, platelet function, and clinical outcomes in Chinese intracranial artery stenosis patients receiving antiplatelet treatment.

Methods Symptomatic intracranial artery stenosis patients (without carrying CYP2C19 loss-of-function alleles) receiving antiplatelet therapy were enrolled in this study. The clinical outcome was the composite events, vascular death, recurrent ischemic stroke, myocardial infarction, or transit ischemic attack. Patients were divided into cases and controls based on the 1-year follow-up. Venous blood samples were collected for methylation level analysis, drug determination, and thromboelastographic assay. The Pearson correlation analysis was used to investigate the association of potential influencing factors and visualize them using three-dimensional plot. Receiver operator curves were applied to compare the diagnostic performance of potential factors and calculate the cut-off values.

Results We assessed 438 patients, 30 (non-carrier of CYP2C19 loss-of-function alleles) experienced adverse clinical events, and 30 patients without clinical events were selected as controls. Total of 34 CpG methylation sites were investigated for ABCB1 methylation. Compared with controls, the cases had significant lower methylation levels (CpG21.22), lower salicylic acid concentration, and lower arachidonic acid inhibition (P value < 0.05). A cut-off point of CpG21.22 0.015 was identified with a specificity of 0.759.

Conclusion ABCB1 hypomethylation is associated with lower drug absorption, higher platelet reactivity, and an increased risk of ischemic events in our patients. This may provide important insights into the research of aspirin resistance.

Keywords ABCB1 · Methylation · Aspirin · Salicylic acid · Thromboelastography · Ischemic events

Introduction
Aspirin is the most widely prescribed drug for the primary and secondary prevention of cardiovascular and cerebrovascular diseases [1, 2], and its clinical efficacy is well established [3, 4]. Despite the development of new antiplatelet drugs, aspirin is still considered as the gold standard of antiplatelet treatment [5, 6]. However, aspirin therapy faces the challenge of reduced responsiveness. A certain proportion of ischemic patients receiving aspirin will experience recurrent ischemic events related to treatment failures. This clinical failure has sometimes
been called aspirin resistance or non-response [7]. Aspirin resistance is defined as the absence of the expected pharmacological effect on platelets or poor clinical outcome. Depending on the population studied, the prevalence of aspirin resistance is estimated to be between 5.5 and 61% [8]. However, the major reason of aspirin poor response is still unclear. Aspirin resistance was previously identified in patients and healthy volunteers with low dose aspirin; therefore, lower bioavailability and poor absorption may result in inadequate platelet inhibition, particularly in heavier subjects [9, 10]. Absorption may be a pivotal factor regarding to aspirin resistance.

DNA methylation is a process by which the methyl groups are added to the DNA molecule. Methylation can change the activity of a DNA segment without changing the sequence. When located in a gene promoter, DNA methylation typically acts to repress gene transcription. In mammalian cells, DNA methylation occurs mainly at the C5 position of CpG dinucleotides. Methylation in the promoter regions correlates negatively with gene expression [11, 12]. ABCB1 is a protein coding gene, and the protein encoded by ABCB1 is an ATP-dependent drug efflux pump for xenobiotic compounds with a broad substrate specificity. It has been reported that aspirin efflux occurs through the ABCB1 transporter [13].

At present, little is known about the association of ABCB1 promoter methylation and aspirin resistance in intracranial artery stenosis. Therefore, our study was performed with an aim to explore the impact of ABCB1 methylation on aspirin exposure, platelet function, and clinical outcome in Chinese patients with symptomatic intracranial artery stenosis.

Methods

Study patient

Chinese symptomatic intracranial artery stenosis patients in Beijing Tiantan Hospital were enrolled into our study. The following inclusion criteria were applied to identify patients: symptomatic intracranial artery stenosis [stroke or transient ischemic attack (TIA)] attributable to 70% or greater stenosis of a major intracranial artery and treatment with dual-antiplatelet (enteric aspirin 100 mg/day plus clopidogrel 75 mg/day) for 3 months, followed by treatment with enteric aspirin alone (100 mg once daily). Patients with hemorrhagic stroke, moyamoya disease, atrial fibrillation, hematological diseases, active peptic ulcer disease, and bleeding tendency were excluded from our study.

