Platelet Surface CD62p and Serum Vitamin D Levels are Associated with Clopidogrel Resistance in Chinese Patients with Ischemic Stroke

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Background: To explore the association of platelet activation markers, vitamin D, and antiplatelet drugs resistance in ischemic stroke patients. *Methods:* A total of 230 patients with ischemic stroke were enrolled in this study. Platelet aggregation, platelet activation marker (CD62p), and vitamin D were measured after 7-14 days of dual antiplatelet treatment (aspirin + clopidogrel). All individuals were divided into a drug resistance group and a drug sensitive group according to the platelet maximum aggregation rate induced by antagonist adenosine diphosphate or arachidonic acid. *Results:* In this study, the prevalence of aspirin resistance was low (1.2%), while the prevalence of clopidogrel resistance (CR) was 24.8%, so we focused on CR. The percentage of CD62p on activated platelet [(25.74 ± 4.61) versus (12.41 ± 3.93), $P < .001$] and the prevalence of hypertension [93.0% (53) versus 79.8% (138), $P = .021$] in CR group were significantly higher than those in clopidogrel sensitive (CS) group, while the vitamin D concentration [(8.96 ± 4.41) versus (13.9 ± 4.84) ng/mL, $P = .003$] in CR group was significantly lower compared with the CS group. No significant difference was found in soluble P-selectin between these 2 groups [(56.2 ± 16.13) versus (54.2 ± 14.87) ng/mL, $P = .258$], neither in calcium [(2.29 ± .12) versus (2.33 ± .13) mmol/L, $P = .821$]. Logistic regression analysis showed that hypertension (odds ratio [OR] = 5.348, 95% confidence intervals [CI] 1.184-23.350, $P = .026$), expression of platelet CD62p (OR = 1.095, 95% CI 1.052-1.201, $P = .018$) and vitamin D level (OR = .832, 95% CI .763-.934, $P = .005$) were associated with CR in ischemic stroke patients. *Conclusions:* CR in ischemic stroke patients is associated with several independent predictors, including increased platelet activation marker CD62p, decreased vitamin D level, and hypertension.

Key Words: Ischemic stroke—clopidogrel resistance—platelet activation—vitamin D

Introduction

The onset of ischemic stroke is closely associated with the enhanced platelet adhesion and aggregation. Therefore, patients with ischemic stroke need to receive oral antiplatelet therapy regularly for long term. Aspirin and clopidogrel are most commonly applied antiplatelet drugs in China.[1] However, the effect of antiplatelet therapy is not always satisfying for a part of ischemic stroke patients, since ischemic stroke and other vascular events may recrudesce.

Recent studies have shown that high residual platelet reactivity (HRPR; namely, antiplatelet drugs resistance) following antiplatelet therapy with aspirin increases the risk of recurrent ischemic vascular events.[2,3] Clopidogrel is widely used in clinical practice. VerifyNow-P2Y12 is a reliable, fast, and sensitive device suitable for monitoring platelet inhibition during clopidogrel therapy in the clinical arena.[4] It is reported that high platelet reactivity was an independent risk factor for adverse events after ischemic stroke, and the VerifyNow-P2Y12 test may be available to guide individualized antiplatelet therapies in

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stroke patients in China.[5] It has been reported that the frequencies of clopidogrel resistance (CR) in Asians varied from 20% to 65%, which highly exceeded the incidence reported in other races.[6] Therefore, assays that identify those patients with an impaired responsiveness or a heightened platelet reactivity may contribute to better risk stratification and will probably improve clinical outcome when appropriate action is initiated. Although numerous studied and reports on effective antiplatelet drugs have been documented, little is known about the predictors of antiplatelet drugs resistance.

Platelet activation is considered to be one of characteristics of atherothrombosis. Platelet activation leads to enhanced platelet aggregation, increased mean platelet volume (MPV) and upregulated production of reactive oxygen species, thrombin, and platelet activating factor. P-selectin, also called CD62p, is a kind of glycoprotein stored in Weibel-Palade bodies in vascular endothelial cells and α-granules in platelets. CD62p plays a pivotal role in inflammation and thrombosis mainly through mediating the roll of neutrophils and monocytes on the surface of vascular endothelial cells, as well as promoting the adhesion of platelets to those rolling cells.[7,8] Platelet activation is affected by a variety of factors, including high glucose,[9] inflammation cytokines,[10] lipopolysaccharides and viral infections. Recently, vitamin D (VitD) has been found to have a wide range of biological effects. Besides the well-known regulation of calcium and phosphorus levels, VitD is also involved in immune adjustment, differentiation, and proliferation of various cells. Mitochondrial in platelet has been reported to express vitamin D receptor (VDR), which indicates that VitD may regulate platelet activation through the pathway of directly modulating nongenomic activity of mitochondrial VDR or calcium signaling.[11,12]

Based on the potential link between VitD, CD62p, and platelet activation, we systematically analyzed their correlation with antiplatelet drugs resistance in ischemic stroke patients, aiming to find out predictors of antiplatelet drugs resistance.

