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A STUDY OF MICRORNA-223 IN EVALUATING PLATELET REACTIVITY IN PATIENTS WITH ACUTE ISCHEMIC STROKE

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To investigate the relationship between plasma microRNA-223 expression and platelet reactivity in patients with acute ischemic stroke (AIS) and to evaluate its predictive value in clopidogrel resistance or high on-treatment platelet reactivity (HTPR). A total of 120 patients with acute ischemic stroke were screened in this study, and 60 patients were included in the acute ischemic stroke group according to the inclusion criteria and platelet reactivity after clopidogrel treatment. Control group was 60 non-ischemic stroke patients hospitalized. The levels of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and adenosine diphosphate-induced platelet aggregation (ADP-PAg) in platelets were detected by flow cytometry. The expression level of plasma microRNA-223 was detected before and after clopidogrel treatment using quantitative real-time polymerase chain reaction (PCR). The AIS group was then divided into the clopidogrel non-HTPR and the clopidogrel HTPR groups based on the relative inhibition rate. We found that: 1) the VASP platelet reactivity index (PRI) was positively correlated with ADP-PAg; 2) before administration, the plasma microRNA-223 expression level and VASP-PRI were higher in the AIS group than in the control group; 3) after administration, the expression level of microRNA-223 was negatively correlated with VASP-PRI; 4) before and after treatment, the plasma microRNA-223 expression level in the clopidogrel HTPR group was lower than in the non-AIS patients; 5) before treatment, there was an interaction between the expression level of microRNA-223 in the plasma and the CYP2C19 loss-of-function (LOF) allele. The study showed that decreased plasma microRNA-223 expression levels in AIS patients indicate an increased risk of clopidogrel HTPR. Carrying CYP2C19 LOF alleles may result in the microRNA-223 expression being more distinct. The combined detection of plasma microRNA-223 and CYP2C19 gene polymorphisms may be effective in predicting the occurrence of clopidogrel HTPR in patients with AIS.

Key words: *microRNA-223, platelet reactivity, ischemic stroke, clopidogrel, CYP2C19, gene polymorphisms, vasodilator-stimulated phosphoprotein, platelet aggregation*

INTRODUCTION

Acute ischemic stroke (AIS) is the most common type of stroke (1). It accounts for 69.6–70.8% of strokes in China (2, 3). National and international ischemic stroke treatment guidelines recommend clopidogrel monotherapy as the preferred antiplatelet drug (4). It has been proved that there are differences in individual responses to the clopidogrel antiplatelet therapy (5). Some patients still experience ischemic events when they receive standardized clopidogrel therapy and can develop clopidogrel resistance, which is also known as high on-treatment platelet reactivity (HTPR) (6). The study found that the factors affecting HTPR after clopidogrel treatment can be grouped into genetic, clinical, and cellular factors. Among these, the factors that regulate the expression of the platelet membrane glycoprotein P2Y12 receptors on the cell membrane cannot be ignored. MicroRNA-223 (miR-223) is the most abundant microRNA in platelets and can affect platelet reactivity by regulating the expression of platelet P2Y12 protein (7, 8). Most of the current studies on miR-223, clopidogrel HTPR, and clinical prognosis focus on patients with coronary heart disease, but there are few studies on patients with AIS. The purpose of this study is to investigate the relationship between plasma miR-223 expression and platelet reactivity in patients with AIS and to evaluate its predictive value in clopidogrel resistance or HTPR.

MATERIAL AND METHODS

Research objects

From November 2017 to November 2018, patients admitted to the Department of Neurology at the Second Hospital of Tianjin Medical University were divided into two groups: a group of patients diagnosed with new-onset AIS and a control group. A total of 120 patients with acute ischemic stroke were screened in this study, and 60 patients were included in the acute ischemic stroke group according to the inclusion criteria and platelet reactivity after clopidogrel treatment. Control group was 60 non-ischemic stroke patients hospitalized. Patients with new-onset AIS were treated with clopidogrel (75 mg/d) after admission.

Inclusion criteria for patients in the AIS group: 1) admitted within three days of onset; 2) aged 18–85 years; and 3) diagnosed with AIS in line with the diagnostic criteria established in the Chinese guidelines for the diagnosis and treatment of AIS 2018 (1), with new lesions showing up on CT scan and/or MRI examination. After the platelet aggregation rate test, patients who met the criteria were screened.

The inclusion criteria for the control group were: 1) aged 18–85 years and 2) no new lesions were confirmed by computed tomography (CT) or magnetic resonance imaging (MRI).

Exclusion criteria: 1) age >85 years; 2) complications with cerebral hemorrhage, hematological diseases, arteriovenous thrombosis, severe heart valve disease, atrial fibrillation, or myocardial infarction; 3) allergic to clopidogrel; 4) using thrombolytic drugs, anticoagulants, antiplatelet aggregation drugs, or non-steroidal anti-inflammatory drugs within two weeks of admission; 5) surgical treatment within six months of the onset of the disease; or 6) active bleeding, liver and kidney disease, severe infections, or malignancies.

