

The effect of Xuefu Zhuyu decoction on clopidogrel resistance and its association with the P2Y12 Gene polymorphisms and promoter DNA methylation

Qinglin Yu¹, Jia Su², Keqi Zhu^{1*}, Shujun Yang³, Hao Zhu⁴ and Jingbo Yu^{2*}

¹Department of Traditional Chinese Internal Medicine, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China

²Department of Gerontology, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China

³Department of Hematology, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China

⁴Department of Anaesthesia, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China

Abstract: Some patients experience lesser degrees of platelet inhibition, which is known as clopidogrel resistance (CR). The goal of our study was to investigate the effects of Xuefu Zhuyu decoction on CR in coronary artery disease patients and whether P2Y12 polymorphisms and its methylation were related to drug response or not. 49 patients diagnosed with CR were randomly divided into control and treatment groups. Platelet functions were measured using Verify-Now P2Y12 assay. By restriction fragment length polymorphism-polymerase chain reaction, the single-nucleotide polymorphisms of rs2046934 and rs6785930 were genotyped. Using bisulphite pyrosequencing assay, we investigated the association of the P2Y12 gene DNA methylation levels and the effects of Xuefu Zhuyu decoction on CR. The results showed that the decoction improved CR ($P=0.005$), and the patients with the TT genotype in rs2046934 received substantial benefits from Xuefu Zhuyu Decoction, in both P2Y12 reaction units (PRU) and inhibition percentage ($P_{PRU}=0.016$; $P_{inhibition\ percentage}=0.028$). And patients with lower methylation levels of CpG1 were more likely to be TT carriers in rs2046934 (CpG1_{TT} Vs. CpG1_{TC+CC}(%): 39.47 ± 6.20 vs. 45.70 ± 8.47 , $P=0.044$). In conclusion, our study indicated that Xuefu Zhuyu decoction might be useful for overcoming CR and the polymorphism of rs2046934 might influence the drug effect.

Keywords: Xuefu Zhuyu decoction, clopidogrel resistance, P2Y12, polymorphisms, promoter DNA methylation.

INTRODUCTION

Thrombus generation (due to platelet activation and aggregation) is the main process in atherosclerotic vascular disease, particularly coronary artery disease (CAD) (Jennings, 2009). Therefore, antiplatelet therapy has been recommended for the prevention of ischemic events in CAD patients, especially for those undergoing percutaneous coronary intervention (PCI) (Wijns *et al.*, 2010). Clopidogrel reduces adenosine diphosphate-induced platelet aggregation by inhibiting ADP receptor P2Y12, so as to decrease the risk of cardiovascular events (Udell *et al.*, 2016, Nguyen *et al.*, 2005). However, some patients still experience cardiovascular events (Udell *et al.*, 2016). This clinical phenomenon has been related to lesser degrees of platelet inhibition, which is known as clopidogrel non-responsiveness or clopidogrel resistance (CR) (Nguyen *et al.*, 2005).

The underlying mechanism is still not very clear. Clinical and demographic factors may affect the antiplatelet efficacy of clopidogrel, including renal dysfunction, diabetes mellitus (DM), smoking, age, reduced left ventricular function, and inflammation (Campo *et al.*, 2010). However, genetic factors, specifically the polymorphisms and the methylation levels of the gene P2Y12, may play a vital role in individual susceptibility

to the clopidogrel (Cuisset *et al.*, 2007). The receptor for adenosine diphosphate (ADP) P2Y12, which is located at 3, q21-25, has two types of polymorphisms, H1/H2 or i-T744C (rs2046934) and C34T (rs6785930). These single nucleotide polymorphisms (SNPs) have been extensively studied, with reports indicating that the P2Y12 T744C polymorphism may affect platelet aggregation (Liao, 2000). However, this finding could not be confirmed in several subsequent studies and the role of DNA methylation in antiplatelet efficacy had not been extensively studied.

