PLOS ONE

RESEARCH ARTICLE

The risk of clopidogrel resistance is associated with ABCB1 polymorphisms but not promoter methylation in a Chinese Han population

Jia Su[1], Qinglin Yu[2], Hao Zhu[3], Xiaojing Li[1], Hanbin Cui[4], Weiping Du[4], Lindan Ji[5], Maoqing Tong[6], Yibo Zheng[1], Hongyu Xu[1], Jianjiang Zhang[1], Yunyun Zhu[1], Yezi Xia[1], Ting Liu[1], Qi Yao[1]*, Jun Yang[1]*, Xiaomin Chen[4]*, Jingbo Yu[1]*

1 Department of Gerontology, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China, **2** Department of Traditional Chinese Internal Medicine, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China, **3** Department of Anaesthesia, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China, **4** Department of Cardiology, Ningbo No.1 Hospital, Ningbo, Zhejiang Province, People's Republic of China, **5** Department of Biochemistry, School of Medicine, Ningbo University, Ningbo, Zhejiang Province, People's Republic of China, **6** The Key Laboratory of Molecular Medicine, Ningbo No. 1 Hospital, School of Medicine, Ningbo University, Ningbo, Zhejiang, China

* nbyaoqi@sina.com (QY); yangjun4499@163.com (JY); chxmin@hotmail.com (XC); nbyujingbo@163.com (JY)

Citation: Su J, Yu Q, Zhu H, Li X, Cui H, Du W, et al. (2017) The risk of clopidogrel resistance is associated with ABCB1 polymorphisms but not promoter methylation in a Chinese Han population. PLoS ONE 12(3): e0174511. https://doi.org/10.1371/journal.pone.0174511

Editor: Andreas Zirlik, Universitatsklinikum Freiburg, GERMANY

Received: June 22, 2016

Accepted: February 28, 2017

Published: March 30, 2017

Data Availability Statement: All relevant data are within the paper.

Funding: The authors received the funding of the Plan of Science and Technology on Medicine and Health in Zhejiang Province (2017KY574) for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

The goal of our study was to investigate the contribution of *ABCB1* expression to the risk of clopidogrel resistance (CR). Platelets functions were measured using the Verify-Now P2Y12 assay. Applying Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP), the single-nucleotide polymorphisms (SNPs) was tested. Using bisulphite pyrosequencing assay, we investigated the association of the *ABCB1* DNA methylation levels and CR. It was shown that female, hypertension, and lower albumin levels increased the risk of CR (P<0.05). If patients did not have hypoproteinaemia or had hypertension, the SNP in rs1045642 was associated with CR (CC vs. TT: albumin ≥35, $P = 0.042$; hypertension, $P = 0.045$; C vs. T: albumin ≥35, $P = 0.033$; hypertension, $P = 0.040$). Additionally, the platelet inhibition of the CT+TT genotype in rs1128503 was larger than that of the CC genotype (P = 0.021). Multivariate logistic regression analysis showed that male, higher albumin and hsCRP decreased the risk of CR, and the stent size maybe positively correlated with CR. The SNP in rs1045642 was related to all-cause mortality ($P = 0.024$). We did not find any relationship between the methylation levels of the *ABCB1* promoter and CR. In conclusions, our study indicated that ABCB1 polymorphisms might be useful in further evaluating the pathogenesis of CR.

Introduction

At sites of vascular injury due to atherosclerotic plaque rupture or erosion, platelets mediate not only haemostasis but also pathologic thrombosis[1]. Thrombus generation (due to platelet activation and aggregation) is the main process involved in atherosclerotic vascular disease, particularly coronary artery disease (CAD)[2]. Therefore, antiplatelet therapy has been the cornerstone therapy in patients with coronary artery disease, especially in those undergoing percutaneous coronary intervention (PCI)[3].

Through inhibiting the purinergic ADP receptor P2Y12, clopidogrel reduces adenosine diphosphate-induced platelet aggregation and decreases the risk of cardiovascular events in CAD patients[4]. However, a large number of patients continue to suffer recurrent ischaemic events[5], and this clinical phenomenon has been correlated with lesser degrees of platelet inhibition[6]. This failure of the antiplatelet drug to inhibit its target of action is called clopidogrel non-responsiveness or clopidogrel resistance[7]. Recently, both prasugrel and ticagrelor, which are novel and stronger antiplatelet agents, were shown to exert more consistent, rapid and effective P2Y12 receptor inhibition in patients with acute coronary syndrome (ACS)[8]. Nevertheless, high incidence of major bleeding in some patients receiving prasugrel was noted [9], and ticagrelor was associated with an 11% increase in combined major and minor PLATO bleeding rates after careful analysis of bleeding events[10]. And cases of inadequate platelet inhibition of prasugrel had been occasionally reported however the incidence of this is less than clopidogrel resistance and this is in keeping with prasugrel being a prodrug[11]. Therefore, clopidogrel remains to be one of the most extensively prescribed antiplatelet drugs in CAD patients, and research focused on the individual susceptibility to clopidogrel is of vital significance.

Several clinical and demographic factors may influence the antiplatelet efficacy of clopidogrel, such as drug-drug interactions (such as Proton pump inhibitors[12]), renal dysfunction, diabetes mellitus (DM), diet, smoking, age, reduced left ventricular function, inflammation and the presence of an ACS[13]. However, genetic factors, specifically the expression of the ABCB1 gene, may significantly influence clopidogrel's response[14].

Clopidogrel is an oral, second-generation thienopyridine irreversible inhibitor of the P2Y12 receptor. It undergoes rapid absorption by the duodenum and is metabolized by hepatic cytochrome P450 enzymes. About 15% of clopidogrel's prodrug is converted into a biologically active thiol metabolite, which, in circulation, irreversibly combines to and inactivates the P2Y12 receptor on the surfaces of platelets, resulting in the inhibition of ADP-induced platelet activation and aggregation[15]. In the above transformation, specific genetic variants are responsible for clopidogrel's transport (ATP-binding cassette subfamily B member 1 [ABCB1]), metabolism (CYP enzymes, paraoxonase-1) and action (P2Y12)[16].