It is known that CYP2C19 loss-of-function variants having an impact on clopidogrel poor response [14]. To eliminate the interference of clopidogrel poor response and the maximal effect of the degree of DNA methylation on platelet function and clinical events, CYP2C19 loss-of-function allele carrier patients (CYP2C19*2 or *3) were also excluded from our study [15, 16]. The clinical characteristics of enrolled patients, such as gender, age, weight, body mass index (BMI), hypertension, diabetes, hyperlipidemia, coronary heart disease, prior cerebral infarction, family history of stroke, and smoking and drinking were recorded or retrieved from the hospital admission records. This study was approved by the Institutional Review Board of Beijing Tiantan Hospital (ID KY2014-051-01), and written informed consents were obtained from patients or their close relatives.

Study design

The clinical outcome was the composite events, vascular death, recurrent ischemic stroke, myocardial infarction, or TIA. Death was defined as vascular mortality due to ischemic stroke, myocardial infarction, or other vascular causes. Ischemic stroke is a new focal neurologic deficit of sudden onset, unassociated with hemorrhage on MRI or CT, and lasting at least 24 h. Myocardial infarction was diagnosed if two of the three criteria, i.e., typical symptoms, increased cardiac enzyme levels, and diagnostic electrocardiographic changes, were met. TIA is a transient episode of neurologic dysfunction caused by ischemia—either at a focal brain location, spinal cord, or retina—without acute infarction, which lasts for at least 10 min, but resolves within 24 h regardless of diffusion-weighted MR images changes [17, 18]. All enrolled patients were followed up, and the occurrence of clinical events was identified during 1-year follow-up visits or through phone interviews by our investigators. Two independent investigators who classified the clinical events were blinded to the results of methylation assay, drug exposure, and platelet function. Based on the presence or absence of ischemic events, patients were stratified into the “case-group” or the “control group.”

Methylation levels should be measured for all the controls in an ideal situation, but the testing cost prohibited this. We, therefore, selected an equal number of patients without clinical events as the control group to match those patients in the case-group. To provide a best match for a “case patient,” we carefully screened control patients and selected a similarly matched “control patient” for every “case patient.” For example, the two pair-matched patients had either the same or similar characteristics with regard to age, gender, BMI, with or without clinical interventions, smoking and/or drinking status, and disease states such as hypertension, hyperlipidemia, diabetes, coronary heart disease, history of stroke, and family history of stroke. Because we only had a limited number of patients in the control group, an exact pair-match might not be possible. However, there is no statistically significant difference between the control patients and case patients with regard to either clinical features or disease states. This process eliminated the interference of other factors and elucidated the
impact of the DNA methylation level on drug exposure, platelet function, and clinical events [19].

**Drug concentration determination**

Venous blood samples were obtained from the cubital vein after 5 days of dual-antiplatelet treatment (45–75 min after administration). The blood samples were centrifuged for 5 min at 12,000 rpm (10,000 g), and the supernatants were taken and stored in a refrigerator (−80 °C) for the analysis of aspirin and salicylic acid (SA) concentrations. Samples were analyzed by a modified ultra-performance liquid chromatographic (UPLC) method (BEH C18 column, 2.1 × 100 mm, 1.7 μm) as previously reported [20]. Water (contains 0.05% trifluoroacetic acid) and acetonitrile (78:22%) were used as the mobile phase, and the flow rate was 0.5 mL·min⁻¹. Tinidazole was used as an internal standard. The temperatures of the column and the autosampler were kept at 30 and 4 °C, respectively. The detecting wavelengths were set at 304 and 277 nm. The calibration curves of aspirin and SA were linear ($r^2 > 0.999$) within the range of 0.1–100 and 0.2–50 μg mL⁻¹, respectively. The intra- and inter-day precisions in all samples were less than 15%, while the accuracy was within ±15% of the nominal value. The UPLC method developed in this study for the determination of aspirin and SA in human plasma is accurate, sensitive, and stable.