Traditional Chinese medicines exhibiting good anti-platelet effects are the most commonly used drugs for CAD patients after PCI to activate blood circulation and thus remove blood stasis (Xue *et al.*, 2009). Xuefu Zhuyu decoction, used to remove blood stasis in the chest, showed good effects in the clinical treatment of CAD in Chinese medicine practice (Kim *et al.*, 2010). However, it remains unknown whether Xuefu Zhuyu decoction can effectively inhibit platelet activation and reduce platelet aggregation to relieve CR in patients after PCI and if P2Y12 gene polymorphisms and its methylation levels are related to this drug response. Thus, in this study, we attempted to assess the effects of Xuefu Zhuyu decoction on CR and the possible genetic variants in terms of drug response.

*Corresponding author: e-mails: nbyujingbo@163.com; zhukeyi@126.com

MATERIALS AND METHODS

Platelet function measurements

We detected the platelet reactivity one month after PCI using the Verify Now P2Y12 assay (Accumetrics Inc., San Diego, CA, USA), which was developed to assess the response to P2Y12 antagonists (Jakubowski *et al.*, 2008). With this test, we obtained the P2Y12 reaction units (PRU) (Marcucci *et al.*, 2009). The inhibition value was equalled to baseline platelet activity (baseline platelet activity - PRU). PRU values above 240 reaction units were considered to reflect clopidogrel resistance (Campo *et al.*, 2010).

Study population

The clinical samples used in this analysis represent a subgroup of samples described previously (Su *et al.*, 2014). Briefly, from 2011 to 2015, a set of patients with clopidogrel resistance was collected at Ningbo No.1 Hospital in eastern China. From 108 patients, we enrolled 49 with clopidogrel resistance and randomly divided them into the control group (24 cases) and treatment group (25 cases). In both the control and treatment groups, the patients continued the conventional Western medicine treatment of clopidogrel 75 mg and aspirin 100 mg daily. We added the Xuefu Zhuyu decoction (twice daily) to the treatment group for one month. The enrolled patients did not take anticoagulant drugs or any other herbs activating blood circulation to eliminate blood stasis during this period. After one month, we evaluated the platelet reactivity again in both patient subgroups. All participants provided written informed consent and the procedures were reviewed and approved by the Ethics Committee of Ningbo No.1 Hospital.

Xuefu Zhuyu decoction preparation

The Xuefu Zhuyu decoction (also called Xuefu Zhuyu Tang) contains many Chinese herbs or ingredients, such as Taoren (Semen Persicae), Honghua (Flos Caryhami), Danggui (Radix Angelicae Sinensis), Shengdihuang (Radix Rehmanniae), Chuanxiong (Rhizoma Ligustici Chuanxiong), Chishao (Radix Paeoniae Rubra), Niuxi (Radix Achyranthis Bidentatae), Jiegeng (Radix Platycodi), Chaihu (Radix Bupleuri), Zhike (Fructus Aurantii), and Gancao (Radix Glycyrrhizae). The above drugs herbs and ingredients are decocted in water for oral application, and the decoction is used to promote blood circulation to remove blood stasis, and to promote the flow of qi to relieve pain (Xue *et al.*, 2009).

Genomic DNA extraction and genotyping

Human genomic DNA was extracted from 3 ml of peripheral blood using the QIAamp DNA BloodMini Kit (Qiagen). DNA concentrations were tested using a NANODROP 1000 and all were above 500ng/uL. The PCR primers for rs2046934 and rs6785930 were designed using the Pyro Mark Assay Design software. Next,

polymerase chain reaction (PCR) amplification (BIO-RAD C1000touch Thermal Cycler PCR) was performed, and the PCR products were purified (SK1141; kit; Sangon Biotech), measured (3500XL sequence analyser; ABI), and sequenced.

Methylation assay

Applied by bisulphite pyrosequencing technology, we evaluated the quantitative DNA methylation of two CpG dinucleotides on the fragment of P2Y12 gene promoter (Mikeska *et al.*, 2007). This process was also combined with sodium bisulphite DNA conversion chemistry (EpiTech Bisulphite Kits; Qiagen), polymerase chain reaction amplification (Pyromark PCR Kit; Qiagen) and sequencing (Pyromark Gold Q24 Reagents; Qiagen) of the target fragment (Su *et al.*, 2014).

