The impact of P2Y12 promoter DNA methylation on the recurrence of ischemic events in Chinese patients with ischemic cerebrovascular disease

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The primary mechanism of clopidogrel resistance is still unclear. We aimed to investigate whether the methylation status of the P2Y12 promoter has effects on platelet function and clinical ischemic events. Patients with ischemic cerebrovascular disease were enrolled into our study. Venous blood samples were drawn for thrombelastography (TEG) and active metabolite assay. Patients were divided into a case- or control-group based on the occurrence of ischemic events during a one year follow-up. Two TEG parameters between the case and control groups were statistically significant [ADP inhibition rate (ADP%): \( P = 0.018 \); ADP-induced platelet-fibrin clot strength (MA_{ADP}): \( P = 0.030 \)]. The concentrations of clopidogrel active metabolite had no significant difference \( (P = 0.281) \). Sixteen CpG dinucleotides on P2Y12 promoter were tested. Three CpG sites (CpG11 and CpG12 \( +13 \)) showed lower methylation status, which correlated with a strong association with increased risk of clinical events. Changes of MA_{ADP} and ADP% were also associated with methylation levels of CpG 11 and CpG 12 \( +13 \). Hypomethylation of the P2Y12 promoter is associated with a higher platelet reactivity and increased risk of ischemic events in our patients. Methylation analysis of peripheral blood samples might be a novel molecular marker to help early identification of patients at high risk for clinical ischemic events.

Stroke is the leading cause of death in China, and the second major cause of death worldwide¹. After a stroke, about 60% of patients receive antiplatelet treatments²³. Aspirin and clopidogrel are the most widely used antiplatelet drugs. Clopidogrel is a prodrug and a thienopyridine derivative. It binds irreversibly and specifically to the P2Y12 platelet receptor, inhibiting ADP-mediated platelet aggregation. High inter-individual variability was observed in patients taking clopidogrel⁴. The approved clopidogrel labels from US Food and Drug Administration, European Medicines Agency, Japan Pharmaceuticals and Medical Devices Agency, and Health Canada state that CYP2C19 poor metabolizers may be at an increased risk for experiencing cardiovascular events when taking the drug at the recommended dose compared to those normal CYP2C19 metabolizers. Alternative medications or treatment strategies should be considered in these patients⁵⁶.

A genome-wide association study of clopidogrel showed that the common loss-of-function CYP2C19*2 variant is a major marker of treatment response, but could only explain about 12% of the variance⁷. Poor drug response was associated with increasing age (3.8% of variance), greater body mass index (BMI) (2.3% of variance), higher triglyceride levels (1.3% of variance) and nominally lower levels of high-density lipoprotein cholesterol (1.0% of variance)⁷. However, the major reason of clopidogrel poor response remains unclear.

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Received: 10 June 2016  
Accepted: 15 September 2016  
Published: 30 September 2016
DNA methylation is a crucial epigenetic marker and regulator of gene expression. Methylation typically occurs at CpG sites (cytosine-phosphate-guanine sites, where a cytosine is directly followed by a guanine in the DNA sequence) in vertebrates. Cytosine is converted to 5-methylcytosine after methylation. There is an inverse relationship between CpG methylation and transcriptional activity. Hypermethylation of promoter DNA is related to transcriptional silencing of gene expression, resulting in decreased protein activity.

The study of DNA methylation represents a new direction to identify undiscovered mechanisms on poor antiplatelet response. The current available information about DNA methylation related to poor clopidogrel responses is limited. Methylation of the P2Y12 promoter may be one potential reason for clopidogrel poor response and its subsequent clinical ischemic events. The aim of our study is to assess the relationship between P2Y12 promoter methylation levels and clinical responses in patients with ischemic cerebrovascular disease.