The control group was tested for vasodilator-stimulated phosphoprotein (VASP), and a real-time quantitative polymerase chain reaction (PCR) was performed to detect plasma miR-223 expression levels. All patients with AIS were treated with adenosine diphosphate-induced (ADP-induced) platelet aggregation (ADP-PAg) and VASP before and 7±1 days after treatment with clopidogrel. The relative inhibition (RI) rate of platelet reactivity was calculated based on the platelet aggregation rate before and after ADP-PAg administration (9-11). Formula RI = (platelet aggregation rate before medication – platelet aggregation rate after medication) ÷ platelet aggregation rate before medication × 100. The RI values were grouped into quantiles. The upper quartile became the clopidogrel high-response group (non-HTPR group after clopidogrel treatment), the lower quartile became the clopidogrel low-response group (HTPR group after clopidogrel treatment), and plasma miR-223 expression levels before and after clopidogrel treatment were detected using real-time fluorescence quantitative PCR. In this study, 60 control patients and 60 extreme cases were selected as research objects.

This study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University (KY2017K016), and all patients or their families signed informed consent before the trial.

Collection of clinical information

Personal information from all subjects was taken and recorded from the electronic medical record system of inpatients, including name, age, gender, personal history, family history, current medical history, history of antiplatelet medication, skull CT or MRI examination results, and laboratory tests, *i.e*., blood glucose, routine blood, liver and kidney function, and lipids. The combined medications administered during the hospital stay were also recorded, including statin lipid-lowering drugs, calcium channel blockers (CCB), and proton pump inhibitors (PPI).

Blood collection

In the control group, blood was taken on the first day of enrollment. In the AIS group, blood was collected before clopidogrel treatment and 7±1 days after treatment. From all patients, 3 ml of blood was collected in a sodium citrate anticoagulation tube. From the control group, blood was kept at –20°C for genotype and plasma miR-223; from the AIS group, it was kept at room temperature, and platelet aggregation rate detection was performed within half an hour. The samples were mixed gently to avoid violent shaking, to prevent the hemolysis of blood cells, and to avoid activating the platelets.

Platelet aggregation rate detected by flow cytometry

This experiment was performed in the flow cytometry room of the central laboratory at the Second Hospital of Tianjin Medical University. Platelet aggregation rate was measured using a VASP detection kit (BioCytex, Marseille, France) to detect the phosphorylation level of VASP, and the platelet aggregation rate was detected using flow cytometry to detect platelet aggregation induced by ADP.

Plasma ribonucleic acid (RNA) extraction and determination

Blood (5 ml) was collected on sodium citrate anticoagulation tube, and using RNA extraction Kit (miRNeasy Serum/Plasma Kit; catalog number: 217184; Qiagen, Hilden, Germany) extract RNA, reverse transcription of mirRNA was performed using One Step PrimeScript miRNA Cdna Synthesis Kit (catalog number:D350A; Takara Bio Inc. Shiga, Japan). The reaction system of reverse transcription of miRNA followed the recommendation of the Kit. UltraSYBR Mixture (High ROX, catalog number: CW2602; CWBIO, Beijing, China) was used to perform real-time fluorescence quantitative PCR reaction with human-5SRRNA as internal reference in a 96-well plate. Three multiple Wells were set for quantification of internal reference genes and miRNA in each sample to calculate the mean value.

CYP2C19 loss-of-function (LOF) allele typing

The PCR-restriction fragment length polymorphism method was used for typing CYP2C19 LOF alleles (common polymorphic sites *2 [G681A] and *3 [G636A]).

MiRNA reverse transcription and real-time PCR reaction

Reverse transcription of miRNA was performed using Takara Bio Inc. Shiga, Japan One Step PrimeScript miRNA Cdna Synthesis Kit, and the reaction system for reverse transcription of miRNA was performed following the manufacturer's recommendations.

Detection of CYP2C19 gene polymorphism

The PCR product fragments of CYP2C19*2 and CYP2C19*3 were 192 bp and 234 bp in length, respectively. CYP2C19*2 and CYP2C19*3 were mutated at the SmaI restriction site and BamHI restriction site. After digestion with SmaI, CYP2C19*2/*2 mutant homozygotes showed a single band of 192 bp; CYP2C19*1/*2 mutant heterozygotes were cut into three fragments of 192, 111, and 81 bp; and CYP2C19*1/*1 wild type was cut into two fragments of 111 and 81 bp. After BamHI digestion, CYP2C19*3/*3 mutant homozygotes showed a single band of 234 bp; CYP2C19*1/*3 mutant heterozygotes were cut into three fragments of 234, 135, and 99 bp; and CYP2C19*1/*1 wild type was cut into two fragments of 135 and 99 bp.