STATISTICAL ANALYSIS

Categorical variables were expressed as the mean $\pm$ standard deviation and analysed with either Pearson's chi-square test or Fisher's exact test. Continuous variables were described as median with interquartile range (IQR) and were used to compare the mean values from t-tests. Non-parametric continuous variance was analysed using the Wilcoxon rank-sum test. All statistical analyses including Hardy-Weinberg equilibrium evaluation were performed using the PASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA). A P value less than 0.05 was considered a statistically significant difference.

RESULTS

Study population

From May 2012 to October 2015, through Verify-Now P2Y12 evaluation, 76 patients whose PRU was greater than 240 were defined as showing clopidogrel resistance. Among them, 49 CAD patients who met all of the requirements were recruited for this study (Su *et al.*, 2014). These patients were randomly divided into the control group (24 cases) and the treatment group (25 cases). The demographic and clinical characteristics of these patients (such as age, gender and, body mass index) are summarized in table 1. All clinical data were well matched.

Improvement of clopidogrel resistance before and after Treatment

Table 2, that 95.8% of patients in the control group retained clopidogrel resistance after conventional treatment, whereas only 60% in the treatment group retained resistance after combination therapy with Xuefu Zhuyu decoction ($P=0.005$).

Further, we found that the PRU in the treatment group was slightly lower than in the control group (treatment group vs. control group: 252.32 $\pm$ 29.15 vs. 275.83 $\pm$ 32.09;

Table 1: Comparison of characteristics among study participants

	Treatment group (n = 25)	Controls group (n = 24)	P value
Male gender, n (%)	16 (64.0)	13 (54.2)	0.484
Hypertension, n (%)	18 (72.0)	16 (66.7)	0.686
Diabetes mellitus, n (%)	5 (25.0)	6 (24.0)	0.675
Dyslipidemia, n (%)	6 (24.0)	7 (29.2)	0.682
Current smoking, n (%)	11 (44.0)	9 (37.5)	0.644
Alcohol abuse	4 (16.0)	4 (16.7)	0.950
Age, y	63.20±10.50	60.74±10.11	0.118
BMI, kg/m ² ,	23.59±2.68	24.05±2.74	0.673

Table 2: Improvement of clopidogrel resistance before and after treatment

	After treatment n (%)		
	CR	NCR	p value
Treatment group (25)	15 (60.0)	10 (40.0)	0.005
Control group (24)	23 (95.8)	1(4.2)	

Table 3: Change in platelet function before and after treatment (1)

	Treatment group	Control group	p value
Before treatment			
PRU	280.16±32.76	278.83±31.53	0.886
Inhibition	0.114±0.097	0.141±0.061	0.255
After treatment			
PRU	252.32±29.15	275.83±32.09	0.01
Inhibition	0.195±0.111	0.150±0.066	0.098

Table 4: Change in platelet function before and after treatment (2)

Treatment group	Before treatment n (%)	After treatment n (%)	p value
PRU	280.16±32.76	252.32±29.15	0.003
Inhibition	0.114±0.097	0.195±0.111	0.009
Control group			
PRU	278.83±31.53	275.83±32.09	0.745
Inhibition	0.141±0.061	0.150±0.066	0.62

Table 5: Hardy-Weinberg equilibrium test of rs2046934 and rs6785930

	P2Y12 i-744 T>C(rs2046934)					P2Y12 34C>T (rs6785930)				
	TT	TC	CC	Chi-square	HWE	CC	CT	TT	Chi-square	HWE
N	15	8	2	0.377	0.539	15	9	1	0.060	0.807

$P=0.01$). In the treatment group, after patients had received Xuefu Zhuyu decoction for one month, the PRU was clearly decreased (Before treatment vs. after treatment: 280.16 ± 32.76 vs. 252.32 ± 29.15 ; $P = 0.003$), and the inhibition percentage was clearly increased (Before treatment vs. After treatment: 0.114 ± 0.097 vs. 0.195 ± 0.111 ; $P = 0.009$). However, we did not observe any significant results in the control group (tables 3 and 4). These findings indicated that Xuefu Zhuyu decoction improves clopidogrel resistance.