**Methylation assay**

Human genomic DNA (gDNA) was extracted from leucocytes in the venous blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA concentrations were determined using a NanoDrop 1000 Spectrophotometer (PEQLAB, Erlangen, Germany), and all concentrations were >500 ng/μL. The gDNA samples were bisulfite treated using EpiTect Bisulfite Kits (Qiagen, Hilden, Germany). After bisulfite conversion, only unmethylated cytosines were converted to uracils, while methylated cytosines remained unchanged [21]. The bisulfite treated gDNA was subjected to polymerase chain reaction (PCR) amplification of the target regions (GRCh38.p7: 87600640-87600208, 432 bp with coverage 34 CpG sites) in ABCB1 gene promoter, and the primer pairs are listed in supplementary material. A total of 34 CpG methylation sites were investigated for ABCB1 methylation using the Sequenom EpiTYPER technology. A cytosine incorporation indicated a methylated CpG whereas a thymine incorporation indicated an unmethylated CpG. CpG methylation was assessed by determining the ratio of cytosine to thymine. Quantification of the methylation status was performed by the EpiTYPER software (Version 1.0, Sequenom, San Diego, CA, USA), generating methylation levels for each CpG dinucleotide or an aggregate of multiple CpG dinucleotides.

**Platelet function analysis**

Thromboelastography (TEG) is used clinically to assess platelet function and to provide a comprehensive assay of the overall clotting process [22]. Platelet function was assessed using the TEG platelet mapping system (Haemoscope Corporation, Niles, Illinois, USA). Whole blood from the cubital vein after 5 days treatment was pipetted into each channel, which consists of an oscillating cup into which a pin is suspended. Four channels were used to detect the effects of antiplatelet treatment acting through the arachidonic acid (AA) and the adenosine diphosphate (ADP) pathways. The thrombin channel contains Kaolin which causes maximal activation of thrombin, and $\text{MA}_{\text{Thrombin}}$ was defined as maximum amplitude and obtained by transferring blood to this channel. The fibrin channel contains Activator F (a mixture of reptilase and factor XIIIa). To detect the $\text{MA}_{\text{Fibrin}}$, representing the fibrin contribution to clot strength, heparinized blood was transferred to this channel. The third was the AA channel which contains Activator F and AA. The contribution of cyclooxygenase-1 (COX-1) pathway to clot formation was determined by the addition of blood to this channel, and $\text{MA}_{\text{AA}}$ reflects the platelet activity to AA-induced clot strength. The last was the ADP channel that contains Activator F and ADP. Likewise, the ADP-induced clot strength ($\text{MA}_{\text{ADP}}$) was assessed by pipetting blood into this channel. The AA inhibition (AA%) and ADP inhibition (ADP%) were calculated by the following equations [19]:

\[
\text{ADP} = 100\% - \frac{\text{MA}_{\text{ADP}} - \text{MA}_{\text{Fibrin}}}{\text{MA}_{\text{Thrombin}} - \text{MA}_{\text{Fibrin}}} \times 100\% \quad (1)
\]

\[
\text{AA} = 100\% - \frac{\text{MA}_{\text{AA}} - \text{MA}_{\text{Fibrin}}}{\text{MA}_{\text{Thrombin}} - \text{MA}_{\text{Fibrin}}} \times 100\% \quad (2)
\]

Finally, three parameters ($\text{MA}_{\text{Thrombin}}$, AA%, and ADP%) were obtained and all parameters were automatically calculated by the TEG software.

**Statistical analysis**

Continuous data were presented as mean ± standard deviation (SD), and the one-sample Kolmogorov-Smirnov test was applied to test for a normal distribution. Continuous data was analyzed using $T$ test (normal distribution) or Mann-Whitney $U$ test (non-normal distribution). Categorical variables were presented as numbers and percentages, and they were analyzed using Pearson’s $\chi^2$ test or Fisher’s exact test (when an expected value of any cell is less than 5). Pearson correlation analysis was used to assess the correlations between ABCB1 promoter methylation, drug concentration, residual platelet reactivity, and clinical events. A receiver operator curve (ROC) analysis was applied to test the ability of influencing factors to predict clinical endpoints. A $P$ value < 0.05 was
considered to be statistically significant. Statistical analyses were performed using SPSS Statistics for Windows (Version 19.0, Armonk, NY IBM Corp. USA).