The *ABCB1* gene, which is also called *MDR1* or *TAP1*, encodes the intestinal efflux transporter pump P-glycoprotein and modulates the absorption of clopidogrel[17]. The effect of different levels of ABCB1 expression is unclear. Simon *et al.*[18] first analysed the influence of the ABCB1 C3435T (rs1045642) polymorphism on clinical outcomes in persons treating with clopidogrel and found that homozygous patients had a higher risk of cardiovascular events. One recent study was found that exposition to clopidogrel, measured by AUC0-t of the drug, was significantly lower in TT homozygotes comparing to CC and CT genotypes (ABCB1 3435C>T), which showed that the presence of 3435C>T allele had an impact on clopidogrel pharmacokinetics[19]. However, these findings could not be confirmed in several subsequent studies. Additionally, the effect of the rs1128503 polymorphism and the DNA methylation of selected CpG islands in the ABCB1 gene on clopidogrel's response are poorly understood. Thus, in the present study, we attempted to assess whether the rs1045642 and rs1128503 polymorphisms and DNA methylation in the ABCB1 promoter are involved in clopidogrel resistance in Chinese CAD patients treated with clopidogrel.

Methods

Study population

From 2010 to 2015, a total of 180 patients with acute coronary syndromes (ACS) were enrolled in present study in the Ningbo No. 1 Hospital. All of them were Han Chinese from Ningbo

city in Eastern China. The inclusion criteria were as follows: (1) According to the most recent guidelines (ACC/AHA guidelines), all individuals were undergoing PCI through the radial route via a drug-eluting stent. The patients we tested the platelet function were mostly existed multi-vessel or left main vessel disease of the coronary artery. (2) The patients received clopidogrel (300 mg) and aspirin (300 mg) before PCI and were administered clopidogrel 75 mg and aspirin 100 mg daily. (3) The patients' age was required to be ≥18 years. The exclusion criteria were as follows: (1) the therapy of concomitant glycoprotein IIb/IIIa inhibitor or warfarin, (2) hepatic or renal insufficiency, (3) history of active bleeding diathesis, (4) hepatic and renal insufficiency, and (5) total platelet count $< 150\,000\ \mu l^{-1}$ or $> 500\,000\ \mu l^{-1}$. All patients provided written informed consent before participation in the research. The study protocol was reviewed and approved by the Ethics Committee of Ningbo No1. Hospital and conformed to the guide lines of the Declaration of Helsinki.

Collection of blood samples and clinical data

Blood samples were obtained overnight via venipuncture in the antecubital area. Serologic markers, such as the concentrations of TC, TG, LDL, GLU, HbA1c, BUN, and CREA, were detected. All the measures applied the standard procedures, and data were collected.

Platelet function measurements

With the administration of clopidogrel for 3 to 5 days, the platelet reactivity may have been stable or not significantly changed in AMI patients undergoing PCI[20]. We detected the platelet reactivity one month after PCI. By the double-syringe technique, blood samples were gathered and the first 5 ml was discarded to avert unprompted platelet activation. The platelet function measurements were analysed by the VerifyNow *P2Y12* assay (Accumetrics Inc., San Diego, California), which was developed to assess the response to *P2Y12* antagonists[21]. The VerifyNow *P2Y12* assay reported *P2Y12* reaction units (PRU), and a PRU more than 240 reaction units suggested the existence of clopidogrel resistance[22].

Genomic DNA extraction, genotyping, and methylation assay

Human genomic DNA was extracted from 3 ml peripheral blood by QIAamp DNA BloodMini Kit (Qiagen). Samples were stored at -100˚C until use. PCR primers were planned through PyroMark Assay Design software. The sequences of primers used in the SNP genotyping and DNA Methylation Assay are described in ***Table 1***.

Polymerase chain reaction (PCR) amplification (BIO-RAD C1000touch Thermal Cycler PCR) was performed as follows: 50 μl reaction volume containing 1 μl template DNA, 1.5 μl 10 mM dNTP, 5 μl Taq Buffer, 1.0 μl25 mM $MgCl_2$, 1.5 μl upstream and downstream primers, 1 μl platinum Taq polymerase 1 U and 37.5 μl water. PCR cycling conditions consisted of 2

Table 1. The primers for SNP genotyping and DNA methylation assay.

	Group	DNA sequence
SNP genotyping	rs1045642-F	5-'GTGTGCTGGTCCTGAAGTTG-3'
	rs1045642-R	5'- TGGAGCCTCAAGCCTATAGC -3'
	rs1128503-F	5-'GTTCACTTCAGTTACCCATCTCG-3'
	rs1128503-R	5'- CGTGGTGGCAAACAATACAGG -3'
DNA Methylation Assay	ABCB1-F1	GGATATGGAAGTTAAGATTTTAGAGATA
	ABCB1-R1	ATTTCAAATATCCCATTACCACATATAAC
	ABCB1-S1	ATGATTAATGAGGTAGAAAAAAG

https://doi.org/10.1371/journal.pone.0174511.t001

cycles of 94˚C denaturation for 2 min, 30 cycles of 94˚C denaturation for 30 sec, 55˚C annealing for 45 sec, and 68˚C extension for 3 min, and one cycle of 68˚C repair extension for 10 min. PCR products were purified (SK1141; kit Sangon Biotech), measured (3500XL sequence analyser; ABI), and sequenced.

The bisulphite pyrosequencing technology was applied to evaluate the quantitative DNA methylation of 2 CpG dinucleotides on the fragment of ABCB1 gene promoter[23]. 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[24].

Statistical analysis

Quantitative data are described as the mean ± standard deviation for categorical variables or the median with interquartile range (IQR) for continuous variables. We carried out a suite of statistical analyses to investigate the association among genetic variables, various confounding factors and clopidogrel resistance. Categorical variables were analysed with either Pearson's chi-square test or Fisher's exact test when appropriate. Continuous variance with normality and homogeneity were applied to compare the mean values from *t*-tests. Non-parametric continuous variance was performed using the Wilcoxon rank-sum test. Logistic regression was used to test the interaction of ABCB1 SNP and confounding variables. All the statistical analyses were conducted by PASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA). A value of P less than 0.05 was considered to indicate a statistically significant difference.

