A SNP involved in alternative splicing of *ABCB1* is associated with clopidogrel resistance in coronary heart disease in Chinese population

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ABSTRACT

Although many scientists are studying the association between genetic polymorphism of *ABCB1* and CR in patients, the molecular mechanism has not been further studied in patients with CHD. This study investigated the relationship between SNP of the *ABCB1* gene in patients with CHD and CR, and whether the polymorphism of the *ABCB1* gene affects the AS of the gene. 741 patients were enrolled in the study, 316 CR cases and 425 NCR cases. The correlation between CR risk and clinical-pathological characteristics were studied. Additionally, the five SNPs were analysed by PCR and Mass Array genotyping methods. Furthermore, silicon analysis was used to predict whether the polymorphism affects the process of AS. Results showed that there was a significant correlation between rs1045642 polymorphism and CR in genotyping and allele analysis. The rs1045642 polymorphism of the *ABCB1* gene of CHD patients carrying the A allele are more likely to develop CR. Silicon analysis showed that rs1045642 generated a new ESE sequence which might affect AS of *ABCB1* gene. We hypothesize that the mechanism of CR might be caused by a change in the AS caused by the polymorphism of the gene. Thus, this work provides guidance for the clinical use of clopidogrel.

INTRODUCTION

Coronary heart disease (CHD) is usually caused by the lipid accumulation in the walls of the heart arteries [1, 2]. CHD is a high-risk cardiovascular disease in the population and the main cause of death in patients with cardiovascular disease [3, 4]. Percutaneous coronary intervention (PCI) is a treatment for CHD, which might lead to stent thrombosis. Therefore, antithrombotic therapy plays a crucial role in the treatment of CHD [5]. Clopidogrel is mainly prescribed for antiplatelet therapy [6] and ultimately converted into active clopidogrel metabolites in the liver [7]. Although clopidogrel is the cornerstone of antiplatelet therapy, some patients will

develop into clopidogrel resistance (CR). Genetic polymorphism is one of the main factors affecting the CR of individuals. Therefore, we can use genetic testing to guide the clinical individualization of CR patients [8–10].

At present, there are many genes in clopidogrel pharmacogenomics research, among which *CYP2C19* and ATP binding cassette subfamily B member 1 (*ABCB1*) genes are particularly concerned. We have selected the *ABCB1* gene for this experimental study of CR and polymorphisms. The p-glycoprotein (p-gp) encoded by the *ABCB 1/MDR 1* (multidrug resistance-1) gene, physiologically, which can transport hydrophobic and hydrophilic compounds through the placenta, intestine and other parts, and protect the blood-brain barrier [11]. In addition, steroid hormones, immunosuppressants and antimetabolites or antibiotics can be discharged, as well as metabolites to protect cells from cytotoxic substances [12]. It causes a variety of drugs to be excreted outside the cancer cells, leading to chemotherapy resistance of tumour [13]. *ABCB1* gene is a highly polymorphic gene with multiple single nucleotide polymorphisms (SNPs). Many studies in recent years had shown that genetic polymorphism affects the process of alternative splicing (AS) and leads to the development and progression of many diseases in humans, such as thalassemia, Alzheimer's disease, male infertility, retinitis pigmentosa and cancer [14–24]. More than 95% of protein coding genes occurs AS that increases the diversity of the human genome [25]. The complex process of AS is affected by many elements. Not only the branch point, the sequence of 3′ and 5′ splice sites but also cis-elements and trans-acting elements are required. Mutations or polymorphisms in cis-regulatory factors might affect mechanical damage of the exon splicing complexes and cause many diseases [16].

Although a large number of scientists around the world are studying the association between genetic polymorphism of *ABCB1* and CR, the results between the genetic polymorphisms and the risk of clopidogrel are still controversial. Diverse results of the above studies might be caused by differences in sample selection requirements, geographical distribution and ethnic differences, sample size, and different genotyping methods [26, 27]. Recently, mutations at specific sites of the gene have caused certain disease and affected the process of the gene's AS [23, 28–31]. As far as we know, the relationship between gene polymorphism and AS in Chinese population with CR risk has not been studied. Therefore, our present study aims to investigate the molecular mechanism of CR caused by the polymorphism of the gene. Our work might provide theoretical guidance for the clinical individualization of clopidogrel in Chinese population with CHD.

RESULTS

Distributions of selected variables in cases and controls

The demographic information and clinical characteristics of the CR group and NCR groups were showed in Table 1. The relationship between age and sex in the CR group and NCR group has not been found, indicating that frequency matching of age and gender was appropriate. The mean platelet volume (MPV) was different between the CR group and the NCR group (*P*=0.036). At the same time, the rest of the variables were not found that significant correlation between CR group and NCR group.

HWE equilibrium test

Sequenom MassArray typing was performed on the above five polymorphisms. We calculated the specific genotype frequencies of the five SNPs and ensured that each site was in HWE equilibrium (Table 2).

Sequence analysis

In order to verify the accuracy of the Sequenom MassArray genotyping method, we randomly selected some samples to verify the sequence of 5 SNPs. The image of agarose gel electrophoresis of the polymerase chain reaction (PCR) amplification products of some samples of DNA samples were shown in Figure 1. The wild homozygous sequence, heterozygous sequence and mutant homozygous sequence of the rs1045642 polymorphic site were listed in Figure 2.

Statistical analysis of genotyping results of five SNPs

Sequenom MassArray typing technique showed genotypes of 741 participants and we performed a statistical analysis of the genotype results. All clinical data and classification results were entered into SPSS22.0 software. The results were expressed as mean ± standard deviation, and the count data was analysed by chi-square test. Expressed by the number and frequency of distribution, the *P* value reflects the difference between each variable in the CR group and NCR group. The chi-square test of five polymorphisms of *ABCB1* gene was performed to compare the distribution of different genotypes between NCR group and CR group and the results were illustrated in Table 3. Chi-square test showed that the rs1045642 locus of *ABCB1* gene was significantly different between the CR group and the NCR group, while the four

Table 1. Distributions of selected variables in CHD cases and controls.