Results

Patient characteristics

Through clinic visits or telephone calls, we followed up 438 patients for 1 year. According to the inclusion/exclusion criteria, 30 patients experienced adverse clinical events and were enrolled in our study and they were categorized as the case-group. Among patients who did not develop ischemic events, 30 patients were selected as matched-controls. However, we failed to extract gDNA from 2 participants (in case-group) and they were excluded. Finally, only 28 patients were in the case-group. The methylation levels of ABCB1 gene promoter were performed for these 58 patients with CYP2C19*1/*1 (wild-type homozygotes). The baseline characteristics and demographics of the enrolled patients are provided in Table 1. The majority of the patients were male in both case (89.3%) and control (93.3%) groups. Given that body weight, BMI, diabetes, hypertension, hyperlipidemia, coronary artery disease, prior cerebral infarction, family history of stroke, and smoking and/or drinking may be risk factors for ischemic stroke, the baseline characteristics of cases and controls were balanced. Of the 28 cases, 3 patients died, 12 had a recurrence of ischemic stroke, 6 had coronary ischemic events, and 7 had TIA during the 1-year follow-up.

Influencing factors selection

In the fragment we selected, many CpG sites were continuous detection sites (such as CpG6.7.8.9, CpG10.11.12, and CpG13.14.15.16) and only the average of the methylation levels at these CpG sites could be measured by Sequenom EpiTYPER technology. To identify the potential influencing factors for the recurrent of clinical events, the differences of the methylation level, aspirin concentration, SA concentration, and TEG parameter (MAthrombin, ADP%, and AA%) between cases and controls were analyzed using T test or Mann-Whitney U test (Table 2). The methylation levels in the two CpG sites (CpG21.22), SA concentrations, and AA% were significantly related to clinical endpoints (P value < 0.05). Methylation status, SA concentration, and AA% may be potential influencing factors for developing adverse clinical events, and the following statistical analysis will focus on them.

Correlation of CpG methylation, drug concentration and platelet function

Pearson correlation analysis of potential factors was performed to explore the relationship of the identified three factors (Table 3). A significant positive correlation was observed between the CpG21.22 methylation level and SA concentration (R = 0.561, P value < 0.001). Likewise, SA concentration and AA% also had a positive correlation (R = 0.327, P value = 0.015). In our data, the control and the case was defined as 1 and 2, respectively. AA% and cases had a significant negative correlation (R = -0.388, P value = 0.003), suggesting higher AA% associated with the lower risk of recurrent clinical events. To visualize the data, we created a scatter three-dimensional plot using the CpG21.22 methylation level, SA concentration, and AA% (Fig. 1). A marker is plotted at each point defined by the points in the vectors x-axis (SA), y-axis (CpG21.22), and z-axis (AA%). According to distribution of markers, AA% increased with the increase of CpG methylation level and SA concentration, which agreed well with Pearson correlation analysis.

Receiver operating characteristic curve analysis

ROC curve is a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold is varied. The curve is created by plotting the true positive rate (sensitivity) against the false positive rate (1—specificity) at various threshold settings. The closer the ROC curve is to the upper left corner, the higher the overall accuracy of the test; therefore, the area under the ROC curve (AUC) is a measure
of how well a parameter can distinguish between two diagnostic groups. Our ROC found that methylation of CpG21.22, SA concentration, and AA% had good predictive value of clinical endpoints: AUC 0.796 (95%CI = 0.677–0.916, P value < 0.001), 0.751 (95%CI = 0.622–0.879, P value = 0.001), and 0.705 (95%CI = 0.566–0.844, P value = 0.009), respectively (Fig. 2). Each point on the ROC curve represents a sensitivity/specificity pair corresponding