Results

Study population

From May 2010 to October 2015, 180 CAD patients who met all requirements were recruited for the present study. UsingVerifyNowP2Y12 assay, 81 patients whose PRU was greater than 240 were defined as having a poor clopidogrel response or clopidogrel resistance. The demographic and clinical characteristics of the cases and controls are summarized in ***Table 2***. The clinical information was well matched except for albumin and the ratios of gender and hypertension. Patients with poor clopidogrel response were more likely to be female (cases versus controls: 32.1% versus 15.15%, $P = 0.007$) and have hypertension (cases versus controls: 75.31% versus 58.59%, $P = 0.018$) and lower albumin levels (cases versus controls:38.11 ± 4.38 versus 39.83 ± 5.64, $P = 0.026$). These data indicated that female sex, hypertension and lower albumin levels might increase the risk of clopidogrel resistance.

Clopidogrel resistance and thers1045642 and rs1128503 polymorphisms

It was well known that the variance of rs1045642 and rs1128503is common in Chinese populations. We searched the genetic information of CHB on the HAP-MAP and found that the frequency of the C allele in ABCB1 3435C>T (rs1045642) and the C allele in ABCB1 1236C>T (rs1128503) in Chinese populations are 0.613 and 0.293. We calculated the frequency of these two SNP in our study and ensured that each of the target polymorphisms was in Hardy–Weinberg equilibrium (***Table 3***).

Through PCR-HPCE, we explored the association of clopidogrel resistance and the rs1045642 and rs1128503 polymorphisms. As shown in ***Table 4***, our results showed that the variation of rs1045642 and rs1128503 was not significantly associated with clopidogrel resistance ($P_{rs1045642} = 0.288$; $P_{rs1128503} = 0.644$).

Table 2. Comparison between CR and non-CR characteristics.

	CR (81)	Non-CR (99)	*P* value
Hypertension, n(%)	61 (75.31)	58 (58.59)	0.018
Diabetes mellitus, n(%)	16 (19.75))	22 (22.22)	0.686
Hyperlipidemia, n(%)	32 (39.51)	44 (44.44)	0.505
Current smoking, n(%)	31 (38.27)	42 (42.42)	0.572
Alcohol abuse, n(%)	17 (20.99)	24 (24.24)	0.604
Gender (male), n(%)	55 (67.90)	84 (84.85)	0.007
Age, y	65.69 ±11.85	63.01 ±12.06	0.136
BMI, kg/m^2	23.29 ±4.58	24.41 ±10.15	0.363
TC, mg/dL	4.36 ±3.72	4.77 ±1.48	0.273
TG, mg/dL	1.67 ±1.16	1.70 ±1.33	0.880
HDL, mg/dL	0.96 ±0.27	1.00 ±0.28	0.370
LDL, mg/dL	2.77 ±0.98	2.86 ±1.02	0.533
GLU, mmol/L	6.09 ±2.10	5.88 ±2.42	0.535
HbA1c, %	6.17 ±1.08	6.49 ±3.36	0.420
ALT, μmol/L	36.67 ±31.47	37.36 ±29.52	0.879
AST, μmol/L	109.75 ±162.84	113.29 ±167.46	0.887
TBIL, μmol/L	15.29 ±8.51	14.77 ±8.03	0.676
Album, g/L	38.11 ±4.38	39.83 ±5.64	0.026
BUN, mmol/L	5.98 ±2.27	5.46 ±1.97	0.101
CREA, mmol/L	75.37 ±24.93	72.71 ±22.44	0.453
UA, μmol/L	350.88 ±129.78	322.37 ±80.42	0.073
hsCRP, mg/L	12.24 ±19.81	14.07 ±26.21	0.604
PLT, $*10^9/L$	194.30 ±58.05	201.43 ±73.93	0.480
MPV, fL	8.38 ±1.32	8.09 ±0.93	0.088
PCT, %	0.16 ±0.04	0.16 ±0.05	0.953
PDW, %	16.23 ±1.19	16.33 ±0.58	0.488
LEVF, %	59.40 ±9.40	61.28 ±7.54	0.137
Stent, n	1.57 ±0.95	1.31 ±0.79	0.051

https://doi.org/10.1371/journal.pone.0174511.t002

Additionally, we determined the relationship by the comparison of the different SNP modes (homozygotes and dominant, recessive and heterozygous models), and the results were insignificant (*Table 5*).

Subgroup analysis

Due to the differences in the baseline characteristics between the CR and NCR patients, we carried out subgroup analysis according to the ratios of males, hypertension and albumin. Overall, there was no significant difference for any subgroup (*Table 6*).

However, we tested the relationship between platelet activity and the four SNP modes, producing new significance values. For rs1045642, in patients with hypertension or a value of albumin greater than 35, patients with the CC genotype had a higher risk of CR than those with the TT genotype (albumin≥35, P = 0.042; hypertension P = 0.045). At the same time,

Table 3. Hardy-Weinberg equilibrium test of rs1045642 and rs1128503.

	ABCB1 3435C>T (rs1045642)					ABCB1 1236C>T (rs1128503)				
	CC	CT	TT	Chi-squre	HWE	CC	CT	TT	Chi-squre	HWE
N	76	79	25	0.374	0.54	39	92	59	0.082	0.76

https://doi.org/10.1371/journal.pone.0174511.t003

Table 4. Comparison of the SNP in rs1045642 and rs1128503 between cases and controls.

	ABCB1 3435C>T (rs1045642)				ABCB1 1236C>T (rs1128503)			
	CC	CT	TT	*P value*	CC	CT	TT	*P value*
CR	38	35	8	0.228	13	40	28	0.644
NCR	38	44	17		16	52	31	

https://doi.org/10.1371/journal.pone.0174511.t004

patients with the C allele were more likely to have an increased risk of CR than those with the T allele (albumin≥35 P = 0.033; hypertension P = 0.040)(***Table 7***).

Different SNP modes and the index of platelet function

The Verify-Now assay measures the agonist-induced activation of platelets and their binding to fibrinogen-coated polystyrene beads. The assay uses ADP as an agonist and PGE1 as an antagonist, and the results are reported as P2Y12 reaction units (PRU)[25].With this test, we obtained the value of PRU, the baseline of platelet activity, and the inhibition value. The inhibition value is equivalent to (baseline-PRU)/baseline. We compared the relationship between the SNP and PRU, as well as inhibition, and did not find any significant results (***Table 8***).