Materials and Methods

Study Population

In total, 230 patients with ischemic stroke were enrolled in the study from June 2017 to October 2018. The inclusion criteria were as follows: (1) clinical diagnosis of ischemic stroke according to the 2014 Chinese guidelines for secondary prevention of ischemic stroke,[1] with computed tomography or magnetic resonance imaging verification; (2) trial of Org 10172 in Acute Stroke Treatment classification[13] of large artery atherosclerosis or small blood vessel with occlusion ischemic stroke; (3) signed informed consent prior to the study. The exclusion criteria were as follows: (1) patients with Alzheimer's disease; (2) occurrence of fever, hypoxia, disorder of consciousness or hemodynamic disorder; (3) patients who were allergic to aspirin or clopidogrel; (4) treatment of anti-coagulation therapy with low-molecular-weight heparin, warfarin or other antiplatelet drugs except aspirin within 1 week; (5) platelet count $>450 \times 10^9$/L or $<150 \times 10^9$/L; (6) severe hepatic or renal dysfunction, malignant diseases, respiratory or immune system diseases at study entry; (7) the presence of trauma or surgery within the preceding 1 month. This study was approved by the Ethics Committee of Beijing Bo'ai Hospital, China Rehabilitation Research Center.

Information Collection

Clinical data on general condition (age, gender, height, and weight), medical history (hypertension, diabetes, stroke, or coronary atherosclerotic heart disease), personal history (smoking or drinking), medication history, family history, laboratory tests, and medical imaging data (computed tomography or magnetic resonance imaging) were obtained from all patients.

Management of Antiplatelet Drugs

During the admission period, all enrolled patients were treated with oral antiplatelet therapy according to the 2014 Chinese guideline[1]: noncardiac ischemic stroke patients were given short-range aspirin combined with clopidogrel in early days and subsequently received aspirin or clopidogrel for the long term. In the case of dual antiplatelet drugs, patients took a 300 mg loading dose of clopidogrel (Sanofi, Paris, France) and 300 mg aspirin (Bayer S.p.A, Leverkusen, Germany) on the day of admission and maintained 75 and 100 mg daily, respectively. After 3 weeks, patients received a 75 mg maintenance dose of clopidogrel or a 100 mg maintenance dose of aspirin daily for single antiplatelet therapy. Venous whole blood samples were obtained from patients treated with dual antiplatelet therapy for 7-14 days and were transfused into 2 tubes, one was anticoagulated with 3.8% sodium citrate, the other was procoagulant. Platelet aggregation and biochemical tests were then measured.

Platelet Function Assay

Platelet aggregation was measured by light turbidimetric platelet aggregation analyzer (Techlink biomedical, Beijing, China) to evaluate antiplatelet responses. The whole blood was centrifuged to separate the platelet-rich plasma (PRP) and the platelet-poor plasma (PPP), platelets in PRP were counted by hematology analyzer and were diluted to 200-300 $\times 10^9$/L using the PPP sample. To measure platelet aggregation, adenosine diphosphate (ADP, 5 μmol/L; Sigma, America) or arachidonic acid (AA, .5 mg/L; Sigma, America) was added to the PRP sample to induce platelet aggregation. The change in light transmission in the PRP sample was recorded until the response reached a plateau. The maximal platelet

aggregation rate (MPAR) was then calculated and recorded, with PPP sample as a reference. MPAR ≥50% induced by ADP was defined as CR, and MPAR <50% induced by ADP was defined as clopidogrel sensitive (CS).[14] MPAR ≥20% induced by AA was defined as aspirin resistance, and MPAR <20% induced by AA was defined as aspirin sensitive.[15] All experiments were performed within 3 hours.