Characteristics		NCR	CR	*P*
Total		425	316	
Sex	M (%)	233 (55.1)	190 (44.9)	0.149
	F (%)	192 (60.4)	126 (39.6)	
History of smoking	Y (%)	90 (59.6)	61 (40.4)	0.531
	N (%)	335 (56.8)	255 (43.2)	
Drinking history	Y (%)	34 (66.7)	17(33.3)	0.163
	N (%)	391 (56.7)	299 (43.3)	
History of diabetes	Y (%)	35 (57.4)	26 (42.6)	0.997
	N (%)	390 (57.4)	290 (42.6)	
History of hypertension	Y (%)	114 (56.2)	89 (43.8)	0.686
	N (%)	311 (57.8)	227 (42.2)	
Combined with APC	Y (%)	354 (56.1)	277 (43.9)	0.098
	N (%)	71 (64.5)	39 (35.5)	
Age		63.50±10.68	64.03±9.28	0.479
BMI/kg/m^2		24.27±3.43	24.16±3.54	0.661
Systolic pressure/mmHg		122.52±16.18	124.38±14.84	0.108
Diastolic pressure/mmHg		74.78±10.20	75.26±9.21	0.509
Glycated hemoglobin/%		5.48±0.71	5.41±0.52	0.151
Triglyceride/mmol/L		1.26±0.62	1.23±0.61	0.561
Total cholesterol/mmol/L		4.37±0.93	4.45±0.96	0.241
HDL-C/mmol/L		1.34±0.26	1.35±0.25	0.850
LDL-C/mmol/L		2.93±0.64	2.98±0.64	0.330
C-reactive protein/mg/L		2.20±3.97	2.54±5.31	0.315
PLT/10^9		215.42±67.11	219.00±63.35	0.461
MPV/fL		12.55±1.73	12.28±1.68	0.036*
PCT/%		0.23±0.07	0.23±0.07	0.831
PDW/%		17.99±3.78	18.03±3.88	0.900

Abbreviation: CR, Clopidogrel resistance; M, Male; F, Female; Y, Yes; N, No; PLT, Platelet count; MPV, Mean platelet volume; PCT, Plateletocrit; PWD, Platelet volume distribution width.
*: *P* value <0.05 was considered as significant.

Table 2. Hardy-Weinberg equilibrium test of five SNPs.

SNP	Frequency *n* (%)		*P*(NCR)	*P*(CR)
	NCR	CR		
rs1045642	GG (37.9%)/GA(47.5%)/AA(14.6%)	GG (29.7%)/GA(47.5%)/AA(22.8%)	0.916	0.413
rs4148727	TT (81.6%)/TC(17.9%)/CC(0.5%)	TT (82.6%)/TC(17.1%)/CC(0.3%)	0.315	0.302
rs2032582	CC (28.7%)/CA(51.3%)/AA(20.0%)	CC (30.0%)/CA(47.5%)/AA(22.5%)	0.487	0.422
rs3789243	GG (43.5%)/GA(46.4%)/AA(10.1%)	GG (44.6%)/GA(42.1%)/AA(13.3%)	0.369	0.236
rs1858923	AA (27.8%)/AG(50.8%)/GG(21.4%)	AA (26.3%)/AG(49.4%)/GG(24.3%)	0.671	0.827

polymorphisms (rs4148727, rs2032582, rs3789243, rs1858923) were not significantly different between the groups. The rs1045642 of *ABCB1* gene genotype was distributed in the NCR group at 37.9% (GG, 161), 47.5% (GA, 202), 14.6% (AA, 62), and the genotype distribution frequency in the CR group was 29.7% (GG, 94), 47.5% (GA, 150), 22.8% (AA, 72). In the genotype analysis, we found that rs1045642 AA genotype and
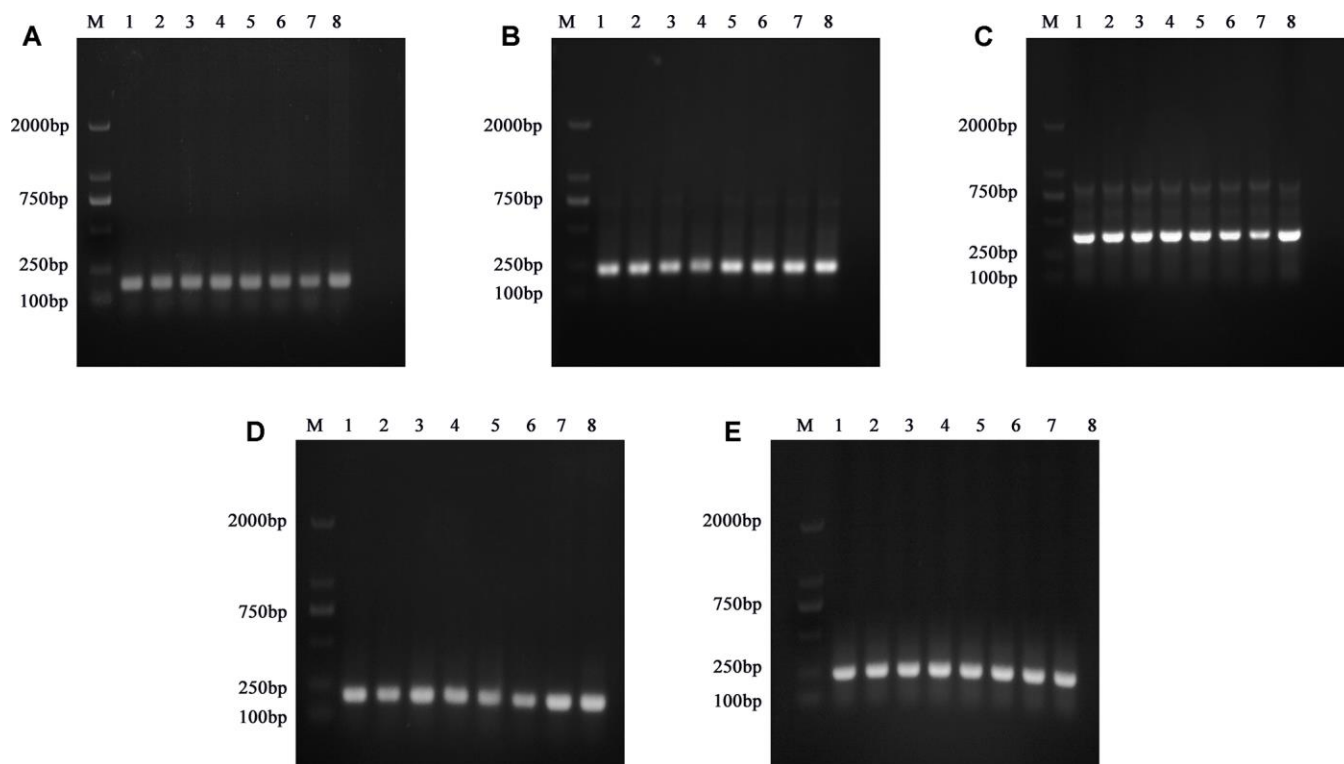