Because we tested the relationship between platelet activity and the four SNP modes, it became clear that a significant association existed. For ABCB1236C>T (rs1128503), compared with the genotype of CC, the inhibition of platelets in patients with CT+TT was much higher (P = 0.021), which contributed to clopidogrel resistance (***Table 9*** and Fig 1).

The relationship between clopidogrel resistance and methylation levels

In the present study, we chose 106 patients at random and found 49 with a poor response to clopidogrel after the platelet function test. We selected a fragment (GRCh37.p13: 87344039–87341039) that contained 2 CpG dinucleotides. Through the bisulphite pyrosequencing assay, we investigated the association of clopidogrel resistance and ABCB1 gene promoter DNA methylation levels in these 106 CAD patients. Additionally, as shown in Fig 2 and ***Table 10***, the methylation levels of CpG1 in ABCB1 in selected fragments were not significantly related to clopidogrel resistance (cases versus controls(%): 90.84±3.42 versus 91.18±2.27, $P = 0.545$) along with ABCB1 CpG2 (cases versus controls (%): 92.65±4.52 versus93.53±4.12, $P = 0.408$). Although we tried to perform a breakdown analysis according to clinical characteristics, we could not find any relationship between the methylation levels of the ABCB1 gene promoter (including CpG1 and CpG2) and poor response to clopidogrel.

Multivariate logistic regression

Respecting the influence of confounding factors, we implemented logistic regression analysis with clinical and genetic variables. The results showed that the TT genotype (rs1045642) was a

Table 5. Comparison of the different SNP modes in two loci between cases and controls.

	CC+TT vs. TT		*P value*	CC vs. CT+TT		*P value*	CC vs. TT		*P value*	C vs. T		*P value*
ABCB13435C>T (rs1045642)												
CR	73	8	0.159	38	43	0.249	38	8	0.117	111	51	0.012
NCR	82	17		38	61		38	17		110	88	
ABCB11236C>T (rs1128503)												
CR	53	28	0.644	13	68	0.984	13	28	0.816	66	96	0.747
NCR	68	31		16	85		16	31		84	114	

https://doi.org/10.1371/journal.pone.0174511.t005

Table 6. Subgroup analysis for SNP in rs1045642 and rs1128503 between cases and controls.

		cases			controls			
		CC	CT	TT	CC	CT	TT	*P value*
*ABCB1*3435C>T (rs1045642)								
Hypertension	Yes	33	26	5	20	27	11	0.075
	No	8	9	3	18	17	16	0.357
Gender	Male	24	26	5	34	36	14	0.445
	Female	14	9	3	4	8	3	0.238
Albumin	≥35	35	50	5	34	39	15	0.123
	<35	3	5	3	4	5	2	0.842
*ABCB1*1236C>T (rs1128503)								
Hypertension	Yes	9	29	23	8	31	19	0.806
	No	4	11	5	8	21	12	0.938
Gender	Male	8	27	20	16	43	25	0.684
	Female	5	13	8	0	9	6	0.192
Albumin	≥35	12	32	26	12	46	29	0.655
	<35	1	8	2	4	6	2	0.359

https://doi.org/10.1371/journal.pone.0174511.t006

protective factor of clopidogrel resistance in the homozygotes mode (TT vs. CC:OR = 0.073, P = 0.024). Additionally, the indexes (such as male sex, hyperlipidaemia, HDL, hsCRP, and EF) were inversely correlated with CR, whereas the values of LDL, UA, and the number of stents were associated with CR (***Table 11***).

Clinical events and genotype in rs1045642 and rs1128503

For these 180 patients, we performed clinical follow-ups for one year. In this year, there were 30 non-fatal myocardial infarctions, 7 non-fatal strokes, 7 cardiovascular deaths, 2 deaths from other causes (1 acute pancreatitis and 1 renal insufficiency), 29 cases of stent thrombosis, and 10 cases of minor or major bleeding. We explored the association between the genotype of rs1045642 and rs1128503 and the risk of cardiovascular events after receiving clopidogrel for one year. The results indicated that the SNP of rs1045642 was related to all-cause mortality (P = 0.024) (***Table 12***). However, we could not identify the susceptible genotype due to the limited sample size.

Discussion

The ABCB1gene, located at 7, p21–21.1,[26], encodes the intestinal efflux transporter P-glycoprotein. The P-glycoprotein plays an important role in the bioavailability of multiple endogenous and xenobiotic compounds (such as clopidogrel) from the intestinal lumen via duodenal enterocytes[17]. Recent research revealed that C3435T SNP (rs1045642) was related to an

Table 7. Subgroup analysis for the different SNP modes in rs1045642 between cases and controls.

	CC+TT vs. TT		*P value*	CC vs. CT+TT		*P value*	CC vs. TT		*P value*	C vs. T		*P value*
Albumin≥35												
CR	65	5	0.063	35	35	0.153	35	5	0.042	100	40	0.033
NCR	75	15		34	54		34	15		103	69	
Hypertension												
CR	56	5	0.085	30	31	0.104	30	5	0.045	86	36	0.040
NCR	47	11		20	38		20	11		67	49	

https://doi.org/10.1371/journal.pone.0174511.t007

Table 8. Comparison of platelet function values and the SNP at the two loci.

		PRU	*P value*	Inhibition	*P value*
*ABCB1*3435C>T (rs1045642)	CC (76)	234.75±75.84	0.525	0.49±0.26	0.552
	CT (79)	220.80±61.05		0.25±0.17	
	TT (25)	212.32±57.95		0.25±0.13	
*ABCB1*1236C>T (rs1128503)	CC (29)	225.21±77.50	0.879	0.23±0.19	0.667
	CT (92)	227.82±67.99		0.45±0.21	
	TT (59)	222.07±65.61		0.26±0.17	

https://doi.org/10.1371/journal.pone.0174511.t008

increase in ulcerative colitis, whereas it was associated with tacrolimus pharmacokinetics and the response to platinum-based chemotherapy in lung cancer[27]. Simon *et al.*[18] first analysed the influence of the ABCB1 C3435T polymorphism on clinical outcomes in persons on clopidogrel and found that homozygous patients had an increasing risk of cardiovascular events. However, many other studies showed controversial findings in several subsequent periods. It was reported that the genotype of ABCB1 C1236T (rs1128503) might influence the incidence of breast cancer[28], osteonecrosis[29], and multiple myeloma[30], and the methylation level of the partial ABCB1 gene promoter is closely linked to the efficacy of interventional embolism chemotherapy for cervical cancer[31]. However, there is little information regarding the influence of thers1128503polymorphism and the DNA methylation of selected CpG islands in the ABCB1 gene on clopidogrel's response.