Statistical methods

Statistical analysis was performed using the IBM SPSS 23.0 software. The Pearson (normally continuous variables) or Spearman (ordered categorical variables or skewed continuous variables) coefficient was used to calculate the correlations between variables. One-sample Kolmogorov-Smirnov tests were used to test for normality of quantitative data, such as age, red blood cell count, white blood cell count, hemoglobin, platelet count, total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, creatinine, uric acid, and plasma miR-223 expression levels. If they showed a

normal distribution, an independent sample t-test was used to compare the quantitative data between the two groups. Variances in uniformity needed to be checked before comparing the quantitative data between the three groups. To compare the quantitative data between the three groups, the homogeneity of variance test (Levene statistic) was performed. If the variances were uniform, the Student-Newman-Keuls multiple test comparison was used to analyze the differences between the groups. If the variances were not uniform, the rank sum test in non-parametric detection was used to compare the differences between the groups. Qualitative data, such as gender, hypertension, smoking history, diabetes, coronary heart disease, hyperlipidemia, combination medications (statins, PPI, and CCB), and previous stroke or transient ischemic attack, were expressed as n (%). The CYP2C19 LOF allele carrier status was divided into the carrier group and the non-carrier group. Independent sample t-tests were used to evaluate CYP2C19 gene polymorphism and platelet reactivity after clopidogrel treatment. Potential relationships between plasma miR-223 expression levels were analyzed, and GraphPad Prism 5 was used for data analysis and processing.

The relationship between plasma miR-223 and the CYP2C19 LOF allele in clopidogrel HTPR was determined using the non-conditional logistic regression interaction function. All statistics were tested bilaterally, with $P<0.05$ being statistically significant.

RESULTS

Plasma miR-223 expression levels in the acute ischemic stroke and control groups

Patients with cerebral infarction were treated with clopidogrel and underwent ADP-PAg and VASP before and 7±1 days after the clopidogrel treatment. Patients were tested for platelet aggregation rate. A correlation analysis of the 60 patients with AIS showed that VASP-platelet reactivity index (PRI) was positively correlated with ADP-PAg before (r=0.190, $P=0.037$), and after (r=0.323, $P<0.001$) treatment. This shows a correlation between the two detection methods to evaluate platelet aggregation rate after clopidogrel administration (*Figs. 1* and *2*). In this study, 60

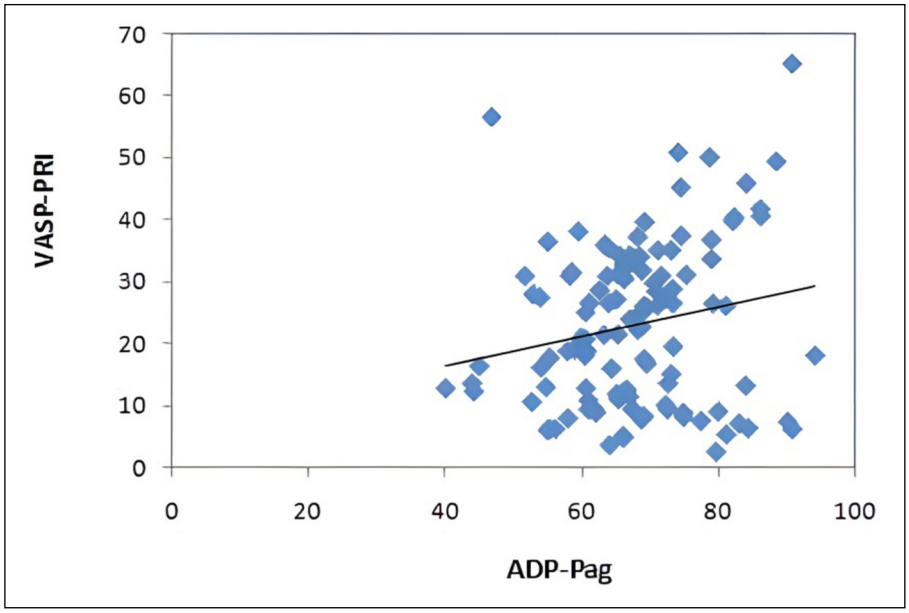

Fig. 1. Correlation analysis between vasodilator-stimulated phosphoprotein-platelet reactivity index (VASP-PRI) and adenosine diphosphate-induced platelet aggregation (ADP-PAg) before medication in acute ischemic stroke patients.