Figure 1. Electropherograms of PCR product fragments verified by sequence verification of 5 SNPs of partial DNA samples. (**A**) Electropherogram of PCR product of rs1045642 polymorphism. (**B**) Electropherogram of PCR product of rs4148727 polymorphism. (**C**) Electropherogram of PCR product of rs2032582 polymorphism. (**D**) Electropherogram of PCR product of rs3789243 polymorphism. (**E**) Electropherogram of PCR product of rs1858923 polymorphism.
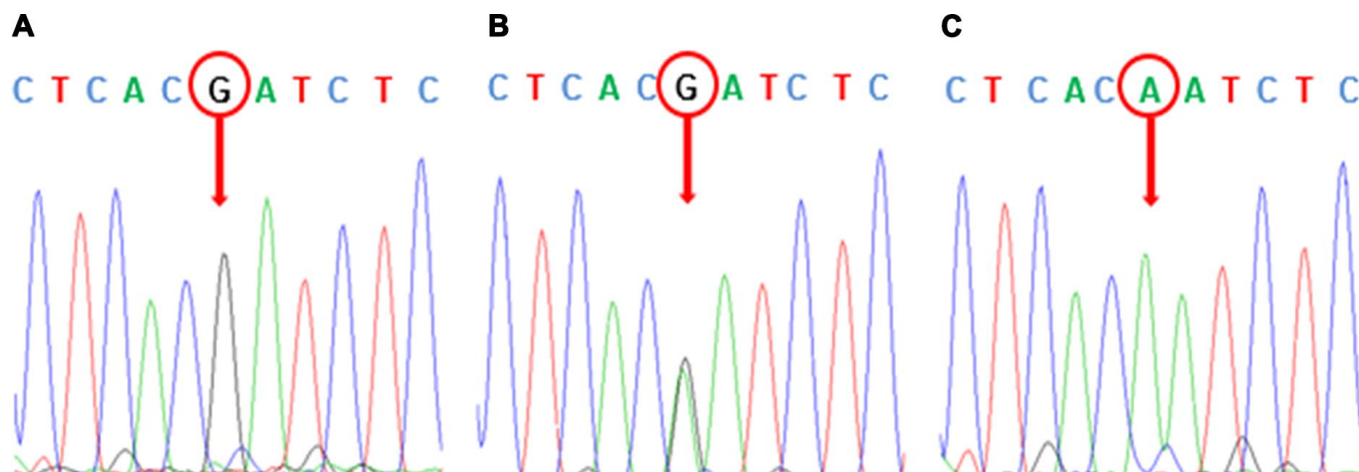

Figure 2. Sequence verification of rs1045642 polymorphism. (**A**) GG-wild type homozygous. (**B**) AG-heterozygote. (**C**) AA-SNP homozygous. The arrow in the picture points to the specific bases of the rs1045642 polymorphic sequence.

Table 3. Statistical analysis of the association between five polymorphisms of the *ABCB1* gene and the risk of CR.

SNP	Model	Genotype	NCR	CR	OR (95% CI)	*P*
rs1045642	Heterozygous model	GG	161(44.4%)	94(38.5%)		
		GA	202(55.6%)	150(61.5%)	1.272(0.914-1.770)	0.154
	Homozygous model	GG	161(72.2%)	94(56.6%)		
		AA	62(27.8%)	72(43.4%)	1.989(1.301-3.040)	0.001*
	Dominant model	GG	161(37.9%)	94(29.7%)		
		GA+AA	264(62.1%)	222(70.3%)	1.440(1.056-1.965)	0.021*
	Allele genetic model	G	524(61.6%)	338(53.5%)		
		A	326(38.4%)	294(46.5%)	1.398(1.135-1.723)	0.002*
rs4148727	Heterozygous model	TT	347(82.0%)	261(82.9%)		
		TC	76(18%)	54(17.1%)	0.945(0.643-1.387)	0.771
	Homozygous model	TT	347(99.4%)	261(99.6%)		
		CC	2(0.6%)	1(0.4%)	0.665(0.060-7.370)	0.738
	Dominant model	TT	347(81.6%)	261(82.6%)		
		TC+CC	78(18.4%)	55(17.4%)	0.937(0.641-1.372)	0.739
	Allele genetic model	T	770(90.6%)	576(91.1%)		
		C	80(9.4%)	56(8.9%)	0.936(0.645-1.339)	0.716
rs2032582	Heterozygous model	CC	122(35.9%)	95(38.8%)		
		CA	218(64.1%)	150(61.2%)	0.884(0.629-1.241)	0.475
	Homozygous model	CC	122(58.9%)	95(57.2%)		
		AA	85(41.1%)	71(42.8%)	1.073(0.709-1.622)	0.740
	Dominant model	CC	122(28.7%)	95(30.1%)		
		CA+AA	303(71.3%)	221(69.9%)	0.937(0.681-1.289)	0.688
	Allele genetic model	C	462(54.4%)	340(53.8%)		
		A	388(45.6%)	292(46.2%)	1.023(0.832-1.257)	0.832
rs3789243	Heterozygous model	GG	185(48.4%)	141(51.5%)		
		GA	197(51.6%)	133(48.5%)	0.886(0.649-1.208)	0.444
	Homozygous model	GG	185(81.1%)	141(77.0%)		
		AA	43(18.9%)	42(23.0%)	1.282(0.794-2.068)	0.309
	Dominant model	GG	185(43.5%)	141(44.6%)		

		GA+AA	240(56.5%)	175(55.4%)	0.957 (0.714-1.283)	0.767
	Allele genetic model	G	567(66.7%)	415(65.7%)		
		A	283(33.3%)	217(34.3 %)	1.048(0.843-1.302)	0.678
rs1858923	Heterozygous model	AA	118(35.3%)	83(34.7%)		
		AG	216(64.7%)	156(65.3%)	0.974(0.688-1.380)	0.882
	Homozygous model	AA	118(56.5%)	83(51.9%)		
		GG	91(43.5%)	77(48.1%)	0.831(0.550-1.257)	0.381
	Dominant model	AA	118(27.8%)	83(26.3%)		
		AG+GG	307(72.2%)	233(73.7%)	0.927(0.667-1.287)	0.650
	Allele genetic model	A	452(53.2%)	322(50.9%)		
		G	398(46.8%)	310(49.1%)	0.915(0.744-1.124)	0.396

*: *P* value <0.05 was considered as significant.

GG genotype were significantly different (OR=1.989, 95% CI=1.301-3.040, *P*=0.001); AA genotype and GA + AA genotype were also significantly different (OR=1.440, 95% CI=1.965-1.056, *P*=0.021). In the allele analysis, there was a statistical difference between the allele A and the G allele (OR=1.398, 95% CI=723-1.135, *P*=0.002). The rs1045642 polymorphism of *ABCB1* gene of CHD patients carrying the A allele is more prone to CR.