P2Y12 Polymorphism and the effects of Xuefu Zhuyu decoction on CR

We searched the genetic information of CHB on the HAP-MAP and found that the frequency of the T allele in i-T744C and the C allele in C34T in Chinese populations are 0.795 and 0.762. We tested the frequency of these two

SNPS and verified that these two SNPs (rs2046934 and rs6785930) were in Hardy-Weinberg equilibrium (table 5). Through PCR-HPCE, we explored the association of the improvement in platelet activity and the rs2046934 and rs6785930 polymorphisms. As shown in fig. 1 and table 6, our results showed that the patients with the TT genotype in rs2046934 received substantial benefit from Xuefu Zhuyu decoction, both in PRU and inhibition percentage ($P_{PRU} = 0.016$; $P_{inhibition\ percentage} = 0.028$). The other variations of SNPs in rs2046934 were not significantly associated with improvement of PRU and inhibition percentage and the SNP of rs6785930 did not affect the change in platelet function after using Xuefu Zhuyu decoction (fig. 2 and table 6).

Additionally, due to limited samples, we were unable to determine relationship between platelet activity and the

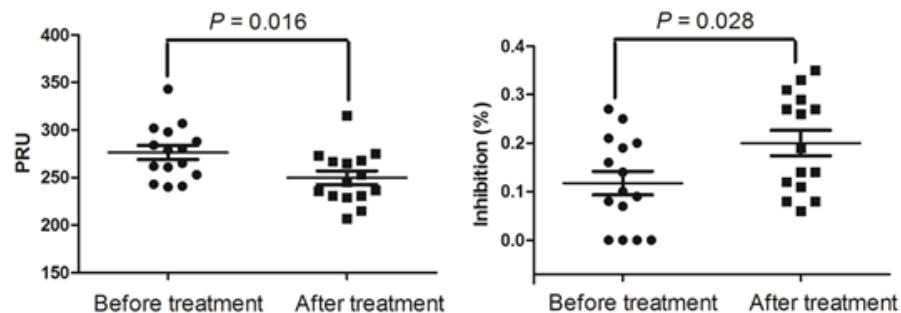


Fig. 1: Comparison of platelet function value before and after treatment in TT homozygotes in rs2046934. (1) PRU (Before treatment vs. after treatment: 276.40 ± 28.69 vs. 249.80 ± 27.84 ; $P=0.016$). (2) Inhibition (Before treatment vs. after treatment: 0.117 ± 0.094 vs. 0.200 ± 0.101 ; $P=0.028$).

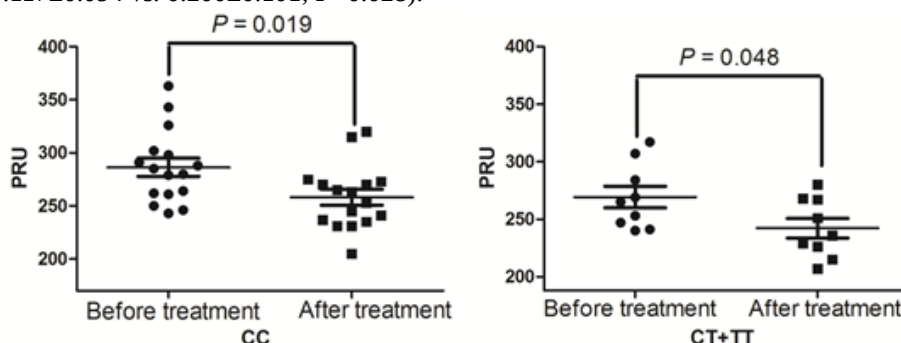


Fig. 2: Comparison of PRU before and after treatment in CC and CT+TT genotypes in rs6785930. (1) PRU of the CC genotype (Before treatment vs. after treatment: 286.31 ± 34.37 vs. 258.06 ± 30.21 ; $P=0.019$). (2) PRU of the CT+TT genotype (Before treatment vs. after treatment: 286.22 ± 28.18 vs. 242.11 ± 25.60 ; $P=0.048$).