Results
Characteristics of enrolled patients. A total of 448 patients were recruited from post-stroke patients who consented to the study, and we were able to follow up 438 patients for one year either through clinic visits or telephone calls (10 patients lost follow-ups due to wrong contact information provided). Thirty patients developed ischemic events and were categorized as the case-group. Among patients who did not experience ischemic events, 30 patients were selected as matched-controls. The P2Y12 promoter DNA methylation analyses were performed for these pair-matched 60 patients. Patients’ baseline and clinical characteristics are listed in Table 1. Given that BMI, hypertension, diabetes, hyperlipidemia, coronary artery disease, family history of stroke, prior cerebral infarction, drinking, and/or smoking may be risk factors for ischemic stroke, the baseline and clinical characteristics of enrolled patients were balanced between the case and control groups. A total of 4 patients died (vascular-related mortality: cerebral infarction or cerebral hemorrhage secondary to infarction) during one year follow-up, and they were categorized into the case group. The detailed clinical characteristics of both cases and controls can be found in the Supplementary Material.

Table 1. Clinical characteristics of patients with events and without events. *Paired t-test or χ² test. #SD: standard deviation.

| Variable                        | Case (n = 30) | Control (n = 30) | P-value* |
|---------------------------------|---------------|-----------------|----------|
| Age (year), mean ± SD          | 61.03 ± 7.22  | 60.43 ± 7.54    | 0.589    |
| Male, n (%)                    | 27 (90.0)     | 27 (90.0)       | 1.000    |
| Stenting, n (%)                | 18 (60.0)     | 18 (60.0)       | 1.000    |
| Risk factors                   |               |                 |          |
| BMI, mean ± SD                 | 25.68 ± 3.35  | 25.98 ± 3.14    | 0.649    |
| Hypertension, n (%)            | 17 (56.7)     | 18 (60.0)       | 0.793    |
| Diabetes, n (%)                | 10 (33.3)     | 10 (33.3)       | 1.000    |
| Hyperlipidemia, n (%)          | 10 (33.3)     | 9 (30.0)        | 0.781    |
| Coronary artery disease, n (%) | 3 (10.0)      | 3 (10.0)        | 1.000    |
| Family history of stroke, n (%)| 3 (10.0)      | 7 (23.3)        | 0.166    |
| Prior cerebral infarction, n (%)| 4 (13.3)     | 7 (23.3)        | 0.317    |
| Smoking, n (%)                 |               |                 |          |
| Never                           | 9 (30.0)      | 11 (36.7)       | 0.855    |
| Current                         | 14 (46.7)     | 13 (43.3)       |          |
| Ex-smoker                       | 7 (23.3)      | 6 (20.0)        |          |
| Drinking, n (%)                 |               |                 |          |
| Never                           | 14 (46.7)     | 14 (46.7)       | 0.102    |
| Social drinker                  | 10 (33.3)     | 15 (50.0)       |          |
| Regular drinker                 | 6 (20.0)      | 1 (3.3)         |          |
| Outcome, n (%)                  |               |                 |          |
| Death                           | 4 (13.3)      | 0 —             |          |
| Ischemic stroke                 | 13 (43.3)     | 0 —             |          |
| Coronary ischemic event         | 5 (16.7)      | 0 —             |          |
| Transient ischemic attack       | 8 (26.7)      | 0 —             |          |

DNA methylation is a crucial epigenetic marker and regulator of gene expression. Methylation typically occurs at CpG sites (cytosine-phosphate-guanine sites, where a cytosine is directly followed by a guanine in the DNA sequence) in vertebrates. Cytosine is converted to 5-methylcytosine after methylation. There is an inverse relationship between CpG methylation and transcriptional activity. Hypermethylation of promoter DNA is related to transcriptional silencing of gene expression, resulting in decreased protein activity.

The study of DNA methylation represents a new direction to identify undiscovered mechanisms on poor antiplatelet response. The current available information about DNA methylation related to poor clopidogrel responses is limited. Methylation of the P2Y12 promoter may be one potential reason for clopidogrel poor response and its subsequent clinical ischemic events. The aim of our study is to assess the relationship between P2Y12 promoter methylation levels and clinical responses in patients with ischemic cerebrovascular disease.

CpG methylation, platelet function and metabolite difference. CpG12 and CpG13 were continuous detection sites, and Sequenom EpiTYPER technology can only measure these two sites simultaneously with the result being the average of the methylation levels at these two CpG sites. To visualize the data, we drew Heatmaps using the TEG parameters (Fig. 1) and CpG methylation levels (Fig. 2) obtained from the case group and the control group. The scale used in the figures denotes high data values with bright red tones and low data values with light green tones. Mixed tones depict values in-between. As can be noted from Fig. 1, among the control group patients, the ADP% values have more high data values compared to the MAADP values, which have more low data values, but we could not accurately determine which parameter differed significantly between the two groups. Figure 2 is the Heatmap depicting the differences of CpG methylation levels between the two groups. It
is clear that CpG11 and CpG12 + 13 in the control group have higher methylation levels (darker red indicates a higher level of methylation).