Table 2: The differences of the methylation level, aspirin concentration, SA concentration, thromboelastography parameters ($MAT_{thrombin}$, ADP%, and AA%) between cases and controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case-group (N)</th>
<th>Control group (N)</th>
<th>Kolmogorov-Smirnov test, P value</th>
<th>T test or Mann-Whitney U test, P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG1</td>
<td>0.008 ± 0.014 (28)</td>
<td>0.009 ± 0.014 (30)</td>
<td>&lt;0.05</td>
<td>0.993</td>
</tr>
<tr>
<td>CpG2</td>
<td>0.005 ± 0.011 (28)</td>
<td>0.003 ± 0.008 (30)</td>
<td>&lt;0.05</td>
<td>0.638</td>
</tr>
<tr>
<td>CpG3</td>
<td>0.008 ± 0.009 (28)</td>
<td>0.010 ± 0.013 (30)</td>
<td>&lt;0.05</td>
<td>0.694</td>
</tr>
<tr>
<td>CpG4.5</td>
<td>0.001 ± 0.003 (28)</td>
<td>0.001 ± 0.004 (30)</td>
<td>&lt;0.05</td>
<td>0.678</td>
</tr>
<tr>
<td>CpG6.7.8.9</td>
<td>0.048 ± 0.040 (27)</td>
<td>0.039 ± 0.013 (30)</td>
<td>&lt;0.05</td>
<td>0.584</td>
</tr>
<tr>
<td>CpG10.11.12</td>
<td>0.048 ± 0.033 (27)</td>
<td>0.041 ± 0.017 (30)</td>
<td>&lt;0.05</td>
<td>0.868</td>
</tr>
<tr>
<td>CpG13.14.15.16</td>
<td>0.054 ± 0.038 (27)</td>
<td>0.053 ± 0.017 (29)</td>
<td>&lt;0.05</td>
<td>0.214</td>
</tr>
<tr>
<td>CpG17</td>
<td>0.073 ± 0.064 (28)</td>
<td>0.093 ± 0.079 (30)</td>
<td>0.119</td>
<td>0.310</td>
</tr>
<tr>
<td>CpG18</td>
<td>0.005 ± 0.011 (28)</td>
<td>0.003 ± 0.008 (30)</td>
<td>&lt;0.05</td>
<td>0.638</td>
</tr>
<tr>
<td>CpG19.20</td>
<td>0.001 ± 0.003 (28)</td>
<td>0.004 ± 0.011 (30)</td>
<td>&lt;0.05</td>
<td>0.316</td>
</tr>
<tr>
<td>CpG21.22</td>
<td>0.010 ± 0.010 (28)</td>
<td>0.023 ± 0.013 (30)</td>
<td>0.066</td>
<td>0.040*</td>
</tr>
<tr>
<td>CpG23</td>
<td>0.035 ± 0.028 (28)</td>
<td>0.027 ± 0.015 (30)</td>
<td>&lt;0.05</td>
<td>0.388</td>
</tr>
<tr>
<td>CpG24.25</td>
<td>0.036 ± 0.031 (27)</td>
<td>0.048 ± 0.029 (29)</td>
<td>0.238</td>
<td>0.118</td>
</tr>
<tr>
<td>CpG26.27</td>
<td>0.119 ± 0.087 (28)</td>
<td>0.155 ± 0.069 (30)</td>
<td>0.885</td>
<td>0.082</td>
</tr>
<tr>
<td>CpG28.29.30</td>
<td>0.079 ± 0.079 (27)</td>
<td>0.073 ± 0.073 (30)</td>
<td>&lt;0.05</td>
<td>0.879</td>
</tr>
<tr>
<td>CpG31</td>
<td>0.244 ± 0.044 (27)</td>
<td>0.244 ± 0.025 (30)</td>
<td>0.259</td>
<td>0.934</td>
</tr>
<tr>
<td>CpG32</td>
<td>0.000 ± 0.000 (28)</td>
<td>0.001 ± 0.003 (29)</td>
<td>&lt;0.05</td>
<td>0.083</td>
</tr>
<tr>
<td>CpG33.34</td>
<td>0.085 ± 0.032 (28)</td>
<td>0.088 ± 0.030 (30)</td>
<td>0.253</td>
<td>0.745</td>
</tr>
<tr>
<td>SA</td>
<td>2.162 ± 1.005 (26)</td>
<td>3.906 ± 2.653 (29)</td>
<td>&lt;0.05</td>
<td>8.012E-9**</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.415 ± 0.376 (25)</td>
<td>0.597 ± 0.505 (28)</td>
<td>&lt;0.05</td>
<td>0.372</td>
</tr>
<tr>
<td>$MAT_{thrombin}$</td>
<td>58.976 ± 12.446 (28)</td>
<td>62.220 ± 4.539 (30)</td>
<td>0.138</td>
<td>0.202</td>
</tr>
<tr>
<td>ADP%</td>
<td>49.579 ± 29.079 (28)</td>
<td>44.840 ± 25.280 (30)</td>
<td>0.483</td>
<td>0.510</td>
</tr>
<tr>
<td>AA%</td>
<td>57.325 ± 37.353 (28)</td>
<td>82.142 ± 20.978 (30)</td>
<td>&lt;0.05</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