Table 6: Effects of Xuefu Zhuyu Decoction on platelet function with P2Y12 polymorphisms

Genotype (n)	PRU			inhibition		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
i-T744C						
TT (15)	276.40 ± 28.69	249.80 ± 27.84	0.016	0.117 ± 0.094	0.200 ± 0.101	0.028
TC+CC (10)	285.80 ± 39.02	256.10 ± 32.15	0.080	0.110 ± 0.107	0.187 ± 0.130	0.166
C34T						
CC (16)	286.31 ± 34.37	258.06 ± 30.21	0.019	0.110 ± 0.103	0.187 ± 0.122	0.064
CT+TT (9)	286.22 ± 28.18	242.11 ± 25.60	0.048	0.122 ± 0.092	0.209 ± 0.093	0.065

rs2046934 and rs6785930 polymorphisms based on comparison of the different SNP modes (homozygous, recessive and heterozygous models).

The DNA methylation levels of P2Y12 and the effects of Xuefu Zhuyu decoction on CR

In this research, we selected a fragment (GRCh37. p13:151103600-151101600), which was containing 2 CpG dinucleotides (fig. 3). Through the bisulphite pyrosequencing assay, we investigated the association of the DNA methylation levels of two CpGs in P2Y12 promoter and the effects of Xuefu Zhuyu decoction on CR. As shown in table 7, the methylation levels of CpG1 in P2Y12 in selected fragments were not significantly related to the improvement of Xuefu Zhuyu decoction on clopidogrel resistance along with P2Y12 CpG2.

The relationship between P2Y12 polymorphism and its methylation levels

It was said above that the patients with the TT genotype in rs2046934 received substantial benefits from Xuefu Zhuyu decoction. We studied the relationship between the methylation levels of two CpGs in P2Y12 promoter and the gene expression from two SNPs. Interestingly, we found that the patients with lower methylation levels of CpG1 were more likely to be a TT carrier in rs2046934 (CpG1_{TT} Vs. CpG1_{TC+CC}(%): 39.47 ± 6.20 vs. 45.70 ± 8.47 , $P=0.044$) (table 8).

DISCUSSION

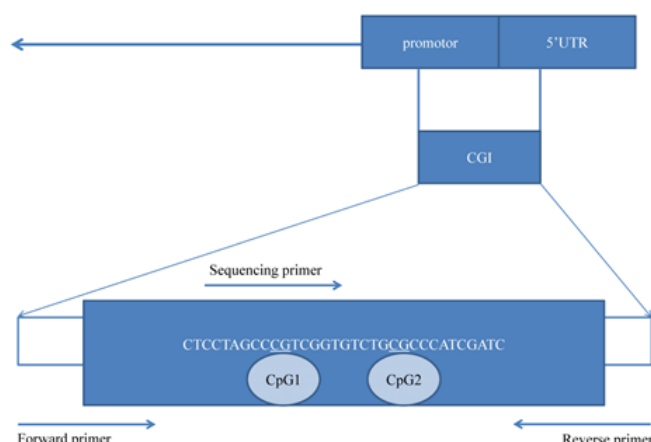
Since 2003, many studies have noted that the level of platelet inhibition varies greatly among patients on

Table 7: The DNA methylation levels of *P2Y12* and the effects of Xuefu zhuyu decoction on CR

After treatment	Treatment group (25)		
	CR (15)	NCR (10)	P value
CpG1	42.87±10.49	44.60±9.41	0.678
CpG2	39.93±7.29	37.50±6.54	0.846