To determine if there is any significant methylation level difference for the remaining sites, a quantitative statistical method would be required. To identify predictors for developing clinical ischemic events, the differences of the metabolite concentration, TEG parameters [maximum amplitude (MA), ADP%, MAADP and arachidonic acid inhibition rate (AA%)] and methylation levels between cases and controls were analyzed using paired t-test. As shown in Table 2, the methylation levels in the three CpG sites (CpG11 and CpG12 + 13) were significantly associated with clinical ischemic events ($P < 0.001$). The differences for ADP% and MAADP in both cases and controls were statistically significant (ADP%: $P = 0.018$; MAADP: $P = 0.030$). The concentrations of clopidogrel active metabolite had no significant difference ($P = 0.281$). This showed that clinical ischemic events could not be predicted by detecting drug concentration of clopidogrel.

**Relationship of active metabolite concentration and platelet function.** Clopidogrel is metabolized to an active metabolite in vivo and exert its antiplatelet effect. Theoretically, there should be a good correlation between the active metabolite concentration and platelet function. We made a scatter plot based on the relationships between clopidogrel activity-related TEG parameters (ADP% and MAADP) and the concentrations of the active drug metabolite (Fig. 3). Figure 3 shows there is no good correlation between the two TEG parameters and metabolite concentration. The correlation coefficient $R$ is $0.067$ ($P = 0.613$) and $-0.054$ ($P = 0.681$), respectively. This suggests that drug exposure cannot accurately predict the anti-platelet effect of clopidogrel. There may be other factors affecting the efficacy of the drug.

**Correlation of P2Y12 methylation level and platelet function.** To investigate the importance of the P2Y12 gene promoter DNA methylation in platelet function, we analyzed the association of methylation levels of three CpG sites (CpG11 and CpG12 + 13) and TEG parameters (ADP% and MAADP). The methylation level correlated inversely with MAADP [CpG11: $R = -0.430$ ($P = 0.001$); CpG12 + 13: $R = -0.294$ ($P = 0.025$)], and positively with ADP% [CpG11: $R = 0.454$ ($P < 0.001$); CpG12 + 13: $R = 0.261$ ($P = 0.048$)] in the linear regression model. The scatter plot of TEG parameters and methylation status is shown in Fig. 4. This correlation suggested that lower methylation level resulted in a higher residual platelet reactivity.

**Discussion**

Theoretically, a causal relationship should exist within the context of drug exposure to platelet activity and to clinical events. However, our study showed that the concentration of the active clopidogrel metabolite had no significant effects on platelet activity and ultimately clinical events, suggesting that other factors might affect the final clinical outcome in our patients. We therefore focused on the role of methylation regulation on the target, P2Y12. A promoter is a region of DNA where transcription of a gene initiates. It is located near the transcription start sites of genes. In our current study, −5000 to 1000 base pairs (Chromosome 3, GRCh38.p2: 151383812–151389812) were selected to cover the P2Y12 promoter. Three regions containing rich CpG sites were selected for the methylation analysis (Table 3). A total of 60 patients (30 cases and 30 matched-controls) were selected to identify the relationship between P2Y12 promoter methylation and clinical ischemic events. Sixteen CpG dinucleotides on P2Y12 promoter were tested among the 30 cases and the 30 matched-controls. Lower methylation levels of three CpGs (CpG11 and CpG12 + 13) showed a strong association with an increased risk of clinical events. A methylation analysis of peripheral blood samples may help early identification of those patients having high risk of clinical ischemic events.