SA salicylic acid
$MAT_{thrombin}$ maximum amplitude
ADP% ADP inhibition
AA% arachidonic acid inhibition
*P value < 0.05
**P value < 0.001

Table 3: Pearson correlation analysis of potential factors

<table>
<thead>
<tr>
<th></th>
<th>CPG21.22</th>
<th>SA Concentration</th>
<th>AA%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA concentration</td>
<td>Pearson correlation</td>
<td>0.561</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Significance</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>AA%</td>
<td>Pearson correlation</td>
<td>0.328</td>
<td>0.327</td>
</tr>
<tr>
<td></td>
<td>Significance</td>
<td>0.012*</td>
<td>0.015*</td>
</tr>
<tr>
<td>Case vs. control</td>
<td>Pearson correlation</td>
<td>−0.479</td>
<td>−0.397</td>
</tr>
<tr>
<td></td>
<td>Significance</td>
<td>&lt;0.001**</td>
<td>0.003**</td>
</tr>
</tbody>
</table>

SA salicylic acid
AA% arachidonic acid inhibition
*P value < 0.05
**P value < 0.01
to a particular decision threshold. The optimal criterion value was calculated using Youden index (J) which is defined as:

\[
J = \text{max} \text{sensitivity}_c + \text{specificity}_c - 1
\]

where \(c\) ranges over all possible criterion values. Graphically, \(J\) is the maximum vertical distance between the ROC curve and the reference line. A cut-off point of CpG21.22 0.015 was identified with a specificity of 0.759. The cut-off points of AA% and SA concentration were 44.95% and 1.94 μg mL\(^{-1}\), respectively. SA concentration and AA% can be influenced by sampling time, and methylation level was relatively stable. Therefore, CpG21.22 methylation level <0.015 had the best predictive value of clinical endpoints.

Discussion

At present, information is limited about the impact of gene methylation on aspirin resistance in intracranial artery stenosis patients. It has been reported that aspirin efflux occurs through the ABCB1 transporter [13]. We aimed to explore the impact of ABCB1 promoter methylation on aspirin resistance in Chinese patients receiving antiplatelet therapy. To eliminate the interference of CYP2C19 loss-of-function alleles, poor metabolizers were excluded from our study. Compared with the controls, the cases had significant lower methylation levels, lower SA concentration, and lower AA% (\(P\) value < 0.05). We speculated that the lower methylation of ABCB1 promoter was associated with overexpression and an increased activity of ABCB1. More aspirin efflux resulted in lower AA% and an increased risk of clinical events. Methylation analysis might be a novel molecular marker to help an early identification of patients at high risk for clinical ischemic events. This may provide important insights into the research of aspirin resistance.

Once absorbed and introduced into the circulation, aspirin (acetylsalicylic acid) is rapidly deacetylated primarily in the liver to SA, which is conjugated with glycine (forming salicyluric acid) and glucuronic acid, and excreted largely in the urine. Aspirin has a plasma half-life of about 20 min in whole blood [23]. In contrast to aspirin, SA has a longer half-life of about 3 h [20]. In our patients, the SA concentration was significantly higher than aspirin concentration, but a significant correlation was observed between them (Pearson correlation analysis, \(R = 0.436, P\) value = 0.001). Considering the stability of SA in plasma, SA levels are a direct measure of aspirin intake and could be a suitable marker of compliance in patients treated with aspirin [20].

ABCB1 is a kind of drug efflux transporter, the multidrug resistance protein 1. It is expressed in a polarized manner in the plasma membrane of cells in barrier and elimination organs, where it has protective and excretory functions [24]. It plays an important role in the elimination of orally administered drugs to limit their bioavailability by effluxing at the lumen-facing epithelia of the small intestine and colon, and the bile-facing canaliculi of the liver. Researchers have assessed the involvement of ABCB1 in intestinal epithelial cell injury caused by aspirin using ABCB1 gene-transfected Caco2 cell, and the results indicated that aspirin efflux occurs through the ABCB1 transporter [13]. In our patients, ABCB1 methylation correlated with SA concentration, suggesting a decreased drug efflux and
an increased bioavailability. Although aspirin resistance is multifactorial, poor drug absorption has been shown to be a cause of the apparent aspirin resistance [9, 10, 25].