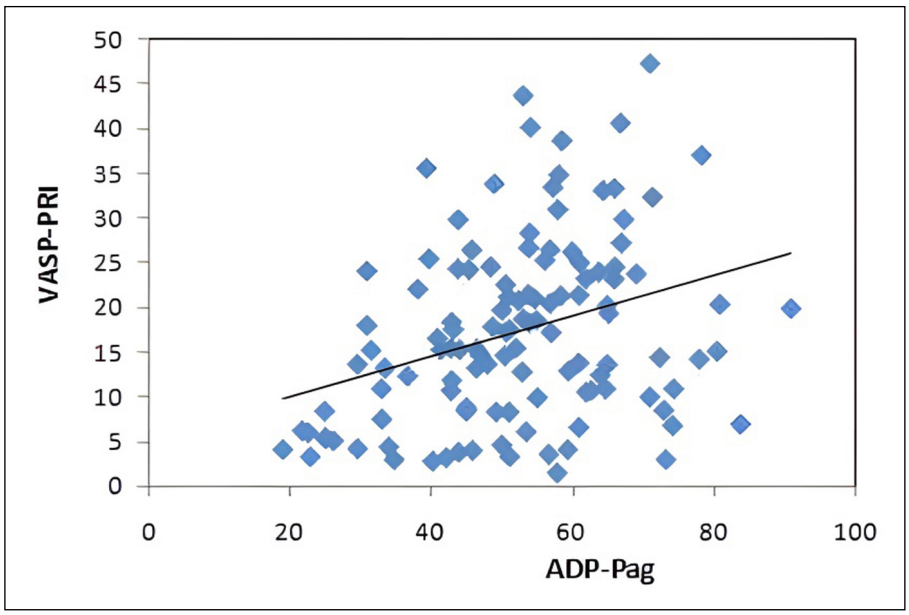

Fig. 2. Correlation analysis between vasodilator-stimulated phosphoprotein-platelet reactivity index (VASP-PRI) and adenosine diphosphate-induced platelet aggregation (ADP-PAg) after medication in patients with acute ischemic stroke.

Table 1. Comparison of basic clinical data of patients in the control group and acute ischemic stroke (AIS) group.

	Control group (n=60)	**AIS group** (n=60)	***t/x²/z***	***P***
Age (year, x±s)	71.18±9.81	69.32±11.05	0.978	0.33
Gender (male, n, %)	30 (50)	40 (66.7)	3.429	0.095
Hypertension	37 (61.7)	41 (68.3)	0.586	0.444
Coronary heart disease	21 (35)	27 (45)	1.250	0.264
Diabetes	18 (30)	23 (38.3)	0.926	0.336
Hyperlipidaemia	5 (8.3)	3 (5)	0.536	0.464
Drinking history	12 (20)	20 (33.3)	2.727	0.099
Smoking history	15 (25)	32 (53.3)	10.11	0.001
Previous stroke/transit ischemic attack	32 (53.3)	24 (40)	2.143	0.143
Statin lipid-lowering drugs	40 (66.7)	41 (68.3)	0.038	0.845
Proton pump inhibitors	41 (68.3)	32 (53.3)	2.833	0.092
Calcium channel blockers	35 (58.3)	36 (60)	0.034	0.853
Fasting blood-glucose (mmol/l)	6.19±1.97	6.71±3.00	–1.107	0.271
Alanine transaminase (U/L, x±s)	15.11±8.51	17.06±12.50	–0.991	0.324
Aspartate aminotransferase (U/L, x±s)	17.19±5.96	19.99±19.88	–1.026	0.307
Total cholesterol (mmol/l, x±s)	4.99±1.23	4.80±1.25	0.858	0.393
Triglycerides (mmol/l, x±s)	1.52±0.96	1.54±0.80	–0.162	0.872
High-density lipoprotein cholesterol (mmol/l, x±s)	1.17±0.26	1.09±0.34	1.495	0.137
Low-density lipoprotein cholesterol (mmol/l, x±s)	3.15±1.02	3.04±1.04	0.622	0.535
White blood cell count ($\times 10^9$/L, x±s)	6.96±2.45	7.23±2.28	–0.64	0.523
Platelet count ($\times 10^9$/L, x±s)	205.77±67.83	201.97±72.29	0.297	0.767
Hemoglobin (g/dl, x±s)	133.77±21.02	135.23±20.01	–0.391	0.696
Red blood cell count ($\times 10^{12}$/L, x±s)	4.29±0.59	4.33±0.59	–0.384	0.701
Uric acid (umol/l, x±s)	328.38±132.33	315.81±81.81	0.624	0.534
Creatinine (umol/l, x±s)	90.70±111.85	84.80±61.29	0.358	0.721
miR-223 before treatment	1.001±0.51	1.254±0.80	–2.063	0.041
VASP-PRI before treatment	34.77±10.69	66.23±11.32	–15.65	<0.001
VASP-PRI after treatment		51.105±18.51		
miR-223 after treatment		0.614±0.357		

Table 2. Related factors of acute ischemic stroke (AIS) in logistic regression analysis.