Platelet activation was determined by quantifying CD62p on platelet and soluble P-selectin in plasma. Platelet CD62p was detected by flow cytometer (BD FACSCalibur, America). PE-conjugated anti-CD61 (BD, America) which bound to the glycoprotein IIb/IIIa of platelet was used to identify the platelets. Twenty microliter PE-conjugated anti-CD61 and 20 μL fluorescein isothiocyanate-conjugated anti-CD62p (BD, San Jose, CA, USA) were added to 5 μL whole blood (anticoagulated with 3.8% sodium citrate). Meanwhile, 20 μL PE-conjugated anti-CD61 and 20 μL FITC-conjugated mouse IgG1 (isotype) were added to the counterpart as negative control. Blood mixture was incubated at room temperature in the dark for 15 minutes. Then, 1% paraformaldehyde in phosphate buffered saline was added for 30 minutes in the dark at 2°C-8°C. The result was expressed as the percentage of CD62p-positive platelets in the CD61-identified platelets. The concentration of soluble P-selectin in PPP was measured by enzyme-linked immunosorbent assay (CUSABIO, Wuhan, China).

Biochemical Measurements

Serum was obtained by centrifugation at 3000 rpm for 10 minutes, and numerous biochemical items were subsequently determined by automated biochemical analyzer (Mindray SM2000, Mindray, Shenzhen, China). The serum level of 25-hydroxy VitD (25-OH VitD) was measured by automated electrochemiluminescence immuno analyzer (Roche Cobase e601, Switzerland). Criteria of VitD status: (1) VitD deficiency: 25-OH VitD < 10 ng/mL; (2) VitD insufficiency: 10 ng/mL ≤ 25-OH VitD < 20 ng/mL; (3) VitD critical range:20 ng/mL ≤ 25-OH VitD ≤ 30 ng/mL; (4) VitD sufficiency: 25-OH VitD > 30 ng/mL.

Statistical Analysis

The statistical analysis was performed by IBM SPSS version 17.0 software. Quantitative data were presented as the mean ± standard deviation. The significant differences between groups were evaluated using χ^2 test (when variables were categorical) or independent-samples t test (when variables were continuous). Multivariate logistic regression analysis was performed to determine independent risk factors of antiplatelet drug resistance. Two-sided P value <.05 was considered to be statistically significant. Variables in univariate logistic regression (P < .05) were taken into consideration in the multivariate model.

Results

Clinical Data and Biochemistry Measurements of Research Population

A total of 230 ischemic stroke patients (119 males and 111 females) were enrolled in this study. All of the patients were divided into the CR or CS group according to the definitions described in the methods section. The main demographic, clinical, and laboratory features of our population are listed in Table 1. CR was observed in 57 patients (24.8%). The prevalence of aspirin resistance was low (1.2%), so we focused on CR. Among all these variables, hypertension rate in CR group was significantly higher than that in CS group ($\chi^2 = 5.316$, $P = .021$), while no significant differences in the distribution of age, gender, body mass index, smoking, alcohol, coronary heart

Table 1. *Clinical characteristics and biochemical indexes of the patients*

	CR (n = 57)	CS (n = 173)	t or χ^2 value	P value
Age (year)	68.5 ± 7.2	67.6 ± 8.5	−.491	.629
Gender (male/female)	30/27	89/84	.024	.876
BMI (n, %)	24.29 ± 2.92	23.81 ± 3.3	.457	.529
Smoke (n, %)	17, 29.8%	55, 31.8%	.077	.781
Drink (n, %)	5, 8.8%	7, 4.0%	1.936	.164
Hypertension (n, %)	53, 93.0%	138, 79.8%	5.316	.021
Coronary heart disease (n, %)	10, 17.5%	19, 11.0%	1.675	.196
Diabetes (n, %)	33, 58.0%	88, 51.0%	.849	.357
Triglycerides (mmol/L)	1.72 ± .79	1.5 ± .74	−1.675	.096
Cholesterol (mmol/L)	4.21 ± 1.42	4.08 ± 1.09	−.442	.663
LDL (mmol/L)	2.89 ± .74	2.74 ± .74	−1.431	.175
UA (μmol/L)	369.8 ± 95.63	341.2 ± 99.8	−1.327	.187
HCY (μmol/L)	17.2 ± 9.16	15.7 ± 4.68	.714	.482
FBG (mmol/L)	6.52 ± 2.64	6.3 ± 1.96	−.813	.419
HbA1c (%)	7.22 ± 1.95	6.9 ± 1.64	.908	.365

Abbreviations: BMI, body mass index; FBG, fast blood glucose; HCY, homocysteine; LDL, low-density lipoprotein; UA, uric acid.

disease, diabetes or biochemical indexes were found between the CR and CS group.

Comparison of Platelet Function and VitD Concentration between CR and CS Groups

The expressions of platelet CD62p in CR group were significantly higher than those in CS group ($P < .001$), while VitD concentration in CR group was markedly lower than that in CS group ($P < .01$). No significant difference was found in P-selectin or Ca^{2+} between the 2 groups (Table 2).