Assessing aspirin resistance is a hotly debated subject, and many in vitro assays are available [Light transmittance aggregometry (LTA), VerifyNow, TEG, etc.]. The Food and Drug Administration-approved TEG relies on the measurement of thrombin-induced clot strength to enable a quantitative analysis of platelet function [26, 27]. The TEG was able to identify a subtherapeutic response that highly correlated with the more labor intensive method of LTA [28]. These findings are concordant with the results of the PREPARE POST-STENTING study [29]. Therefore, TEG parameters may serve as a tool to investigate personalized antiplatelet treatment designed to reduce ischemic events [18, 30]. In China, TEG is the most widely used approach assessing the platelet function due to (1) the well-known association of TEG parameters and clinical events [31, 32]; (2) an overall assessment of ex vivo hemostatic function (thrombin, platelets, fibrin, and clotting); and (3) medical payment and reimbursement. Aspirin achieves its antithrombotic effect through the inactivation of COX-1, thereby preventing the generation of thromboxane A2 from AA. Clopidogrel inhibits platelet function via the ADP pathway. TEG can be used to determine the degree to which a patient is anticoagulated due to any of the two medications. In this assay, a standard TEG is conducted using patient’s venous blood. Then, separate assays are performed using the patient’s blood with AA or ADP added. The contribution of fibrin to the maximum amplitude is subtracted using a mathematical formula. This allows a determination of the MAAA and MADD, respectively. The difference between the patient’s whole blood result and AA/ADP added results is used to calculate the percent inhibition. AA % is the AA-induced inhibition rate of platelet aggregation, and ADP% represents the ADP receptor-induced inhibition rate.

Up until recently, most of the studies regarding antiplatelet resistance focused on clopidogrel, and CYP2C19 loss-of-function alleles may be dependent risk factors for developing clinical events in patients with acute coronary syndromes undergoing percutaneous coronary intervention [15]. Among patients with ischemic stroke or TIA treated with clopidogrel, carriers of CYP2C19 loss-of-function alleles are at increased risk of new stroke and composite vascular events compared with non-carriers [14]. However, our patients received dual-antiplatelet therapy for only 3 months, and all of them received long-term mono-antiplatelet therapy (aspirin 100 mg once daily). Most of the clinical events occurred after 3 months, suggesting aspirin resistance may be more important in our patients, and therefore, we focused our study on aspirin. To eliminate the interference of clopidogrel, CYP2C19 loss-of-function allele carrier patients were excluded from our study.

In our study, the duration of follow-up was 1 year and the clinical outcome was the composite events. A total of 438 patients were followed up, and only 174 were non-carriers of CYP2C19 loss-of-function alleles (*2 and/or *3), of those 30 experienced ischemic clinical events. The rate of recurrent ischemic events was 17.2% (30/174), and the total rate of recurrent events was 15.3% (67/438). In the previous published data (1-year follow-up and composite clinical events), the total rates of recurrent ischemic events ranged from 14.1 to 21.1%, and the rates of recurrent ischemic events in CYP2C19 wild-type patients ranged from 7.9 to 17.9% [17, 33–36]. Their incidence of ischemic events were close to our results.

Several limitations should be addressed: (1) the information of ABCB1 gene expression was not available, and the relationship between gene expression and methylation status in our patients needs to be investigated; (2) we performed the TEG test only once at the beginning of the study. TEG parameters may be affected by environment; however, it is difficult to control the environmental factors (such as diet, smoking, and concomitant medications) for each patient; (3) theoretically speaking, increasing aspirin dose could overcome effects of methylation status and this hypothesis need to be confirmed by recruiting healthy volunteers and separating them into two groups based on methylation status.

In summary, the influence of ABCB1 promoter methylation on aspirin exposure, platelet function, and clinical endpoints were explored in Chinese intracranial artery stenosis patients. ABCB1 hypomethylation is associated with lower drug absorption, higher platelet reactivity, and an increased risk of ischemic events in our patients. The lower methylation of ABCB1 promoter may be related to overexpression and an increased activity of ABCB1. Our study may provide important insights into the research of aspirin resistance.

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Authors’ contribution Z. Zhao and Z. Miao conceived and designed the study. X. Li designed the statistical analysis and drafted the manuscript. X. Li and S. Sun performed the analysis and wrote the manuscript. K. Zhao and N. Ma performed the experiments and follow-up. N. Ma and Z. Miao supervised the quality of the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

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