	Odd ratio (OR) (95% CI)	***P***
Gender (male)	0.780 (0.329–1.851)	0.573
Drinking history	0.958 (0.331–2.774)	0.936
Smoking history	2.924 (1.131–7.557)	0.027
Proton pomp inhibitors	0.712 (0.319–1.588)	0.407
miR-223 before treatment	1.159 (0.880–1.526)	0.294
VASP-PRI before treatment	1.011 (1.004–1.019)	0.002

CI, confidence interval.

patients with AIS, who underwent ADP-PAg and VASP before and after clopidogrel treatment, were used to detect platelet aggregation rate. The RI value was calculated, based on the ADP-PAg method, to detect the platelet aggregation rate and grouped in quantile form. In the upper quartile, 30 patients were in the clopidogrel high-response group (clopidogrel non-HTPR group), while in the lower quartile, 30 patients were in the clopidogrel low-response group (clopidogrel HTPR group). For these 60 patients, miR-223 expression levels were quantified using real-time PCR. In the control group, 60 patients were tested for platelet aggregation rate (VASP) and plasma miR-223 expression levels.

Table 1 shows the comparison between the basic clinical data of plasma miR-223 in patients in the control and AIS groups. The results show that smoking rates were higher in the AIS group than in the control group ($P<0.05$). Before administration, the plasma miR-223 expression levels and VASP-PRI were higher in the AIS group than in the control group ($P<0.05$). Through logistic regression analysis (*Table 2*), we found that smoking was associated with an increased risk of AIS (OR 2.924, 95% CI 1.131–7.557, $P=0.027$). The increase in VASP-PRI before medication was associated with an increased risk of AIS (OR 1.011, 95% CI 1.004–1.019, $P=0.002$). There

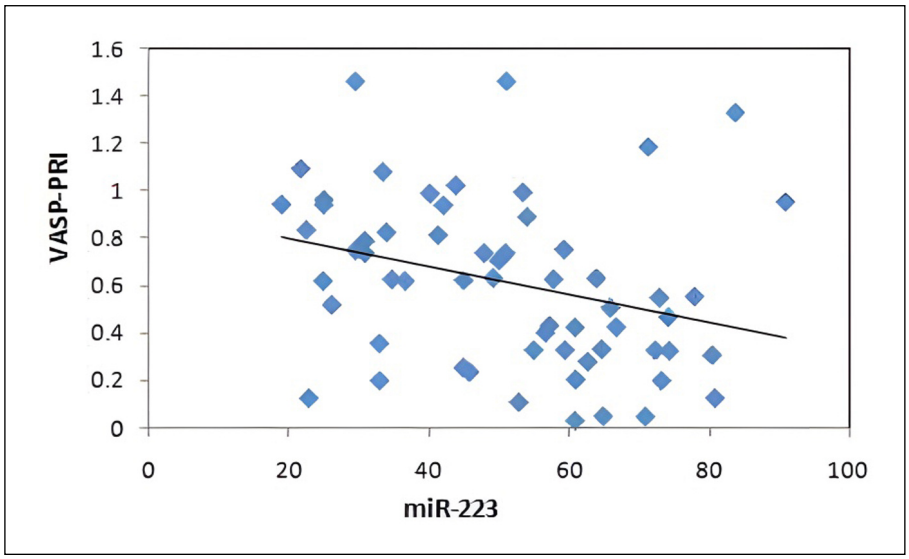

Fig. 3. Correlation analysis between vasodilator-stimulated phosphoprotein-platelet reactivity index (VASP-PRI) and microRNA-223 expression levels after medication in patients with acute ischemic stroke.

was no significant relationship between plasma miR-223 expression levels and an increased risk of AIS before treatment (OR 1.159, 95% CI 0.880–1.526, *P*=0.294).

Expression of miR-223 in the clopidogrel low- and high-response groups

After patients with AIS had received clopidogrel maintenance therapy for 7±1 days, 30 cases in the clopidogrel non-HTPR group and 30 cases in the clopidogrel HTPR group were selected. Plasma miR-223 levels were quantified in both groups using real-time PCR and CYP2C19*2, and *3 genotyping tests were performed. In the 60 patients with genotypes, the allele frequency of CYP2C19*1/*1 was 36%, CYP2C19*1/*2 was 48%, CYP2C19*2/*2 was 16%, and CYP2C19*1*3 was 10%. The baseline data of the two groups of patients are shown in *Table 3*.

Correlation analysis showed that the expression level of miR-223 and VASP-PRI were negatively correlated (r= –0.304, *P*=0.018). This revealed a good correlation between miR-223 and VASP-PRI after administration (*Fig. 3*). The results showed that the level of miR-223 expression in the clopidogrel HTPR group before and after treatment was lower than in the non-HTPR group, and the difference was statistically significant. *Table 3* shows that the percentage of patients with hypertension and the CYP2C19 LOF allele was significantly higher in the clopidogrel HTPR group than the non-HTPR group (*P*<0.05). Logistic regression analysis (*Table 4*) showed that having both the CYP2C19 LOF allele and hypertension was associated with an increased risk of clopidogrel HTPR (OR 0.304, 95% CI 0.100–0.922, *P*=0.035). A decrease in plasma miR-223 expression levels before and after administration was associated with an increase in risk of clopidogrel HTPR (miR-223 before treatment: OR 2.219, 95% CI 1.030–4.781, *P*=0.042; miR-223 after treatment: OR 5.109, 95% CI 1.082–24.133, *P*=0.039).