CR was observed in 57 patients, and the rate decreased with higher VitD levels (48.8% (41) versus 10.9% (14) versus 11.1% (2), $\chi^2 = 40.981$, $P < .001$), while the rate of CS (51.2% (43) versus 89.1% (114) versus 88.9% (16), $\chi^2 = 40.981$, $P < .001$) correspondingly increased with higher VitD levels (Fig 1).

Correlation among Hypertension, CD62p, VitD, and CR

Univariate analysis identified several statistically significant independent variables, including hypertension, CD62p, and VitD. Multivariate logistic regression analysis among hypertension, CD62p, VitD, and CR was shown in Table 3. It was observed that hypertension (odds ratio [OR] = 5.348, 95% confidence intervals [CI] 1.184-23.350, $P = .026$), CD62p (OR = 1.095, 95% CI 1.052-1.201, $P = .018$), and VitD (OR =.832, 95% CI .763-.934, $P = .005$) were independent predictors of CR.

Discussion

HRPR is associated with a variety of factors, including proteomics, metabolomic factors (glycometabolism, smoking, renal function, and other factors affecting hepatic enzymes or platelet receptor expression), and genomics factors (genetic polymorphisms of cytochrome enzyme P450 [CYP] and P2Y12).[16] For patients with low response to clopidogrel, increasing the dose of clopidogrel is one of the commonly applied clinical interventions. Wu et al[17] screened acute coronary syndrome (ACS) patients with HRPR after receiving standard dose clopidogrel therapy (75 mg, 1 time/day) for long-term and treated them with double-dose clopidogrel (150 mg, 1 time/day). Then they found that the platelet reactivity after double-dose treatment was significantly reduced compared with baseline, without increased incidence of bleeding events. However, other studies showed that CR could not be suppressed by high-dose clopidogrel

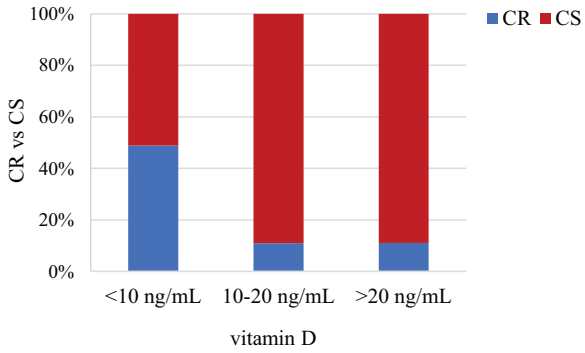

Figure 1. *Bar graph shows the prevalence of CR and CS at ADP test according to vitamin D levels in patients treated with clopidogrel.*

Table 3. *Multivariate logistic regression analysis on clopidogrel resistance*

Variable	OR	95% CI	*P* value
Hypertension	5.348	1.184-23.350	.026
CD62p	1.095	1.052-1.201	.018
Vitamin D	.832	.763-.934	.005

treatment.[18] Based on further studies about factors affecting the diversity of platelet reactivity, developing relevant treatment strategies of CR will benefit more patients.

After platelet activation, α-granules inside platelet rapidly fuse with platelet membrane, and CD62p is translocated to the membrane, with a small part entering blood circulation. CD62p is considered as one of the most characteristic markers of platelet activation, and the elevated level of CD62p in plasma (soluble P-selectin) reflects the activation status of platelet.[19] The expression of CD62p on platelet was positively correlated with platelet aggregation.[20] Platelet activation and aggregation are critical in the pathogenesis of acute ischemic cerebrovascular diseases. It is reported that pretreatment level of platelet CD62p obviously increased during the acute phase in ischemic stroke patients, while the level of platelet CD62p showed a gradually declining tendency after treatment with antiplatelet therapy.[21,22] These findings suggested that platelet CD62p might be associated with HRPR. So far, there have been few reports on the correlation between platelet CD62p, soluble P-selectin, and CR.

In this study, the expression of platelet CD62p in the CR group was significantly higher than that in CS group, indicating that high reactivity of platelet was one of the factors leading to CR. While the level of soluble P-selectin in

Table 2. *Analysis of platelet function and vitamin D concentration in the research population ($\bar{x} \pm s$)*

	CR (n = 57)	CS (n = 173)	*t* value	*P* value
P-selectin (ng/mL)	56.2 ± 16.13	54.2 ± 14.87	−.672	.258
CD62p (%)	25.74 ± 4.61	12.41 ± 3.93	−5.253	<.001
Vitamin D (ng/mL)	8.96 ± 4.41	13.9 ± 4.84	3.057	.003
Ca^{2+} (mmol/L)	2.29 ± .12	2.33 ± .13	.664	.821

plasma had no significant association with CR, probably due to other sources of soluble P-selectin, such as activated endothelial cells.[23] Thus, platelet CD62p was supposed to be more specific as a platelet activation marker compared with soluble P-selectin. It is suggested that particular attention should be paid to platelet specific markers and relevant detection techniques when evaluating platelet function.