In the present study, with the application of the Verify-Now P2Y12 assay for platelet function tests in clinical practice and the use of polymerase chain reaction-high performance capillary electrophoresis (PCR-HPCE) for sequencing, we found that if patients had no hypoproteinaemia or had hypertension, the SNP in rs1045642 was associated with CR (CC vs. TT: albumin $\geq$35, P = 0.042; hypertension, P = 0.045; C vs. T: albumin $\geq$35, P = 0.033; hypertension, P = 0.040). Additionally, the SNP in rs1045642 was related to the all-cause mortality after one-year follow-up. These data were not consistent with previous research[32] and our most recent meta-analysis[33]. These inconsistent results might be due to the variance of gene frequency in different populations in different areas. Searching the HAPMAP, the information showed that the frequency of the C allele in rs1045642 in Chinese patients was slightly higher than that of Caucasians. A recent study from east asians was indicated that CYP2C19 PM along with ABCB1 3435 TT status was a strong independent predictor of the primary end point (deaths, non-fatal MIs and strokes)[34]. Hence, the variance of the cardinal number in patients with three genotypes resulted in the inconsistent findings compared to the former study. Moreover, to some extent, the limited sample size might have led to statistical bias.

Our results showed that the platelet inhibition of the CT+TT genotype in rs1128503 was larger than that of the CC genotype (P = 0.021).This study was the first to include rs1128503 in the association with the degree of platelet inhibition evaluated by Verify-Now P2Y12 assay. The most recent findings may elicit a novel target and help us to screen the susceptible locus with a new perspective. There are more than 50 single-nucleotide polymorphisms (SNP) in ABCB1. Among them, rs1045642 and rs1128503 are the most common in Chinese patients. Here, we selected these two loci, but we were unable to perform an interaction analysis due to

Table 9. Comparison of platelet function value and the recessive hereditary mode in rs1128503.

	CC (29)	CT+TT (151)	*P value*
PRU	225.21±77.50	226.40±65.77	0.689
Inhibition	0.23±0.19	0.34±0.17	0.021

https://doi.org/10.1371/journal.pone.0174511.t009

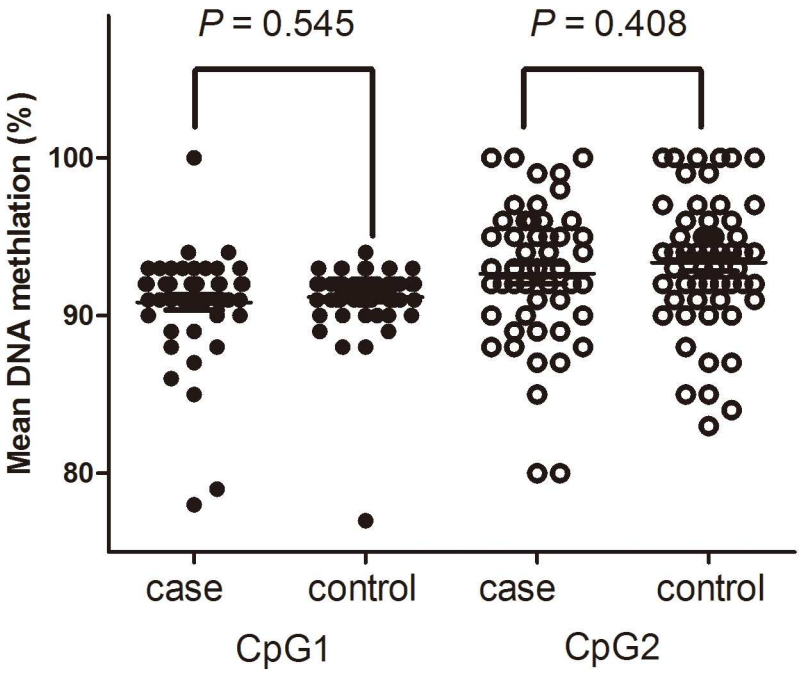

Fig 1. Comparison of platelet function value and the recessive hereditary mode in rs1128503. (1) PRU (CC vs. CT+TT: 225.21±77.50 vs. 226.40±65.77; *P* = 0.689). (2) Inhibition (CC vs. CT+TT(%): 0.23±0.19 vs. 0.34±0.17; P = 0.021).

https://doi.org/10.1371/journal.pone.0174511.g001

the limited sample size. One study showed that coexisting polymorphisms of the P2Y12 and CYP2C19 genes might be related to persistent platelet activation while on clopidogrel[35].