A study by Su et al. reported the association of methylation levels of P2Y12 promoter DNA and the risk of clopidogrel resistance in coronary artery disease patients. Although many CpG dinucleotides in the P2Y12 gene promoter exist, only one fragment (GRCh37.p13: 151103600-151101600) containing two CpG dinucleotides was selected for the methylation assay. We found 11 CpG sites in this region based on the sequencing information.
provided, however, the detected CpG specific location was not identified in that paper. Therefore we could not make comparisons to the site locations detected in our study. The residual platelet reactivity (VerifyNow P2Y12 assay) cutoff value ≥240 reaction units indicated the existence of clopidogrel resistance\(^2\). The results indicated that lower P2Y12 gene promoter DNA methylation increased the risk of clopidogrel resistance in patients with albumin ≤35 g/L, current smoking, or alcohol abuse. Although clopidogrel resistance could be defined according to the platelet functions test\(^15\), platelet function assays are poorly standardized. Clinical ischemic events may be the gold standard for poor clopidogrel response\(^16,17\). Thus, poor or non-responders were identified during follow-up in our study. Results from this study are applicable to coronary artery disease patients with albumin ≤35 g/L, current smoking or alcohol abuse. The residual platelet reactivity for these patients can be predicted through methylation detection; however, whether the residual platelet reactivity is related to clinical events still requires validation from clinical data. In comparison, our study are for patients with an ischemic stroke, and we assess patients’ risk of clinical events by methylation assays.

It is reported that environmental and lifestyle factors could influence epigenetic mechanisms\(^12\). We focused on the impact of methylation on platelet function and clinical events. Matched-controls were selected according to

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**Figure 2. Heatmap generated from CpG methylation levels.** Methylation levels are indicated by a gradient map representing the hypo- to hypermethylated status (from green to red respectively) of different CpG sites. Cpg 11 and Cpg 12 + 13 were significantly hypermethylated in cases as compared to controls. Several methylation levels of CpG sites cannot be tested in our study and are presented using a black color.
to the clinical characteristics of cases. Due to the similarity between cases and controls, we could not detect other factors such as smoking and drinking on the methylation level.

We speculated that hypomethylation of CpG11, CpG12 + 13 on the P2Y12 gene promoter may be associated with an up-regulation of P2Y12 expression. In patients with hypermethylation of the promoter, the P2Y12 receptor can be effectively inhibited by clopidogrel. In contrast to this, in patients with hypomethylation, clopidogrel cannot inhibit the P2Y12 receptor effectively, which leads to high residual platelet reactivity (MAADP and ADP%).

High platelet reactivity indicated an increased recurrent risk of ischemic events. It has been reported that MAADP and ADP% can be used to predict clinical ischemic events. Although our study included a small number of patients, a statistical significant difference between cases and matched-controls for MAADP and ADP% parameters (paired t-test, ADP%: \( P = 0.018\), MAADP: \( P = 0.030\)) was observed. The linear regression analysis showed that there was a correlation between CpG methylation level and MAADP and ADP% (Fig. 4).

There are several laboratory assays for testing platelet function, such as light transmission aggregometry, VerifyNow analysis, vasodilator-stimulated phosphoprotein phosphorylation assay and TEG. In China, TEG is the most widely used approach due to (1) an overall assessment of ex vivo hemostatic function, such as thrombin, platelets, fibrin, and clotting factors; (2) using whole blood for test and processing; and (3) medical payment and reimbursement. This study aimed to observe whether methylation levels affecting the final clinical outcome.

As a routine test in Chinese hospitals, we included TEG parameters in the statistical analysis. We found that methylation levels were in correlation with platelet function (ADP% and MAADP) measured by TEG. But there were relatively large fluctuations of MAADP and ADP% in both case-group and matched-control group patients.