VitD, an important component of bone and mineral metabolism, is a lipid-soluble vitamin, which is mainly present in the form of 25-OH VitD in the body. 25-OH VitD is the best indicator of VitD status, which reflects the dietary intake and skin synthesis of VitD.[24] VDR also exists on platelets,[11] indicating that the function of platelets may also be affected by VitD. The adverse effects of VitD deficiency on hemostasis and thrombosis had been increasingly confirmed in vitro and in animal models. It had been proven that VitD could reduce the expression of plasminogen activator I, tissue factors, and thrombomodulin.[25] By now, few studies on VitD and platelet activation are reported.

We found that VitD deficiency was commonly seen in ischemic stroke patients, particularly in the CR group. VitD deficiency was considered to affect the function of platelets, confirmed in the study of Maya Sultan et al.[26] They found that platelet aggregation induced by ADP was significantly decreased in diabetic patients and healthy individuals both treated with calcitriol (1,25-(OH) 2-D3), compared with normal saline treatment group. It had also been reported that VitD level was negatively correlated with MPV in patients with stable coronary heart disease.[27] MPV reflects platelet reactivity, metabolism, and enzyme activity in platelet. Moreover, MPV is associated with the increased incidence (including mortality) or risks of coronary heart disease, ACS, hypertension, and stroke. Elevated MPV was supposed to be a potential prognostic biomarker for patients with cardiovascular and cerebrovascular diseases.[28] On the basis above, it is suggested that low concentration of VitD may promote platelet activation (as shown by enhanced expression of platelet CD62p), leading to ADP-induced high platelet aggregation and subsequent CR. The role of VitD in suppressing the expression of platelet CD62p remains unknown and the molecular mechanisms need more investigation. In addition, serum calcium was also detected in our study. It was reported that platelet aggregation significantly enhanced in normocalcemic VDR knock-out mice on a high calcium diet, then calcium intake having a great effect on platelet activation was concluded.[29] In our study, no significant difference in calcium was found between CR and CS group, which indicated that VitD interacted with VDR of platelet and regulated platelet activation through some other pathways rather than calcium-dependent way.

Studies on the correlation between serum uric acid concentration and platelet reactivity remained controversial. Elevated level of uric acid is one of the risk factors for atherosclerosis. Several studies had analyzed the correlation between uric acid and antiplatelet drugs resistance, and the results turned out to be different. Yildiz et al suggested that high uric acid level led to aspirin resistance,[30] while Barbieri et al concluded that uric acid concentration did not affect the reactivity of aspirin, clopidogrel, and ticagrelor after analyzing 493 patients with ACS or percutaneous coronary intervention who received dual antiplatelet therapy (aspirin and clopidogrel, or aspirin and ticagrelor),[31] consistent with the present study. We suggested that these conventional risk factors of atherosclerosis were clinically treated and under control during the illness. The baseline concentration of uric acid bad been altered due to medical treatments, therefore leading to differences in results from different populations. In our study, hypertension was found to be an independent risk factor of CR, which was consistent with results from other Chinese populations.[32,33] Studies had shown that VitD was one of the risk factors of hypertension, and deficiency of VitD could significantly increase the risk of hypertension. The antihypertensive properties of VitD included renoprotective effects, suppression of the renin-angiotensin-aldosterone system, direct effects on vascular cells, and effects on calcium metabolism.[34-36] Low level of VitD promoted platelet activation, and platelet CD62p (P-selectin), a marker of platelet activation, acted as direct mediators of vascular inflammation and injury in the development of hypertension. The expression of CD62p was increased in patients with hypertension, and its level was consistent with the severity of blood pressure.[37] In return, hypertension could also promote platelet activation, and the underlying mechanism might include hemodynamic factors or injured vascular endothelial cells caused by hypertension, both leading to platelet activation.[38,39]

In conclusion, the data in our study suggested that lower level of VitD and increased expression of platelet CD62p were both independent risk factors of CR in patients with ischemic stroke. Further investigation would be performed to attenuate the effects of these risk factors on CR and occurrence of adverse events following stroke, so as to furtherly reduce the risk of recurrent stroke and achieve a better prognosis, thus making precision medicine of antiplatelet come true.

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