LD and haplotype analysis

SHEsis software was used to analyze the LD of five polymorphisms of *ABCB1*. In the Figure 3 and Table 4, LD was detected in the rs3789243 and rs1858923 polymorphisms (r^2=0.310, D′=0.815), rs1858923 and rs4148727 polymorphisms (r^2=0.070, D′=0.872). Meanwhile, haplotype analysis was performed in CR and NCR groups. In this study, the CR group and NCR group with haplotype estimated frequency below 3% were excluded from further analysis. We found that TGAGG haplotypes (rs4148727, rs1045642, rs2032582, rs3789243, rs1858923.) can appreciably lower the risk of CR (OR=0.663, 95% CI=0.447-0.983, *P*=0.040), and other haplotypes were not significantly different between CR group and NCR groups (Table 5).

Silico analysis of rs1045642 polymorphism

Mutations in cis-elements and splice sites can affect gene splicing. These effects were detected by HSF 3.1 and ESE finder 3.0. The HSF 3.1 software was used to predict whether the mutation of the rs1045642 SNP of

the *ABCB1* gene has an effect on AS, which predicted that a new ESE sequence was generated by mutation of the rs1045642 SNP of the *ABCB1* gene (Figure 4A). Since the ESE sequence is a cis-acting element of AS, we further used ESE-finder software to predict whether the mutation of the gene would alter the ability to bind the trans-acting factor SR proteins in the AS. The ESE-finder software predicted that the new ESE sequence generated by the mutation will bind to the four SR proteins beyond the binding threshold (Figure 4B). Thus, silicon analysis showed that rs1045642 generated a new ESE sequence which might affect AS of *ABCB1* gene.

DISCUSSION

ABCB1, P-glycoprotein, is an ABC transporter whose main function of ABCB1 is to expel toxic metabolites and xenobiotics from cells [32, 33]. Therefore, the ABCB1 gene has a great development in the mechanism of anticancer drug resistance [34]. Previous studies had reported gene polymorphisms and CR, and the *ABCB1* is one of the hot genes. In our research, we have selected five polymorphisms of rs1045642, rs4148727, rs2032582, rs3789243 and rs1858923 on the *ABCB1* gene to study the relationship between *ABCB1* gene polymorphisms and the risk of CR. Meanwhile, the polymorphism rs1045642 of *ABCB1* gene is more frequently studied in CR. In recent years, there have been different studies between the *ABCB1* gene polymorphism and the occurrence of CR in patients with CHD. Our research showed that four polymorphisms of rs4148727, rs2032582, rs3789243
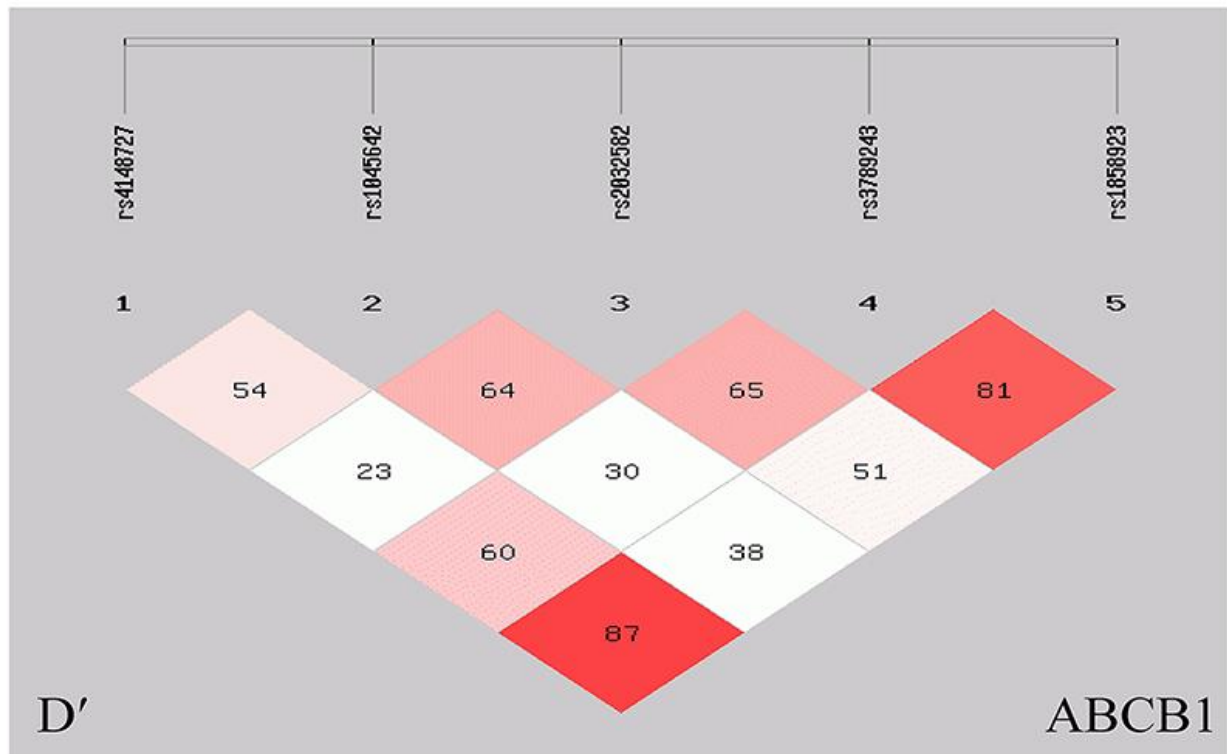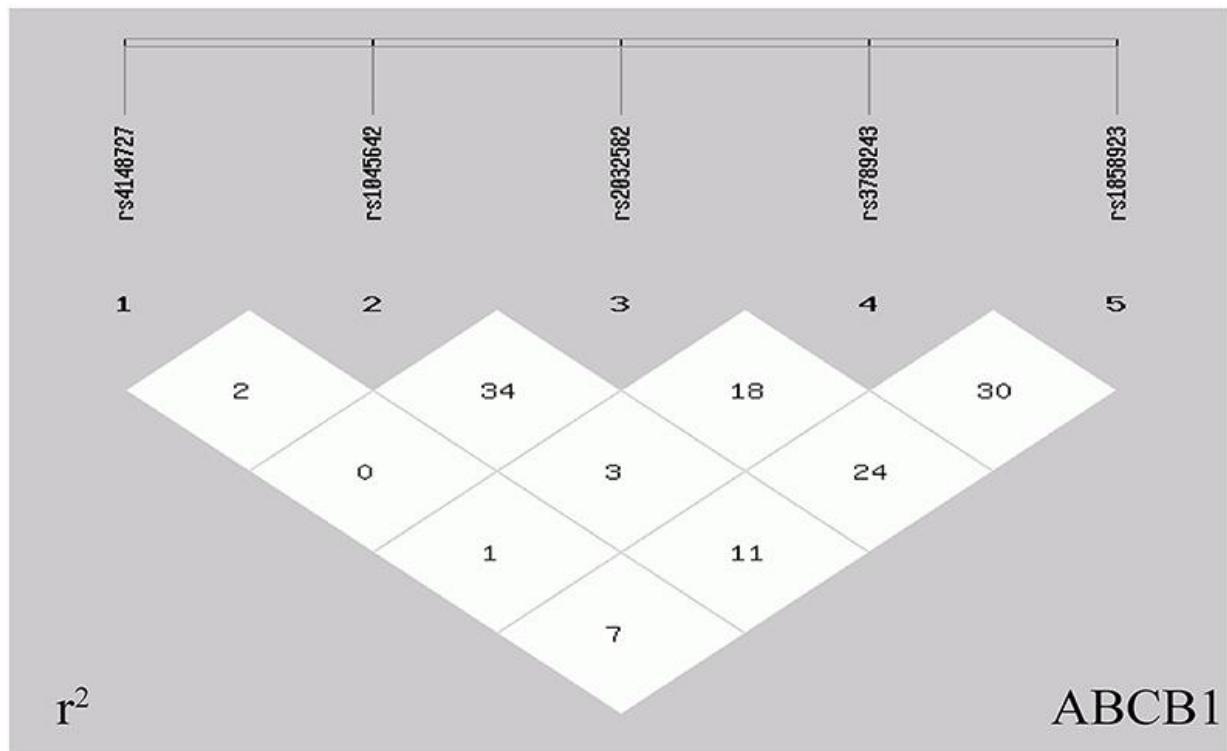
Figure 3. Linkage disequilibrium plot and r^2 values for five polymorphisms of the *ABCB1* gene. (**A**) D′ plot of five polymorphic linkage disequilibrium. (**B**) r^2 plot of five polymorphic linkage disequilibrium. Linkage disequilibrium was detected in the rs3789243 and rs1858923 polymorphisms ((r^2 = 0.310, D′ = 0.815), rs1858923 and rs4148727 polymorphisms (r^2 = 0.070, D′ = 0.872).