| Metabolite Conc. | Mean ± SD (n) | Paired t-test P value |
|------------------|---------------|-----------------------|
| Cases group      | Controls group |                       |
| MA               | 3.90 ± 2.28   | 3.71 ± 1.91           | 0.281 |
| ADP%             | 61.95 ± 5.51 (30) | 62.42 ± 6.14 (30) | 0.699 |
| MAADP            | 41.19 ± 26.06 (30) | 54.58 ± 27.04 (30) | 0.018* |
| AA%              | 40.54 ± 15.40 (30) | 33.47 ± 15.42 (30) | 0.030* |
| CpG 1            | 63.27 ± 30.44 (30) | 76.41 ± 30.96 (30) | 0.159 |
| CpG 2            | 0.68 ± 0.20 (29) | 0.59 ± 0.23 (28) | 0.113 |
| CpG 3            | 0.52 ± 0.06 (28) | 0.51 ± 0.18 (29) | 0.946 |
| CpG 4            | 0.77 ± 0.17 (28) | 0.70 ± 0.28 (29) | 0.324 |
| CpG 5            | 0.91 ± 0.08 (30) | 0.93 ± 0.08 (30) | 0.237 |
| CpG 6            | 0.65 ± 0.12 (30) | 0.66 ± 0.09 (30) | 0.965 |
| CpG 7            | 0.62 ± 0.15 (29) | 0.66 ± 0.11 (28) | 0.115 |
| CpG 8            | 0.87 ± 0.04 (30) | 0.89 ± 0.05 (30) | 0.088 |
| CpG 9            | 0.84 ± 0.15 (30) | 0.87 ± 0.10 (30) | 0.299 |
| CpG 10           | 0.79 ± 0.04 (29) | 0.76 ± 0.09 (30) | 0.194 |
| CpG 11           | 0.88 ± 0.09 (30) | 0.84 ± 0.18 (30) | 0.303 |
| CpG 12 + 13      | 0.48 ± 0.14 (29) | 0.86 ± 0.13 (28) | <0.001* |
| CpG 14           | 0.52 ± 0.15 (29) | 0.79 ± 0.21 (29) | <0.001* |
| CpG 15           | 0.31 ± 0.09 (30) | 0.30 ± 0.11 (30) | 0.791 |
| CpG 16           | 0.33 ± 0.08 (30) | 0.31 ± 0.07 (29) | 0.223 |

Table 2. Statistical results of active metabolite concentration, TEG parameters (ADP% and MAADP) and methylation levels between case and control groups.
Therefore deviations may exist if only TEG parameters were utilized to predict clinical events, and this may be due to the reliability of TEG platelet function test. Compared to TEG, VerifyNow P2Y12 test may be a more reliable platelet function measurement. Since the VerifyNow P2Y12 test is expensive and it is not covered by insurance, it is currently not widely used in China. It is our hope that further research with the VerifyNow test will allow us to confirm the relationship between gene methylation and P2Y12 platelet activity.

Multiple genes may contribute to the clopidogrel poor response. Another study found that ABCB1 promoter methylation status in whole blood appeared to be inversely associated with ABCB1 mRNA expressions and maximum platelet aggregation. However, information concerning a clinical endpoint was not available. A similar study focused on ABCC3. ABCC3 gene promoter methylation correlated inversely with the gene expression, but did not seem to exhibit any impact on maximum platelet aggregation. Based on the clinical ischemic events, research on the methylation status of other potential genes such as CYP2C19, CES1, and PON1 is warranted.

There are several limitations in our study: (1) P2Y12 gene expression was not measured. The association between the CpG methylation level and the quantitative gene expression remains unclear; (2) The sample size was relatively small, and our results need to be confirmed by a larger study.

Table 3. Sequences of primers for P2Y12 gene DNA methylation analysis and chromosome position of CpG sites.

| Fragments | Group        | DNA sequence                  | CpG ID | Chromosome position   |
|-----------|--------------|-------------------------------|--------|-----------------------|
| Fragment 1| Forward primer | 5'-TTTTTTTTAGGAGGTAGGGTAGTG-3' | CpG1   | 151387988             |
|           | Reverse primer | 5'-CCAACCCAAATAATTCTCTACCCA-3' | CpG2   | 151387936             |
|           |               |                                | CpG3   | 151387919             |
|           |               |                                | CpG4   | 151387910             |
|           |               |                                | CpG5   | 151387845             |
|           |               |                                | CpG6   | 151387827             |
|           |               |                                | CpG7   | 151387795             |
| Fragment 2| Forward primer | 5'-TATTTGGAATTTGTTTGATG-3'    | CpG8   | 151386670             |
|           | Reverse primer | 5'-AATTCAACACCAAATACCTACCA-3' | CpG9   | 151386646             |
|           |               |                                | CpG10  | 151386638             |
|           |               |                                | CpG11  | 151386483             |
|           |               |                                | CpG12  | 151386429             |
|           |               |                                |        | 151386429             |
| Fragment 3| Forward primer | 5'-TTTGTGTTAATTAAGGAATTTAT-3' | CpG14  | 151385892             |
|           | Reverse primer | 5'-TCACTACCCCTAATTTATCATTCAA-3' | CpG15  | 151385073             |
|           |               |                                | CpG16  | 151385049             |