Table 8: The relationship between *P2Y12* polymorphism and its methylation levels

	rs2046934			rs6785930		
	TT	TC+CC	P value	GG	GT+TT	P value
CpG1	39.47±6.20	45.70±8.47	0.044	45.38±9.92	40.33±9.58	0.229
CpG2	36.53±6.39	38.10±7.78	0.587	38.38±6.34	35.00±7.60	0.246

**Fig. 3:** Two CpGs in *P2Y12* gene promoter

clopidogrel (Gurbel *et al.*, 2003), Approximately 25 to 50% of patients treated with clopidogrel fail to show adequate pharmacological response and are not protected from major adverse cardiac events (MACE)(Tantry *et al.*, 2013, Komosaal *et al.*, 2015) , such as adverse ischemic events and stent thrombosis in patients after PCI (Geisler *et al.*, 2013). We term this phenomenon as CR, which has been used to reflect the failure of clopidogrel to achieve its antiplatelet effect.

Many clinical trials have suggested possibilities for overcoming Clopidogrel Resistance. The work of Patti *et al* showed that pre-treatment with a 600-mg clopidogrel loading dose before primary PCI was related to the improvement of angiographic results and 30-day MACE compared with a 300-mg loading dose (Patti, *et al.*, 2011). However, as the dose increased, the risk of bleeding became higher. The new platelet aggregation inhibitors prasugrel and ticagrelor were shown to produce more consistent, rapid and effective *P2Y12* receptor inhibition in patients with acute coronary syndrome (ACS) (Kubica *et al.*, 2014). However, it was shown that this phenomenon was not wholly true for clopidogrel treatment. In the acute phase of ST-elevation myocardial infarction (STEMI), high platelet reactivity was exhibited in approximately 37% of patients treated with prasugrel (Bonello *et al.*, 2011) and 46% of patients treated with ticagrelor (Alexopoulos *et al.*, 2012). We should also

consider the high incidence of major and minor bleeding in some patients receiving prasugrel (Wiviott *et al.*, 2007). and ticagrelor (Wiviott *et al.*, 2015). Hence, clopidogrel remains one of the most widely prescribed antiplatelet drugs in CAD patients, and research on how to overcome CR is of great importance.

In traditional Chinese medicine, Xuefu Zhuyu decoction has been used to promote blood circulation to dissipate blood stasis. We found substantial benefit in treating atherosclerosis with Xuefu Zhuyu decoction, especially in CAD patients. A recent meta-analysis indicated that Xuefu Zhuyu decoction might be effective for treating hyperlipidaemia (Liao *et al.*, 2014), and another study demonstrated that it had a superior ability to reverse myocardial fibrosis in a hypertensive rat model (Zhang *et al.*, 2016). In our study, we found that Xuefu Zhuyu decoction improves clopidogrel resistance. To our knowledge, by comparing PRU and inhibition percentage, this study was the first to focus on improving clopidogrel resistance using Xuefu Zhuyu decoction. Although one study showed that Xuefu Zhuyu oral liquid could effectively improve aspirin resistance (Xue *et al.*, 2015), it did not determine the underlying mechanism. However, the sample size in this study was relatively small. Future investigation with larger samples could be performed for further assessment.

The improvement of clopidogrel resistance might result from its main components. One study reported that Taoren and Honghua, which are rich in amygdalin and hydroxysafflor yellow A (HSYA), could significantly reduce platelet aggregation and protect vascular endothelial cells (Liu *et al.*, 2012). It has also been demonstrated that the active ingredients of the Radix Angelica Sinensis, (the dried root of Angelica sinensis (Danggui), another Chinese herb), such as antioxidant and anti-inflammatory agents, could limit platelet aggregation (Wu *et al.*, 2011). Further more, tetramethylpyrazine, one of the active ingredients of the Chinese herbal medicine 'Chuanxiong,' which can selectively inhibit platelet thrombus formation under high shear rates, showed a unique antiplatelet effect (Li *et al.*, 2001). The above Chinese herbal medicine in Xuefu Zhuyu decoction played a vital role in limiting platelet aggregation and producing a specific antiplatelet effect, thus improving clopidogrel resistance.