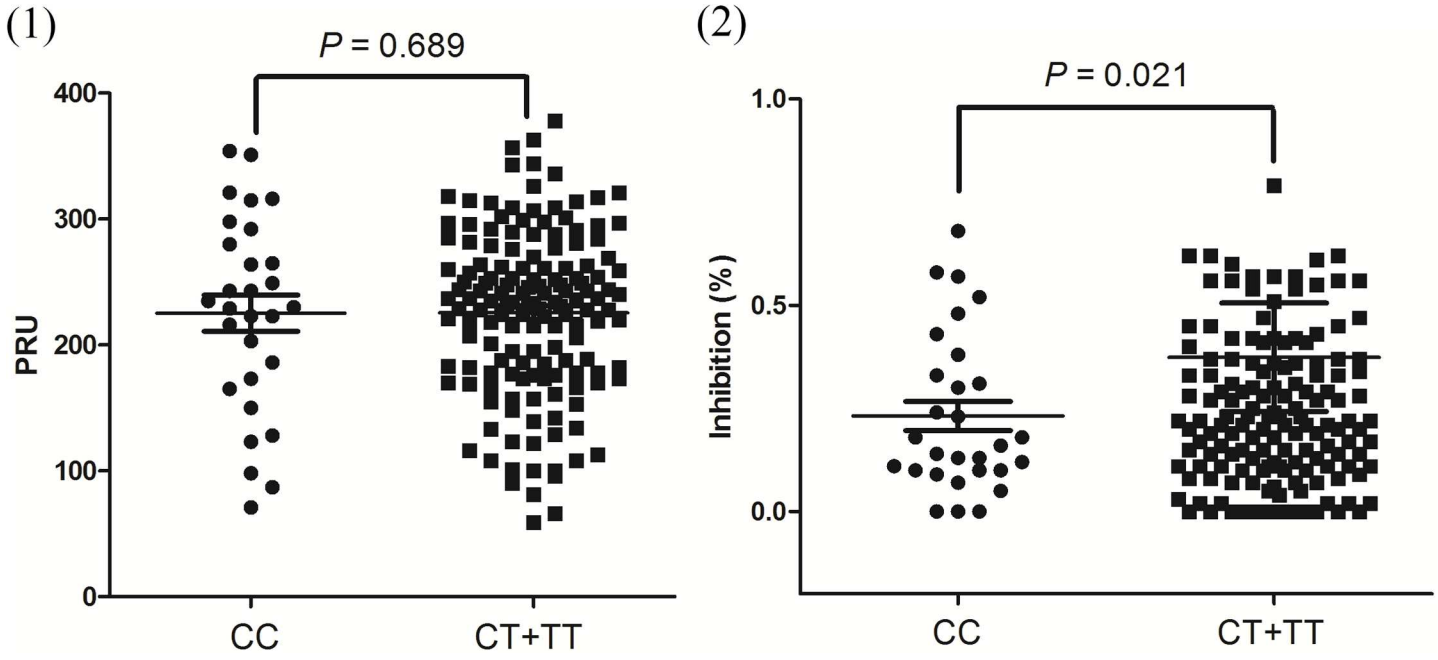

Fig 2. Comparison of ABCB1 methylation levels between cases and controls. ABCB1 CpG1 (cases vs. controls (%):90.84±3.42 vs. 91.18±2.27, *P* = 0.545) and ABCB1 CpG2 (cases vs.controls (%):92.65±4.52 vs. 93.53±4.12, *P* = 0.408).

https://doi.org/10.1371/journal.pone.0174511.g002

Table 10. Comparison of ABCB1 methylation levels between cases and controls.

	Cases (49)	Controls (57)	*P value*
CpG1	90.84±3.42	91.18±2.27	0.545
CpG2	92.65±4.52	93.53±4.12	0.408

https://doi.org/10.1371/journal.pone.0174511.t010

Another recent study reported that the coexistence of QT mutations/polymorphisms in patients with tetralogy of Fallot (TOF) might aggravate abnormal repolarization after cardiac repair and increase the risks of life-threatening events[36]. Therefore, gene-gene interactions, such as those of CYP3A4 or CYP2C19 or PON1 with ABCB1, need to be studied. Additional studies with larger sample sizes might give us a chance to improve and perfect our limitations.

Applying bisulphite pyrosequencing, we evaluated the DNA methylation levels of two CpG dinucleotides on the ABCB1 promoter region. However, no significance was detected between antiplatelet response and methylation status. Various trials have focused on epigenetics, such as miRNA, siRNA, and DNA methylation. DNA methylation is a reliable epigenetic marker and specifically occurs in the context of cytosine-phosphate guanine (CpG) dinucleotide

Table 11. The multiple logistic regression analysis of various and homozygote in rs1045642.

	B	Std. Error	Wald	*P value*	Exp(B)	Exp(B) 95% CI
Constant	18.019	17.480	1.063	0.303		
TT vs. CC	-2.621	1.160	5.130	0.024	0.073	0.01–0.71
Gender (male)	-4.072	1.415	8.275	0.004	0.017	0.00–0.27
Hypertension	-0.267	0.955	0.078	0.780	0.766	0.12–4.98
Diabetes mellitus	-1.509	1.490	1.025	0.311	0.221	0.01–4.10
Hyperlipidemia	-3.449	1.142	9.111	0.003	0.032	0.00–0.30
Current smoking	-0.524	1.000	0.274	0.600	0.592	0.08–4.21
Alcohol abuse	2.010	1.106	3.307	0.069	7.466	0.86–65.18
Age	0.086	0.046	3.591	0.058	1.090	1.00–1.19
BMI	-0.060	0.150	0.161	0.688	0.942	0.70–1.26
TC	0.003	0.016	0.046	0.830	1.003	0.97–1.04
TG	0.432	0.359	1.447	0.229	1.540	0.76–3.11
HDL	-6.232	2.466	6.384	0.012	0.002	0.00–0.25
LDL	1.421	0.601	5.583	0.018	4.140	1.27–13.454
GLU	0.092	0.238	0.150	0.698	1.097	0.69–1.75
HbA1c	-0.293	0.166	3.111	0.078	0.746	0.54–1.03
ALT	0.012	0.029	0.176	0.675	1.012	0.96–1.07
AST	0.000	0.004	0.008	0.930	1.000	0.99–1.01
TBIL	-0.033	0.065	0.259	0.611	0.968	0.85–1.10
A	-0.158	0.132	1.434	0.231	0.854	0.66–1.11
BUN	-0.045	0.223	0.041	0.840	0.956	0.62–1.48
CREA	-0.016	0.018	0.838	0.360	0.984	0.95–1.02
UA	0.017	0.006	8.639	0.003	1.017	1.01–1.03
hsCRP	-0.067	0.026	6.839	0.009	0.935	0.89–0.98
PLT	-0.037	0.021	3.134	0.077	0.963	0.92–1.00
MPV	-0.367	0.713	0.264	0.607	0.693	0.17–2.80
PCT	55.804	28.903	3.728	0.054	1.719	0.43–6.88
PDW	-0.190	0.798	0.057	0.812	0.827	0.17–3.96
LEVF	-0.156	0.061	6.516	0.011	0.856	0.76–0.97
Stent	1.592	0.634	6.306	0.012	4.915	1.42–17.03

https://doi.org/10.1371/journal.pone.0174511.t011

Table 12. Comparison of clinical events and genotypes in rs1045642 and rs1128503.