Figure 4. The relationship between methylation level (CpG11 and CpG12 + 13) and platelet function index (ADP% and MAADP). The impact of methylation status on platelet function was assessed by linear regression model. The lines in the plots represent the regression lines. R: Correlation coefficient.
In summary, the hypomethylation of P2Y12 promoter is associated with higher residual platelet reactivity and an increased risk of ischemic events. Methylation studies are a new direction to identify the mechanism of poor clopidogrel response, and research concerning the methylation status of other clopidogrel pathway genes are necessary.

Methods

Patient selection. The study enrolled Chinese patients with ischemic cerebrovascular disease at Beijing Tiantan Hospital, affiliated with Capital Medical University. The inclusion criteria were: (1) a diagnosis of ischemic cerebrovascular disease with 70 to 99% stenosis of a major intracranial artery or an extracranial artery, confirmed by digital subtraction angiography; (2) enrollment at least five days after dual-antiplatelet therapy (aspirin 100 mg/day plus clopidogrel 75 mg/day); (3) patients received dual-antiplatelet therapy of aspirin and clopidogrel for 3 months; (4) no evidence of cardioembolism, including recent myocardial infarction or atrial fibrillation within one month; and (5) age ≥30 years. Exclusion criteria were: (1) patients with atrial fibrillation, Moyamoya disease, active peptic ulcer disease, severe liver or kidney impairment, or bleeding tendency; (2) known contraindication or allergy to aspirin, clopidogrel or heparin; and (3) enrollment in another study that would conflict with this study. Our 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. All methods were done in accordance with relevant guidelines and regulations.

Study design. Clinical events included vascular-related mortality (death from cerebral infarction or bleeding secondary to infarction), ischemic stroke, transient ischemic attack or myocardial infarction. Ischemic stroke is a new focal neurologic deficit of sudden onset, unassociated with hemorrhage on MRI or CT, and lasting at least 24 h. Transient ischemic attack 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 imaging changes. The occurrence of clinical ischemic events was identified during a one year follow-up visit. Patients who did not attend their follow-up visits within one year were contacted by telephone to obtain the above information.

Patients were divided into the case group if they experienced clinical events during the one year follow-up. Patients who had no recurrence of ischemic events within one year were classified as the control group. Ideally we should measure methylation levels for all patients in the control group; however, the total cost of methylation testing prohibited us to do so. We therefore selected an equal number of patients without clinical events as the control group. To best match a “case patient”, we carefully screened all control patients and selected a similarly matched “control patient” for every “case patient”. For example, the 2 pair-matched patients had same or similar characteristics in terms of age, gender, BMI, with/without clinical interventions, smoking and/or drinking status, and disease states such as hypertension, hyperlipidemia, diabetes, coronary heart disease, history of stroke, family history of stroke, etc. Due to the limited number of patients in the control group, we cannot guarantee that the paired-patients were an exact match. However, there should be no statistical significant difference between the control-group patients and case-group patients with regards to clinical features or disease states. This eliminated the interference of other factors and maximized the effect of the degree of DNA methylation on platelet function and clinical events.

Platelet function testing. TEG is a methodology used clinically to assess platelet function and to provide a comprehensive assay of the overall clotting process. Venous blood samples were obtained from the cubital vein after five days of dual-antiplatelet treatment. The blood samples were analyzed by the TEG Hemostasis System (Haemoscope Corporation, Niles, Illinois, USA). The blood clots were linked to a stationary pin suspended in an oscillating cup containing the whole blood sample. TEG gives a quantitative analysis of platelet function based on the formation, strength, and degradation of clots in whole blood.