Table 4. SHEsis software analyzes the LD relationship of five polymorphisms of *ABCB1*, and uses D′ and r^2 to measure the LD relationship between the five SNPs.

D′	SNP	rs1045642	rs2032582	rs3789243	rs1858923
	rs4148727	0.546	0.237	0.608	0.872
	rs1045642	-	0.642	0.303	0.385
	rs2032582	-	-	0.656	0.516
	rs3789243	-	-	-	0.815
r^2	SNP	rs1045642	rs2032582	rs3789243	rs1858923
	rs4148727	0.022	0.022	0.019	0.070
	rs1045642	-	0.349	0.034	0.116
	rs2032582	-	-	0.186	0.247
	rs3789243	-	-	-	0.310

Abbreviation: LD, linkage disequilibrium.

Table 5. Results of haplotype analysis of five *ABCB1* SNPs in CR and control groups.

Haplotype*	Frequency *n* (%)		*P*	OR (95%CI)
	CR	NCR		
C G C G A	26.78(0.042)	42.98(0.051)	0.456	0.828 (0.505~1.360)
T A A A G	20.89(0.033)	14.85(0.017)	0.053	1.924 (0.980~3.776)
T A A G A	29.78(0.047)	36.67(0.043)	0.718	1.096 (0.667~1.800)
T A A G G	165.44(0.262)	185.45(0.218)	0.05	1.282 (1.002~1.640)
T A C A A	33.67(0.053)	27.79(0.033)	0.05	1.667 (0.996~2.789)
T G A G G	40.77(0.065)	79.64(0.094)	0.04*	0.663 (0.447~0.983)
T G C A A	143.43(0.227)	208.64(0.245)	0.388	0.896 (0.699~1.149)
T G C G A	32.44(0.051	59.72(0.070)	0.132	0.713 (0.458~1.109)
T G C G G	63.03(0.100)	91.22(0.107)	0.626	0.919 (0.653~1.293)

Abbreviation: CR, Clopidogrel resistance; OR, Odds ratio; CI, Confidence interval.
The order of SNPs in haplotypes: rs4148727, rs1045642, rs2032582, rs3789243, rs1858923.
*: *P* value <0.05 was considered as significant.

and rs1858923 were not associated with CR. However, the rs1045642 polymorphism was found to be intensively associated with CR.

Previous studies have shown that the concentration of active clopidogrel metabolites in homozygous 3435TT was significantly lower than that of CT/CC, suggesting that the increased expression of p-glycoprotein associated with 3435TT genotype might mediate the increase of clopidogrel efflux in the intestine [35, 36]. Therefore, it showed that *ABCB1* gene polymorphism was related to the efficacy of clopidogrel in patients. We further studied the *ABCB1* gene polymorphism and CR and found that the TT homozygous rs1045642

mutation has an increased risk of CR compared with CT/CC individuals. People with T alleles have an increased risk of ischemic events. Our results were consistent with those of Jessica L Mega et al. [37]. However, the study on CR and rs1045642 polymorphisms by Jia Su et al. found that there was no significant correlation between rs1045642 mutation and CR [38]. The inconsistency of the research results might be related to different sample selection standards, geographical distribution and ethnic differences, sample size and different genotyping methods. Currently, there are no prescribed standard for CR. Flow cytometry vasodilator-stimulated phosphoprotein phosphorylation (Vasp) analysis and verification-Now P2Y12 detection,

pfa-100, whole TEG and impedance aggregation metrology (multi-plate analyzer) can all be used detect platelet function and clopidogrel reaction [39]. The method of evaluating CR is different, which might have an impact on our further research on CR. In this study, we had used TEG method to measure platelet inhibition rate. The VerifyNow analysis P2Y12 method was used by Jia Su et al. Previous studies had confirmed that allelic variants of the *CYP2C19* gene have ethnic and geographic diversity, but they were not obvious in Asians or mixed races, which indicated that different genetic backgrounds have different frequencies of mutations [40]. In addition, studies had shown that polymorphisms of enzyme genes that metabolize drugs might change the response to drugs and occur at different frequencies in different ethnic groups [41]. This indicates that genetic background might change the effect of mutations on treatment in a specific group. Our samples were collected in Northwest China, and the samples in the study of Jia Su et al. were recruited in East China. Richard C Crist's study concluded that due to the small sample size, many previous studies were insufficient for retrospective drug genetic studies of African Americans [41]. The sample size is too small to detect experimental effect. The difference in sample size might also affect the results of experimental studies. 741 samples were included in our study, and 180 samples were included in the study of Jia Su et al., which was far less than the sample size of our experiment. At present, there are many methods for genotyping, including PCR-RFLP (polymerase chain reaction-restricted fragment length polymorphism) method, Taq-Man probe, ARMS (amplification difficult mutation system), genechip, Sanger sequencing, Mass spectrometry, etc. Different genotyping methods have their advantages and shortcomings. The typing method used in our experiment was Sequenom MassArray, and the PCR-RFLP genotyping method was used by Jia Su et al. The advantages of the Sequenom MassArray method include low cost, no need for special fluorescent primers, only a pair of PCR primers and amplification primers, convenient detection, high sensitivity, and high data accuracy. However, the PCR-RFLP method requires a specific restriction site for genotyping SNPs, and it is impossible to accurately identify false positives. Therefore, our typing method is better than the typing method used by Jia Su et al.