	ABCB1 3435C>T (rs1045642)				ABCB1 1236C>T (rs1128503)			
	CC(76)	CT(79)	TT(25)	*P value*	CC(29)	CT(92)	TT(59)	*P value*
MACE	20	16	6	0.669	8	24	12	0.661
No fatal MI	13	13	4	0.990	4	18	8	0.566
No fatal stroke	2	3	2	0.483	1	3	3	0.845
Cardiovascular Death	5	0	2	0.055	3	3	1	0.129
All-cause mortality	7	0	2	0.024	0	1	1	0.342
Stent thrombosis	12	14	3	0.791	5	13	11	0.750
Bleeding	1	7	2	0.101	1	5	4	0.812

https://doi.org/10.1371/journal.pone.0174511.t012

[37]. The hypermethylation of vertebrate CpG islands (CGIs) is relative to the transcriptional silencing of gene expression and thus controls the protein level[38]. It was found that aberrant methylation plays a vital role in the occurrence and development of diseases, including adrenocortical cancer[39], age-associated cancer[40], coronary artery disease[41], and psychotic disorders[42]. Two years ago, we investigated the contribution of P2Y12 promoter DNA methylation to the risk of clopidogrel resistance (CR) and discovered that the lower methylation of two CpGs indicated that the CR in alcohol abuse and CpG1methylation was inversely correlated to CR in smokers and in the albumin subgroup[24]. To the best of our knowledge, this was the first study on the topic of DNA methylation and CR. The ABCB1 results were not significant, which was inconsistent with another study that showed that hypomethylation of the ABCB1 promoter is related to a poor response to clopidogrel in ischaemic stroke patients[43]. Although there were differences in the study subjects (coronary artery disease and stroke), the population, drug, and the dose were all uniform. These inconsistent findings might be due to the varying regions of CpGIs each study selected. Bisulphite pyrosequencing chose a random region of CpGIs and could not cover the entire area. Thus, sequencing in various regions resulted in inconsistent conclusions. We aim to perform additional studies and a more advanced empirical approach to validate our findings in the future.

The genotype accounts for approximately 2% to 12% of inter-individual variability of the response to clopidogrel[44], and various extrinsic factors (environment, comorbidities, and drug interactions) may also contribute to clopidogrel resistance[45]. We performed logistic regression analysis with confounding variables and showed that male sex and higher levels of albumin and hsCRP decreased the risk of CR, and the number of stents may be positively correlated with CR. These were similar with the study by Gremmel etal, which reported that lower platelet reactivity assessed by the VerifyNow P2Y12 assay were associated with increased levels of hsCRP[46], and the study by Tan etal, which indicated that the number of stents was associated with higher risks of thromboembolic events[47]. Additionally, a recent study showed that chronic kidney disease (CKD) seemed to be associated with poor response to clopidogrel and the high incidence of stent thrombosis in diabetic patients after PCI[48], which might be due to the increased platelet turnover and the up-regulation of the P2Y12 pathway[49]. Hochholzer et al revealed that theCYP2C19*2 polymorphism along with clinical factors (age, diabetes mellitus, body mass index, platelets, verapamil/diltiazem) could replace the phenotyping of platelet function[50]. All these factors demonstrated that the phenomenon of CR was affected by multiple extrinsic and intrinsic factors.

Dual-antiplatelet functions and statins are essential to the proper treatment of CAD, especially after PCI. In follow-up studies, the efficiency of antiplatelet and the influence of statins on atherosclerosis and ischaemic endpoints should be evaluated. If the LDL-C level was greater than 1.3 mmol per litre, the absolute CV risk would decrease in combination with a decrease

in LDL-C [51]. A study on IMPROVE-IT reported a clear benefit in the reduction in major cardiovascular (CV) events with simvastatin and ezetimibe therapy[52]. The expression of the gene could also influence the lipid-lowering efficacy, and our most recent meta-analysis indicated that the ABCB1 C3435T polymorphism might be a pharmacogenomic biomarker for predicting clinical outcomes in patients with statins[53]. Therefore, we should consider the effect of statins in the present study, although we discovered that the ABCB1 C3435T polymorphism was related to the all-cause mortality under treatment with clopidogrel in CAD patients. However, the various types of statins and small sample size in our study limited the exploration of the underlying relationship.

Numerous tests are available for measuring platelet function and predicting clopidogrel response, such as the flow cytometric vasodilator-stimulated phosphoprotein phosphorylation (VASP) analysis, the Verify-Now P2Y12 assay, PFA-100, whole blood thromboelastography and impedance aggregometry (multiplate analyzer)[45]. However, various studies in different areas applied distinct methods and cut-off values. Therefore, a common operation standard and evaluation system were urgently required. We produced a systemic and predictive clinical score, which consisted of extrinsic and intrinsic elements, to screen the CR patients. Recently, Fontana[54] and Geisler[55] provided two novel scoring standards, but both of them require further validation. Moreover, if clopidogrel resistance is present, action would be taken to overcome the high platelet reactivity: increasing the loading dose or/and maintenance dose or adding another antiplatelet drug (cilostazol), or changing clopidogrel to a newer thienopyridine, such as prasugrel or ticagrelor. Despite the decline in ischaemic cardiovascular events, these agents produce a higher risk of bleeding [56].

This study presents inherent limitations. First, the sample size was small. Further study with larger sample sizes could be designed for further evaluation. Second, we chose only one fragment of the CGI from the promoter of the ABCB1 gene, and there might be other region correlated to CR. Thus, the conclusions should be interpreted with caution. Third, in gene-gene or gene-drug or gene-environment interactions, unknown confounding factors might exist and affect the expression of the ABCB1 gene, which led to biased results. The exact interactions remain to be investigated in future studies.

5.Conclusions

In summary, our study found that the SNP in rs1045642 was associated with CR in patients with hypertension or albumin $\geq$35 and was related to all-cause mortality. The platelet inhibition of the CT+TT genotype in rs1128503 was larger than that of the CC genotype. Additionally, the methylation of the ABCB1 gene promoter did not affect clopidogrel's response. Finally, multivariate logistic regression showed that male sex and higher levels of albumin and hsCRP were associated with a decreased risk of CR, and the number of stents may be positively correlated with CR. All these data might provide new insight to elaborate the pathogenesis of CR. However, we aim to perform larger studies with more effective planning and a more advanced empirical approach to validate our findings in further research.

Acknowledgments

We thank Yaqing Wang and Shujun Yang for excellent technical assistance. The authors also thank Dr Jin Xu for editing the manuscript.

Author Contributions

Conceptualization: JS XC JY.