DISCUSSION

Definition and detection of clopidogrel high on-treatment platelet reactivity

Clopidogrel HTPR was once called clopidogrel resistance, which can be divided into clinical resistance and laboratory resistance. In this study, flow cytometry was used to detect the ability of clopidogrel to inhibit platelet aggregation in response to clopidogrel specificity of actin-vasodilation-stimulating phosphoprotein phosphorylation levels in platelets. VASP is in a non-phosphorylated state in the platelet-based state. Clopidogrel mainly inhibits the binding of the P2Y12 receptor to ADP by irreversibly binding to the P2Y12 receptor, activating adenyl cyclase, and causing cyclophosphane. Increased cyclic adenosine monophosphate (cAMP) production and prostaglandin E1 activates the cAMP cascade to control the phosphorylation of VASP. Phosphorylated VASP inhibits platelet aggregation by inhibiting the activation of platelet glycoprotein IIb/IIIa receptors (12). Therefore, the phosphorylation status of VASP responds to the degree of inhibition of the P2Y12 receptor, *i.e.*, the binding of clopidogrel to the P2Y12 receptor (12). VASP defines HTPR after clopidogrel treatment based on PRI≥50% (13, 14). However, this technique requires a flow cytometer, which is expensive, suitable for laboratory testing, and difficult to popularize. Therefore, in this study, the ability of clopidogrel to inhibit platelet aggregation was evaluated by measuring the platelet aggregation rate induced by ADP-PAg using flow cytometry. This method defines HTPR after clopidogrel treatment as a platelet aggregation rate of 5 μmol/L ADP induction ≥50% (15, 16) and 10 μmol/L ADP≥70% (17-20).

In this study, VASP-PRI and ADP-PAg were detected before and after clopidogrel treatment in 60 patients with AIS. ADP-PAg was positively correlated (r=0.190, *P*=0.037), and VASP-PRI was positively correlated with ADP-PAg after medication (r=0.323, *P*<0.001). Therefore, these two methods were used to detect platelet aggregation, and the rates were correlated. A previous study by this research group (16) included a total of 43 patients with AIS and used VASP, ADP-induced flow cytometry, and PL-11 whole blood continuous counting method to measure platelet aggregation rate and compared the correlation and consistency of the three methods. It was found that the three methods had a good correlation and consistency in detecting platelet aggregation rate, which is supported by the results of this study.

MiR-223 expression level and clopidogrel high on-treatment platelet reactivity

Ischemic stroke patients show a differential miRNA expression profile as compared to controls. These new associations between circulating miRNAs and ischemic stroke may help to refine stroke subtype diagnosis and identify novel

Table 3. Comparison of data between clopidogrel non-high on-treatment platelet reactivity (HTPR) group and clopidogrel HTPR group in acute ischemic stroke (AIS) patients.

	Clopidogrel HTPR group (n=30)	**Clopidogrel non-HTPR group** (n=30)	***t/x²/z***	***P***
Age (year, x±s)	69.83±10.52	68.8±11.71	0.36	0.721
Gender (male, n, %)	19 (63.3)	21 (70)	0.300	0.584
Hypertension	25 (83.3)	16 (53.3)	6.239	0.012
Coronary heart disease	13 (43.3)	14 (46.7)	0.067	0.795
Diabetes	11 (36.7)	12 (40)	0.071	0.791
Hyperlipidaemia	3 (10)	0 (0)	3.158	0.076
Drinking history	10 (33.3)	10 (33.3)	0.000	1.000
Smoking history	15 (50)	17 (56.7)	0.268	0.605
Previous stroke/Transit ischemic attack	12 (40)	12 (40)	0.000	1.000
Statin lipid-lowering drugs	23 (76.7)	18 (60)	1.926	0.165
Proton pump inhibitors	14 (46.7)	18 (60)	1.071	0.301
Calcium channel blockers	18 (60)	18 (60)	0.000	1.000
Total cholesterol (mmol/l, x±s)	4.63±1.38	4.98±1.09	–1.10	0.276
Triglycerides (mmol/l, x±s)	1.66±0.87	1.42±0.72	1.14	0.259
High-density lipoprotein cholesterol (mmol/l, x±s)	1.07±0.34	1.11±0.34	–0.38	0.709
Low-density lipoprotein cholesterol (mmol/l, x±s)	2.89±1.15	3.19±0.91	–1.114	0.270
White blood cell count ($\times 10^9$/L, x±s)	6.94±1.95	7.53±2.57	–0.996	0.323
Platelet count ($\times 10^9$/L, x±s)	216.03±88.96	187.9±48.05	1.524	0.133
Hemoglobin (g/dl, x±s)	133.2±20.56	137.27±19.58	–0.784	0.436
Red blood cell count ($\pm 10^{12}$/L, x±s)	4.32±0.64	4.35±0.56	–0.246	0.807
Uric acid (umol/l, x±s)	327.31±66.47	304.31±94.47	1.09	0.28
Creatinine (umol/l, x±s)	89.38±60.73	80.22±62.53	0.576	0.567
miR-223 before treatment	0.962±0.788	1.546±0.711	–3.016	0.004
miR-223 after treatment	0.464±0.329	0.765±0.322	–3.587	0.001
Changes of miR-223 before and after treatment	0.498±0.521	0.781±0.475	–2.196	0.032
Carrying of CYP2C19 loss of function (LOF) allele; (n, %)	23 (76.7)	15 (50)	4.593	0.032