Compared with previous CR-related studies, our study not only analysed five polymorphisms of the gene, but also used bioinformatic software to predict the relationship between rs1045642 polymorphism and AS. More than 50% of all mutations leading to genetic diseases result in abnormal splicing [42]. Different forms of cis-regulatory elements include exon splicing enhancer (ESE), exon splicing silencer, intron splicing enhancer and intron splicing silencer sequences. The trans-acting factors are mainly SR proteins protein and heterogeneous nuclear ribonucleoproteins [16]. The combination of cis-regulatory element mutation/polymorphism and trans-acting factor leads to mechanical cleavage of exon splicing complex, which is
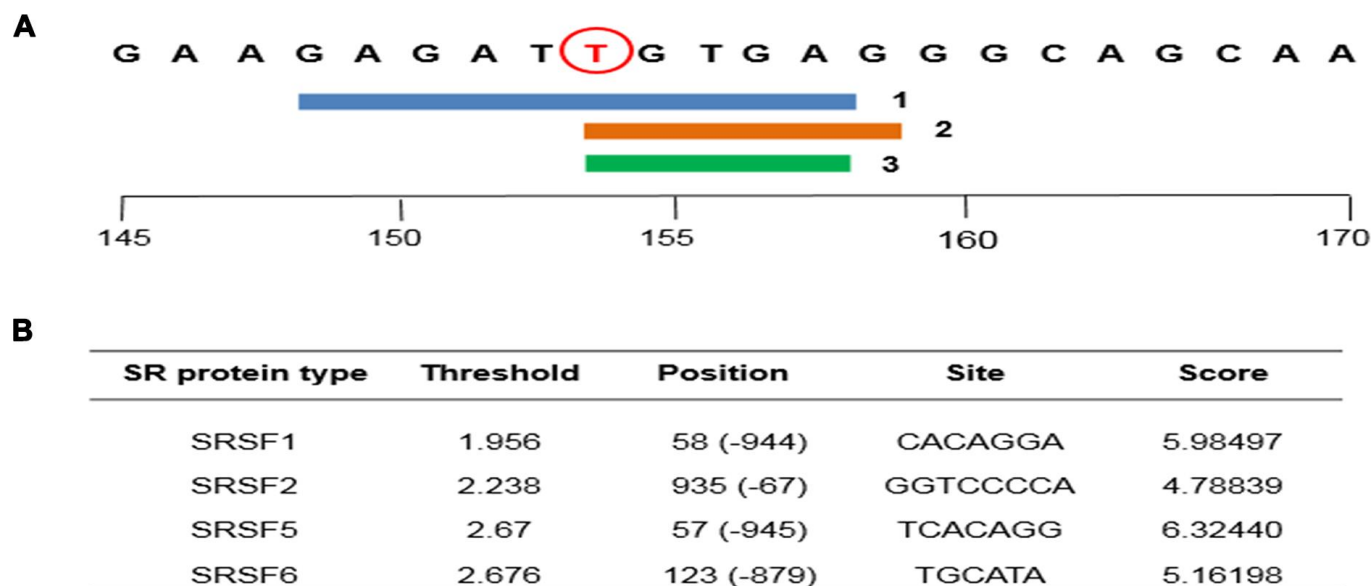

Figure 4. Human Splicing Finder version 3.1 (HSF) and ESE finder 3.0 bioinformatics analysis softwares are used to predict the effect the mutation of the rs1045642 SNP of the *ABCB1* gene on AS. (**A**) The three algorithms of HSF software predict that polymorphism is an ESE site. (**B**) ESE finder software predicts the binding ability of ESE site to SRps.

SR protein type	Threshold	Position	Site	Score
SRSF1	1.956	58 (-944)	CACAGGA	5.98497
SRSF2	2.238	935 (-67)	GGTCCCCA	4.78839
SRSF5	2.67	57 (-945)	TCACAGG	6.32440
SRSF6	2.676	123 (-879)	TGCATA	5.16198

an important mechanism for AS to participate in the development and progression of various diseases. Preliminary studies indicated that exon polymorphism of *CD44* gene was analysed by ESE finder 3.0 demonstrated that mutation generated a new SR proteins binding site [43]. Therefore, we used HSF 3.1 to predict the rs1045642 polymorphism to generate a new ESE sequence. Furthermore, it was analysed by software (ESE finder 3.0) analysis that the binding ability of ESE sequence to SR proteins exceeded their binding thresholds. The bioinformatics software predicted that the rs1045642 polymorphism is a potential splice site, and the altered binding ability of the ESE sequence and the SR protein formed by the mutation affects the abnormal process of the AS of the *ABCB1* gene. Therefore, we suspect that there is a relationship between the risk of CR in patients with CHD and the rs1045642 polymorphism affecting the AS process of *ABCB1* gene.

In our research, there was a significant relationship between the risk of CR in CHD patients and the different genotypes carried by individuals in China. Meanwhile, bioinformatics software has predicted that the *ABCB1* gene rs1045642 polymorphism affects the process of AS and might have a certain relationship with the risk of CR in CHD patients (Figure 5). This study not only provides theoretical support for the individualized administration of clopidogrel after PCI in clinical CHD patients, but also offers a new basis for CR risk in CHD patients and the change of AS process caused by *ABCB1* gene polymorphism.