Several studies have reported that poor response to clopidogrel might be strongly heritable. Many specific genetic variants affect clopidogrel's transport (the gene of ATP-binding cassette subfamily B member 1), metabolism (CYP enzymes, gene of paraoxonase-1) and action (P2Y12 gene) (Sanguhl *et al.*, 2010). For example, genetic polymorphisms of CYP2C19 might disturb the effect of the drug, as carriers of at least one low-function CYP2C19 allele experience a reduction of the active metabolite in plasma by up to 32.4% compared to healthy gene carriers (Varenhorst *et al.*, 2009, Simon *et al.*, 2009). Our meta-analysis also showed that the polymorphism of ABCB1 C3435T might be a risk factor for MACE in patients with a clopidogrel loading dose of 300 mg, whereas TT homozygotes decreased bleeding events (Su *et al.*, 2012). Staritz *et al.* first demonstrated that a homozygote H2 genotype (744 CC) contributed to clopidogrel resistance in clopidogrel-treated patients (Staritz *et al.*, 2009). However, the results from different studies were inconsistent (Angiolillo *et al.*, 2005). In this study, our results indicated that the antiplatelet effect in patients with the TT genotype in rs2046934 was much improved after the application of Xuefu Zhuyu Decoction. This result indicated that the polymorphisms of rs2046934 might influence the ability of Xuefu Zhuyu decoction to overcome CR cases. However, due to the variance of gene frequency in different populations in different areas and the limited sample size, our results should be interpreted with caution.

Several other clinical and genetic elements might influence the antiplatelet efficacy of clopidogrel, such as comorbidities, DNA methylation, and miRNA (Shi *et al.*, 2013). Hyperglycemia has been shown to increase platelet reactivity by inducing P-selectin expression, alters membrane fluidity with subsequent platelet adhesion and activates protein C (Ferreiro *et al.*, 2011). Two years ago, our research found that the lower methylation of two

CpGs on the P2Y12 gene promoter was associated with CR in patients with alcoholism, and CpG1 methylation was inversely related to CR in patients who smoked and in a patient subgroup who had an albumin level of <35 (Su *et al.*, 2014).

In parallel, other polymorphisms or the expression of other genes might also influence the effect of Xuefu Zhuyu Decoction. However, these variables could not be examined more effectively by a separate analysis of these elements due to our sample size. In our study, although we had not detected that the methylation levels of CpG1 and CpG2 in P2Y12 were related to the improvement of Xuefu zhuyu decoction, we found that the lower methylation levels of CpG1 were more likely to affect the gene expression, especially rs2046934. It was reported that the hypermethylation of vertebrate CpG islands (CGIs) is relative to the transcriptional silencing of gene expression and thus controls the protein level (Morita *et al.*, 2012). But the accurate mechanism, the influence on P2Y12 expression by methylation, need to be explored by further basic research. We aimed to perform additional studies focusing on other possible genetic variants and a more advanced empirical method to validate our findings in future work.

The effectiveness of traditional Chinese medicine is affected by various factors. For example, although we chose a Chinese herb decoction, different doctors will use different doses. There was a drug named Xuefu Zhuyu oral liquid, but it was incompatible with TCM treatment based on syndrome differentiation. In addition, when we were decocting the herbs, there were some slight differences in decocting time and drug concentration. If a patient also had other conditions, this would affect the absorbability and metabolism of traditional Chinese medicine. All of the above make it challenging to achieve unified standards in TCM. A more standardized and accurate study would help us to reach a better understanding.

CONCLUSION

In summary, our study found that Xuefu Zhuyu decoction improves clopidogrel resistance and that patients with the TT genotype in rs2046934 received substantial benefit from Xuefu Zhuyu Decoction, both in PRU and inhibition percentage. What was more, the polymorphism of rs2046934 might influence the drug effect. These data might provide new insight to overcome Clopidogrel Resistance. However, we aim to perform larger studies with more effective planning to validate our findings in further research.

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