Maximal clot strength (MAThrombin) is the MA, a direct function of the maximum clot strength, and it was achieved by transferring whole blood to a vial containing kaolin which was then mixed by inversion. The kaolin activated blood was transferred to a TEG cup containing CaCl2. To detect the fibrin contribution to clot formation (MAFibrin) representing the fibrin contribution to clot strength, heparinized blood was transferred to a TEG cup containing Activator F. The contribution of P2Y12 receptor to clot formation was assessed by the addition of heparinized blood to the TEG cup along with ADP and Activator F. MAADP reflects platelet activity to ADP-induced clot strength and assesses an individual patient’s response to antiplatelet therapy. Likewise, the cyclooxygenase-1 pathway contribution to clot formation (MAAA) was assessed by transferring heparinized blood to AA and Activator F to the TEG cup. The ADP% and AA% were calculated as the following equations:

\[
ADP\% = 100\% - \frac{MA_{ADP} - MA_{Fibrin}}{MA_{Thrombin} - MA_{Fibrin}} \times 100\%
\]

(1)

\[
AA\% = 100\% - \frac{MA_{AA} - MA_{Fibrin}}{MA_{Thrombin} - MA_{Fibrin}} \times 100\%
\]

(2)

Finally, four TEG parameters, MA, MAADP, ADP% and AA% were measured. All four parameters are calculated by the TEG software.
Clopidogrel active metabolite determination. After patients took clopidogrel for at least 5 consecutive days (assuming at steady state plasma concentration), a 75 mg clopidogrel tablet was administered to each patient and 2 mL peripheral blood samples were drawn 60 min after drug administration. The samples were placed into tubes containing 2% ethylenediaminetetraacetic acid, and 30 μL of 500 mM 2′-Bromo-3′-O-Methoxycetophenone protectant solution was added immediately. The tubes were vortexed for 60 seconds. After the completion of the reacting room at temperature for 10 min, the tubes were centrifuged for 5 min at 12000 rpm (10000 g). The supernatants were taken and stored in a refrigerator (−80 °C) for the analysis of the clopidogrel active metabolite concentrations. The drug concentration was analyzed using the HPLC-MS/MS assay method as reported in the literature34.

Methylation analysis. Leucocytes from venous blood samples were applied to extract human genomic DNA using a commercial kit (QIAGEN DNA Blood Mini Kit, Qiagen, Hilden, Germany). All DNA concentrations were greater than 500 ng/μL. The genomic DNA was treated with sodium bisulfite (Epitext Bisulfite Kits; Qiagen). Unmethylated cytosines were converted to uracils while methylated cytosines remained unchanged35. Three target regions in the P2Y12 gene promoter were amplified with PCR using bisulfite-treated genomic DNAs as templates. The designed primer pairs for the three fragments are listed in Table 3. A total of 16 CpG dinucleotides in the three regions of the P2Y12 gene promoter were examined using Sequenom EpiTYPER technology (Table 3). The methylation calls were 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.

Statistical analysis. All data analyses were performed using SPSS software (Version 17.0, SPSS Inc., Chicago, Illinois, USA). Categorical data were shown as numbers and percentages, and were analyzed using Pearson’s χ² test or Fisher’s exact test (when expected value of any cell is less than 5). Continuous variables were presented as mean ± SD, and analyzed using paired t-test. A P-value <0.05 was considered to be statistically significant. A linear regression analysis was used to assess correlations between P2Y12 promoter methylation and residual platelet reactivity, and between active metabolite concentration and residual platelet reactivity. Each CpG site methylation levels and TEG parameters (MA, ADP%, MAADP, AA%) for every patient were visualized in the Heatmap, and the Heatmap was produced using HemI software (Version 1.0.3.3, HemI Illustrator, Huazhong University of Science and Technology, Wuhan, China)36.
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Acknowledgements

This study was supported by National Natural Science Foundation of China grants (No. 81503157), a grant from Organization Department of Beijing Municipal Committee (No. 2014000021469G258) and a grant from Capital Medical University (No. 16JL72).

Author Contributions

Z.-G.Z., Z.-R.M., X.-J.W. and W.L. conceived and designed this study. N.M., B.W., X.-Q.L., S.-H.M. and K.Z. conducted the experiment. X.-J.W. performed data analysis and wrote the manuscript. S.-S.S. contributed to editing of the manuscript. All authors read and approved the final version of the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Li, X.-G. et al. The impact of P2Y12 promoter DNA methylation on the recurrence of ischemic events in Chinese patients with ischemic cerebrovascular disease. *Sci. Rep.* 6, 34570; doi: 10.1038/srep34570 (2016).

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