There is still controversial about the study between CR and polymorphism in patients with CHD. Previous studies on CR only performed polymorphic genotyping by collecting basic clinical information, test results, and patient blood samples, and then statistical analysis to obtain genotypic analysis and CR. However, the mechanism of clopidogrel-related drug resistance has not been further studied. In the current study, we not only did the predecessor research, but also predicted the SNP of clopidogrel-related genes through bioinformatic software. It was predicted that rs1045642 SNP site will
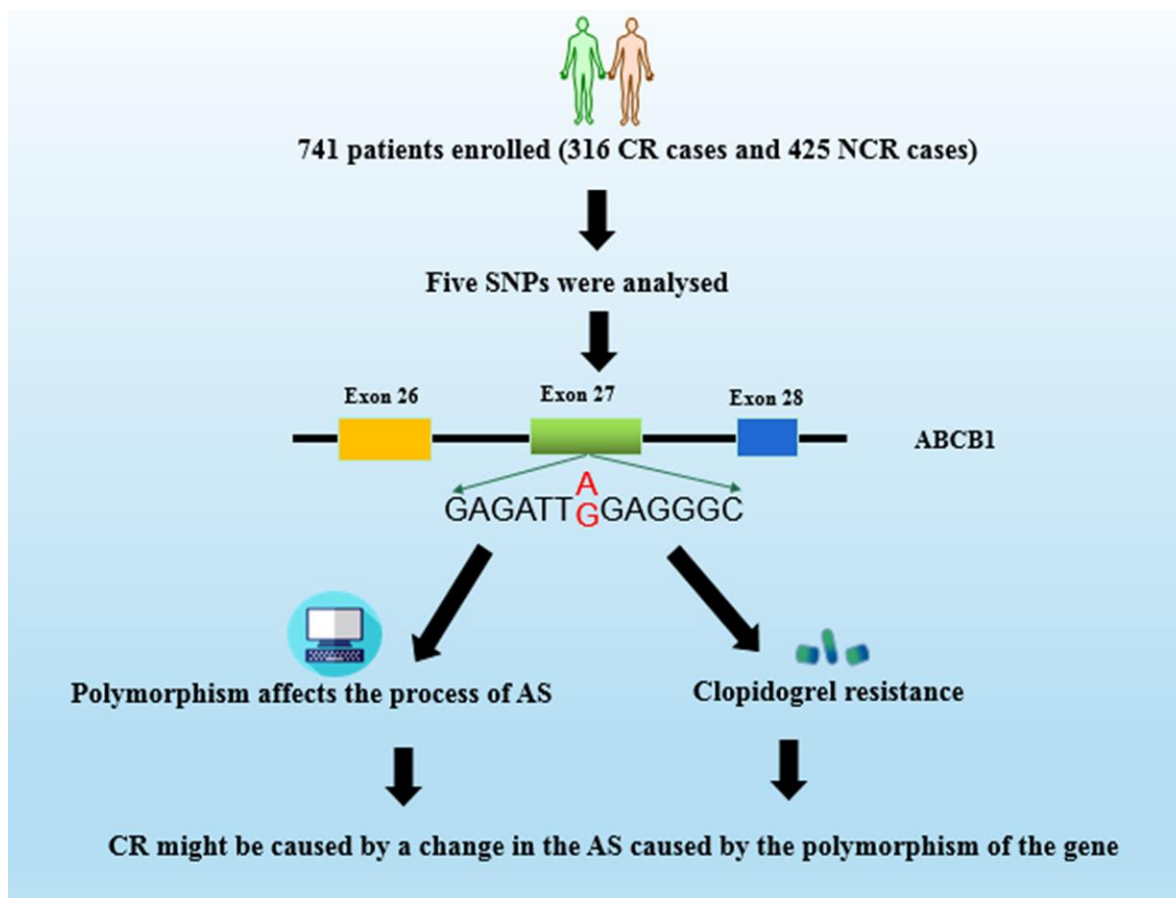

Figure 5. Schematic representation of mechanism of genetic polymorphism of *ABCB1* and clopidogrel resistance (CR) in patients.

potentially affect the AS of the *ABCB1* gene. Therefore, we hypothesize that the mechanism of CR might be caused by a change in the AS caused by the polymorphism of the gene. Our research not only provides theoretical guidance for the clinical individualization of clopidogrel in patients with CHD, but also provides new idea for the study of the mechanism of CR. Meanwhile, specific experiments are needed to support our findings.

MATERIALS AND METHODS

Study population

The subjects of this case-control study were 741 patients with CHD or taking clopidogrel hydrogen sulfate after PCI in the Second Hospital of Lanzhou University and Gansu Provincial People's Hospital in Lanzhou City, Gansu Province, China. Briefly, there were 316 patients in the CR group and 425 patients in the NCR group. The exclusion criteria of this study were as follows: (1) the subjects were under 18 years old; (2) patients with a history of clopidogrel allergy; (3) severe liver and kidney insufficiency; (4) peptic ulcer; (6) major surgery; (7) cerebrovascular accident or recent bleeding history. Clinical data were collected from the electronic records, including demographic characteristics, history of smoking, history of drinking, history of hypertension, history of diabetes and previous history of major medical conditions. Laboratory tests, including triglycerides, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, glycosylated hemoglobin, high-sensitivity C-reactive protein and platelet count.and other biochemical variables, were performed after admission. This study was approved by the Ethics Committee of Lanzhou University School of Basic Medicine.

Thromboelastography test

The platelet inhibition rate was detected by thromboelastography (TEG) method according to the following steps: (1) 1 ml of citrated blood was mixed with kaolin by inversion and 340 μl of the activated blood was added to the 20 μl of calcium chloride; (2) 360 μl heparin anticoagulated blood was added to 10 μl batroxobin mixture; (3) 10 μl batroxobin and 10 μl ADP mixture were added to 360 μl heparin blood; (4) the CFMSLEPU-8800 instrument was used to detect the blood clot elasticity map. The test was performed using the CFMSLEPU-8800 Analyzer System. At present, there is no standardized baseline for CR. In this study, ADP inhibition rate <20% was classified as CR, and ADP inhibition rate ≥50% was defined as NCR [44].

SNPs selection

The *ABCB1* gene is located on human chromosome 7 at q21 [45]. This study referred to NCBI (http://www.ncbi.nlm.nih.gov/ncbisearch), Ensembl (http://asia.ensembl.org/index.html/), the HapMap database of Han Chinese in Beijing, China (http://hapmap.ncbi.nlm.nih.gov) Databases and published articles related to CR when selecting the SNPs of the *ABCB1* gene [46]. Ultimately, five polymorphisms rs1045642, rs4148727, rs2032582, rs3789243 and rs1858923 were selected in the current study.

Extraction of genomic DNA

Genomic DNA was extracted from EDTA-treated peripheral blood using the phenol/chloroform method. The extracted DNA was dissolved in 50 μl of $1 \times$ TE buffer and frozen ($-80°$ C) until required. The NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Lenexa, KS, USA) was used to detect the concentration and purity of DNA. Samples with DNA concentration exceeding 20 ng/ul were sent to biological company (Bomiao Biotechnology Co., Ltd., Beijing, China) for genotyping.

PCR amplification

The extracted genomic DNA was amplified by PCR. The PCR primers were designed using Assay design 3.1 software and submitted to Shanghai Invitrogen Biotechnology Co., Ltd. for synthesis. The amplification primers were shown in Table 6. For amplification of the DNA, the thermal cycling involved initial denaturation at 95° C for 5 min, 45 cycles of 95° C for 20 s, 56° C for 30 s, and 72° C for 1 min, and a final extension at 72° C for 7 min.