therapeutic miRNA targets for the treatment of ischemic stroke (21). Multiple studies have confirmed that microRNA is involved in the regulation mechanism of platelet reactivity. A number of previous clinical studies have shown that the decreased expression of MiR-223 in circulation/platelet is closely related to the hyperplatelet reactivity of patients treated with clopidogrel, but there are few studies on the specific mechanism of MiR-223 in the regulation of platelet reactivity.

MiR-223 is the most abundant microRNA in platelets and is stable in platelets and plasma. Most microRNAs are first transcribed by RNA polymerase II to produce microRNA precursors with 5' caps and 3' polyadenylic acids, namely pre-miRNA. Pre-miRNA is transferred from the nucleus to the cytoplasm through Ran-GTP. Pre-miRNA is used as a template in the cytoplasm to generate mature microRNA under the action of the Dicer-TRBP2 complex. Mature microRNAs, guided by proteins, such as Ago2, bind to RNA-induced silencing complexes (RISC) and are mediated by RISC to the 3'-untranslated region (3'-UTR) of the targeted mRNA to cut the target gene or suppress translation, thereby regulating gene expression (7).

This study found that the smoking rate of patients in the AIS group was higher than in the control group ($P<0.05$). The levels of plasma miR-223 and VASP-PRI in the AIS group were higher than in the control group before administration ($P<0.05$). After logistic regression analysis, we found that smoking and pre-dose VASP-PRI increased with the increased risk of AIS (smoking: OR 2.924, 95% CI 1.131–7.557, $P=0.027$; pre-dose VASP-PRI: OR 1.015, 95% CI 1.003–1.028, $P=0.017$), and that there was no significant relationship between plasma miR-223 expression levels and an increased risk of AIS before treatment (OR 0.823, 95% CI 0.460–1.473, $P=0.512$). Shen *et al*. (22) found a significant association between smoking exposure and the risk of all-cause and cardiovascular disease mortality. This result is consistent with our findings. At the same time, it is possible that in patients with AIS, the platelet aggregation rate and VASP-PRI increased due to the increased binding of ADP to the platelet P2Y12 receptor protein after platelet activation. Therefore, the increase in VASP-PRI before smoking and medication is associated with an increased risk of AIS, but the level of miR-223 expression in plasma before medication has no significant relationship with an increased risk of AIS.

Table 4. Logistic regression analysis of high on-treatment platelet reactivity related factors of clopidogrel in patients with acute ischemic stroke.

	Odd ratio (OR) (95%CI)	***P***
Hypertension	0.229 (0.069–0.758)	0.016
Hyperlipidaemia	0.00 (0.00)	0.999
miR-223 before treatment	3.205 (1.332–7.712)	0.009
miR-223 after treatment	5.109 (1.082–24.133)	0.039
Changes of miR-223 before and after treatment	3.436 (1.040–11.344)	0.043
Carrying of CYP2C19 LOF allele	0.304 (0.100–0.922)	0.035
Interaction between microRNA-223 and CYP2C19 LOF allele before treatment	3.750 (1.078–13.039)	0.038
Interaction between microRNA-223 and CYP2C19 LOF allele after treatment	1.266 (0.297–5.399)	0.750

CI, confidence interval; LOF, loss of function.

Previous literature (9-11) used the method of RI of platelets to classify platelet reactivity after drug treatment, *i.e*., to express the platelet response to the drug by the percentage reduction in platelet aggregation after drug treatment, RI = (platelet aggregation rate before medication - platelet aggregation rate after medication) ÷ platelet aggregation rate before medication × 100, and group them in quantile form. In this study, the RI values obtained by the ADP-PAg method were used to group quantiles. The upper quartile was the clopidogrel non-HTPR group, and the lower quartile was the clopidogrel HTPR group.

In this study, 60 patients with AIS were screened using the above method, and a correlation analysis showed that the expression level of miR-223 in patients was negatively correlated with VASP-PRI after administration (r= –0.304, *P*=0.018). Zhang *et al*. (12) analyzed a total of 62 patients with troponin-negative non-ST-segment elevation acute coronary syndromes. Plasma circulating miR-223 expression levels were quantified using real-time PCR, and platelet reactivity was measured by PRI. It was found that the expression level of miR-223 was negatively correlated with VASP-PRI (r= –0.379, *P*= .002), which is consistent with the results of this study. Plasma miR-223 expression levels were negatively correlated with VASP-PRI and may have complementary binding sites to the 3'-UTR sequences of miR-223 and P2Y12. This forms an Ago2-miR-223 complex to target P2Y12 mRNA, thereby inhibiting the expression of the P2Y12 receptor protein related to platelet aggregation, leading to a decrease in platelet aggregation rate and a decrease in VASP-PRI.

This study also found that plasma miR-223 expression levels before and after treatment in the clopidogrel HTPR group were lower than in the clopidogrel non-HTPR group, and the difference was statistically significant. Using logistic regression analysis, we found that the decrease in plasma miR-223 expression levels before and after administration was associated with an increased risk of clopidogrel HTPR (miR-223 before treatment: OR 2.219, 95% CI 1.030–4.781, *P*=0.042; miR-223 after treatment: OR 5.109, 95% CI 1.082–24.133, *P*=0.039). Landry *et al*. (23) found that miR-223 and the 3'-UTR sequence of P2Y12 have complementary binding sites, forming the Ago2-miR-223 complex to target P2Y12 mRNA, thereby inhibiting the expression of the P2Y12 receptor protein related to platelet aggregation. Similarly, Cheng *et al*. (24) verified the function of miR-223 through a double luciferase reporter assay and found that miR-223 can target the 3'-UTR region of the P2Y12 mRNA. In contrast, other investigators (25-27) believed that miR-223 in platelets regulates the expression of P2Y12 by constituting the miR-223: P2Y12 pairing, and that the increase in miR-223 expression levels can increase the P2Y12 receptor expression on the platelet surface, which makes platelets easier to activate and aggregate. The results of this study are consistent with the research of Landry and Cheng (23, 24). It is possible that due to the reduced expression of miR-223, the binding of P2Y12 mRNA to P2Y12 mRNA is reduced, which leads to the increased expression of P2Y12 mRNA and regulates the expression of the P2Y12 receptor protein. However, clopidogrel mainly works with P2Y12. The irreversible binding of the receptor inhibits the binding of the P2Y12 receptor to ADP, thereby inhibiting platelet aggregation. Due to the increased expression of the P2Y12 receptor protein, ADP binds closely to it and limits the ability of clopidogrel to also bind to it, and platelets are more easily activated and aggregated. Therefore, a decrease in plasma miR-223 expression levels before and after administration is associated with an increased risk of clopidogrel HTPR.

In addition, inflammation is closely related to thrombosis, and C-reactive protein (CRP) is a biomarker of inflammatory response in human body. The study have shown that the monomer form of C-reactive protein associated with membrane microvesicles is related to the improvement of platelet activation and the decrease of anti-platelet response, markers of inflammation, such as monomeric CRP (mCRP) or platelet microvesicles (MVs), may be useful markers of antiplatelet therapy (28). Whether the regulatory mechanism of platelet reactivity changes caused by inflammation in human body is related to microRNA-223 or other microRNAs remains to be further studied.

Except clopidogrel, novel P2Y12 receptor antagonists are widely used in the treatment of cardiovascular and cerebrovascular diseases, a long-term follow-up study suggests (29): prasugrel and ticagrelor have similar effects on major adverse cardiac events in patients with STEMI undergoing

primary PCI, but prasugrel seems more tolerated and less discontinued than ticagrelor. Individualized selection and quantitative evaluation of antiplatelet drug therapy are urgent clinical problems. MicroRNA-223 expression level and inflammatory markers, such as C-reactive protein monomers or platelet-derived microvesicles, may provide markers of antiplatelet therapy response, and normalized monitoring of the effectiveness and safety of antiplatelet therapy may be a useful means of individualized therapy in the future.

In summary, a decrease in plasma miR-223 expression levels before and after administration to AIS patients indicates an increased risk of clopidogrel HTPR. An interaction occurred between the CYP2C19 LOF allele and the plasma miR-223 expression levels. Therefore, the carrying of CYP2C19 LOF alleles may make plasma expression of miR-223 more obvious. The combined detection of plasma miR-223 and CYP2C19 gene polymorphisms may be effective in predicting the occurrence of clopidogrel HTPR in patients with AIS and, at the same time, could provide an objective basis for future research on individualized treatment with clinical antiplatelet drugs.

Deficiencies and prospects of this survey are: 1) this study measured plasma miR-223 expression levels rather than directly detecting platelet-derived miR-223 expression levels, which may have had an impact on the association between platelet-derived miR-223 and platelet reactivity; 2) This study was a single-center, small-sample study. There may have been some bias. These findings need to be further verified by a multi-center, large-sample study.

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