Identification of ABCB1 SNPs by Sequenom MassArray

Extracted DNA samples were sent to a biological company (Bomiao Biotechnology Co., Ltd., Beijing, China) for genotyping. Sequenom MassArray typing technique was used to determine the all individual genotypes. The specific steps of the Sequenom MassArray genotyping method are as follows: (1) The PCR amplification primer sequences and extension primer sequences of genotyping SNPs were designed, and the primer sequences were sent Shanghai Invitrogen Biotechnology Co., Ltd for synthesis; (2) 4 μl PCR master mix and 1 μl template DNA were added to each well of the 384-well plate. The PCR reaction program was 94° C, 5 min; 45 cycles: 94° C, 20 s, 56° C, 30 s, 72° C, 1 min; 72° C, 3 min; 4° C, ∞; (3) The PCR product was treated with shrimp alkaline phosphatase (SAP) to

Table 6. Primers for polymerase chain reaction.

Gene	SNP	Group	Base sequence (5′ → 3′)	Products size
ABCB1	rs1045642	Forward primer	ACGTTGGATGTTGCCTATGGAGACAACAGC	
		Reverse primer	ACGTTGGATGAAGGCATGTATGTTGGCCTC	99bp
	rs4148727	Forward primer	ACGTTGGATGCTTCAATGCTTTGGAGCCAT	
		Reverse primer	ACGTTGGATGTAAGTTTGGGTGGAGGAAGG	98bp
	rs2032582	Forward primer	ACGTTGGATGCATATTTAGTTTGACTCACC	
		Reverse primer	ACGTTGGATGTGTTGTCTGGACAAGCACTG	92bp
	rs3789243	Forward primer	ACGTTGGATGATAAGCCCAAGATCCTGTCC	
		Reverse primer	ACGTTGGATGCTGACTGCTTCAGTTCCAAC	107bp
	rs1858923	Forward primer	ACGTTGGATGTCTGACTCCTGTGATAAGGG	
		Reverse primer	ACGTTGGATGTGCTGATTTTCCTCCAGCTC	109bp

Table 7. Primers for polymerase chain reaction.

Gene	SNP	Group	Base sequence (5′ → 3′)	Products size
ABCB1	rs1045642	Forward primer	GCTGAGAACATTGCCTATGG	
		Reverse primer	GGAAGTGTGGCCAGATGCTTGT	259bp
	rs4148727	Forward primer	CATTCTGCCTATTCTGGCTA	
		Reverse primer	GGATAAGTTTGGGTGGAGGA	232bp
	rs2032582	Forward primer	GCTATAGGTTCCAGGCTTGC	
		Reverse primer	GTCCAAGAACTGGCTTTGCT	390bp
	rs3789243	Forward primer	CCATTCCTTGAGGACTTTGG	
		Reverse primer	CCCAGACAATAAGCCCAAGA	202bp
	rs1858923	Forward primer	TGTCTTGATTTCGCCATTGA	
		Reverse primer	CCTGTGATAAGGGGTTGAGG	246bp

remove free dNTPs in the system, and 2 μl SAP mixture and 5 μl PCR product were added to each well of 384-well plate for reaction. The SAP reaction program was 37° C, 20 min; 85° C, 5 min; 4° C, ∞; (4) 2 μl single base mixture and 7 μl SAP-PCR reaction product were added to each well of 384-well plate. The extension reaction program was 90° C, 30 s; 40 cycles: 94° C, 5 s, (52° C, 5 s, 80° C, 5 s) × 5 cycles; 72° C, 3 min; 4° C, ∞; (5) Purification reaction; (6) Chip spotting; (7) Mass spectrometry detection and data output. After the MALDI-TOF mass spectrometer analyzer detects the SpectroCHIP chip, TYPER4.0 software was used to further analyze and process the raw data.

Sequencing verification

Conclusively, partial PCR products were randomly selected for direct sequencing verification. PRIMER 3.0 software was used for primer design and sent to General Biosystems (Anhui) Co., Ltd. for synthesis. The synthesized primer sequence and PCR product size were shown in Table 7. The PCR reaction program was pre-denaturation 94° C for 3 min, 31 cycles of 94° C for 30 s, 58.6° C for 30 s, and 72° C for 25 s, and a final extension at 72° C for 5 min. In the 1.1% agarose gel pool in 1×TAE electrophoresis solution, the PCR products (5 μl/well) of each sample were electrophoresed at 110 V for 25 min, and the agarose was treated with GoldView I. The target bands were detected on the Tanon 1600 gel imaging system (Tanon Science and Technology Co., Ltd.), and the amplified products were sent to General Biosystems (Anhui) Co., Ltd. for sequencing verification. The sequencing results were analysed by SeqManII software (DNASTAR, Madison, WI, USA).

Statistical analysis

SPSS 22.0 statistical software (IBM Corp. Armonk, NY, USA) was used for data analysis. For the

continuous variables, analysis of variance techniques or Kruskal-Wallis tests were used and expressed as the means ± SD. Categorical variables are summarised by frequency and percentage. Associations between SNPs and CR in CHD were assessed using the Chi-square test. Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and haplotype analysis tests were evaluated using SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) in case and control groups. Odds ratio (OR) with 95% Confidence intervals (CI) were calculated to estimate the strength of relationship between individual genotypes of five polymorphisms and CR. P value < 0.05 for the difference was significant.

Silico analysis

In order to determine whether rs1045642 affects the splicing efficiency, computer analysis was performed using HSF 3.1 and ESE finder 3.0 bioinformatics analysis software [42, 43, 45]. HSF 3.1 detects receptor (3'ss) and donor (5'ss) positions by a position weight matrix. ESE finder 3.0 software was used to estimate ESE sequences that change due to mutations. The default threshold was used to determine the locus responsible for the four Serine/arginine-rich (SR) proteins, including AS factor/splicing factor 2 (ASF / SF2), SR splicing factor 5 (SRp40), SR splicing factor 3 (SC35), SR splicing factor 6 (SRp55). The default thresholds for HSF were 1.956, 2.67, 2.383, and 2.6676, respectively. Only sequences with scores above or equal to the threshold were selected.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Abbreviations

ABCB1: ATP binding cassette subfamily B member 1; CR: Clopidogrel resistance; CHD: Coronary heart disease; SNP: Single nucleotide polymorphism; AS: Alternative splicing; PCR: Polymerase chain reaction; PCI: Percutaneous coronary intervention; ESE: exon splicing enhancer; MPV: mean platelet volume; SAP: Shrimp alkaline phosphatase; CI: Confidence intervals; OR: Odds ratio; HWE: Hardy-Weinberg equilibrium; LD: Linkage disequilibrium; SR: Serine/arginine-rich; ASF / SF2: AS factor/splicing factor 2.

AUTHOR CONTRIBUTIONS

SSZ, JW, CXD and XDX conceived the manuscript. SSZ, JW, AAZ, WKY, YHC and XTZ did experiments

and wrote the initial draft of the manuscript. XWZ, TY and DXX participated in the collection of blood samples. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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