Data curation: WD YZ HX JZ YZ YX TL.

Formal analysis: JS QY HZ LJ.

Funding acquisition: XC JY.

Investigation: JS XL HC MT.

Methodology: JS XL HC MT QY JY.

Project administration: JS XC JY.

Resources: XL HC MT.

Software: JS QY HZ.

Supervision: XC JY.

Validation: HZ JY.

Visualization: JS QY HZ.

Writing – original draft: JS.

Writing – review & editing: XC JY.

References

1. Franchi F, Angiolillo DJ (2015) Novel antiplatelet agents in acute coronary syndrome. Nat Rev Cardiol 12: 30–47. https://doi.org/10.1038/nrcardio.2014.156 PMID: 25286881
2. Jennings LK (2009) Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thromb Haemost 102: 248–257. https://doi.org/10.1160/TH09-03-0192 PMID: 19652875
3. Wijns W, Kolh P, Danchin N, Di Mario C, Falk V, Folliguet T, et al. (2010) Guidelines on myocardial revascularization. Eur Heart J 31: 2501–2555. https://doi.org/10.1093/eurheartj/ehq277 PMID: 20802248
4. Anderson JL, Adams CD, Antman EM, Bridges CR, Califf RM, Casey DE Jr., et al. (2013) 2012 ACCF/AHA focused update incorporated into the ACCF/AHA 2007 guidelines for the management of patients with unstable angina/non-ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation 127: e663–828. https://doi.org/10.1161/CIR.0b013e31828478ac PMID: 23630129
5. Udell JA, Bonaca MP, Collet JP, Lincoff AM, Kereiakes DJ, Costa F, et al. (2016) Long-term dual antiplatelet therapy for secondary prevention of cardiovascular events in the subgroup of patients with previous myocardial infarction: a collaborative meta-analysis of randomized trials. Eur Heart J 37: 390–399. https://doi.org/10.1093/eurheartj/ehv443 PMID: 26324537
6. Hochholzer W, Trenk D, Bestehorn HP, Fischer B, Valina CM, Ferenc M, et al. (2006) Impact of the degree of peri-interventional platelet inhibition after loading with clopidogrel on early clinical outcome of elective coronary stent placement. J Am Coll Cardiol 48: 1742–1750. https://doi.org/10.1016/j.jacc.2006.06.065 PMID: 17084243
7. Nguyen TA, Diodati JG, Pharand C (2005) Resistance to clopidogrel: a review of the evidence. J Am Coll Cardiol 45: 1157–1164. https://doi.org/10.1016/j.jacc.2005.01.034 PMID: 15837243
8. Kubica A, Kasprzak M, Siller-Matula J, Kozinski M, Pio Navarese E, Obonska K, et al. (2014) Time-related changes in determinants of antiplatelet effect of clopidogrel in patients after myocardial infarction. Eur J Pharmacol 742: 47–54. https://doi.org/10.1016/j.ejphar.2014.08.009 PMID: 25199965
9. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. (2007) Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 357: 2001–2015. https://doi.org/10.1056/NEJMoa0706482 PMID: 17982182
10. Wiviott SD, Steg PG (2015) Clinical evidence for oral antiplatelet therapy in acute coronary syndromes. Lancet 386: 292–302. https://doi.org/10.1016/S0140-6736(15)60213-6 PMID: 25777663
11. Alexopoulos D (2012) Prasugrel resistance: fact or fiction. Platelets 23: 83–90. https://doi.org/10.3109/09537104.2011.600478 PMID: 21787175

12. Fernando H, Dart AM, Peter K, Shaw JA (2011) Proton pump inhibitors, genetic polymorphisms and response to clopidogrel therapy. Thromb Haemost 105: 933–944. https://doi.org/10.1160/TH10-11-0715 PMID: 21544314

13. Cayla G, Hulot JS, O'Connor SA, Pathak A, Scott SA, Gruel Y, et al. (2011) Clinical, angiographic, and genetic factors associated with early coronary stent thrombosis. JAMA 306: 1765–1774. https://doi.org/10.1001/jama.2011.1529 PMID: 22028352

14. Campo G, Fileti L, Valgimigli M, Tebaldi M, Cangiano E, Cavazza C, et al. (2010) Poor response to clopidogrel: current and future options for its management. J Thromb Thrombolysis 30: 319–331. https://doi.org/10.1007/s11239-010-0457-5 PMID: 20157839

15. Sangkuhl K, Klein TE, Altman RB (2010) Clopidogrel pathway. Pharmacogenet Genomics 20: 463–465. https://doi.org/10.1097/FPC.0b013e3283385420 PMID: 20440227

16. Beitelshees AL, Voora D, Lewis JP (2015) Personalized antiplatelet and anticoagulation therapy: applications and significance of pharmacogenomics. Pharmgenomics Pers Med 8: 43–61. https://doi.org/10.2147/PGPM.S52900 PMID: 25897256

17. Gros P, Ben Neriah YB, Croop JM, Housman DE (1986) Isolation and expression of a complementary DNA that confers multidrug resistance. Nature 323: 728–731. https://doi.org/10.1038/323728a0 PMID: 3022150

18. Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N, et al. (2009) Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 360: 363–375. https://doi.org/10.1056/NEJMoa0808227 PMID: 19106083

19. Karazniewicz-Lada M, Danielak D, Rubis B, Burchardt P, Komosa A, Lesiak M, et al. (2015) Impact of common ABCB1 polymorphism on pharmacokinetics and pharmacodynamics of clopidogrel and its metabolites. J Clin Pharm Ther 40: 226–231. https://doi.org/10.1111/jcpt.12236 PMID: 25430046

20. Matetzky S, Shenkman B, Guetta V, Shechter M, Beinart R, Goldenberg I, et al. (2004) Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation 109: 3171–3175. https://doi.org/10.1161/01.CIR.0000130846.46168.03 PMID: 15184279

21. Kim IS, Jeong YH, Kang MK, Koh JS, Park Y, Hwang SJ, et al. (2010) Correlation of high post-treatment platelet reactivity assessed by light transmittance aggregometry and the VerifyNow P2Y12 assay. J Thromb Thrombolysis 30: 486–495. https://doi.org/10.1007/s11239-010-0484-2 PMID: 20449634

22. Marcucci R, Gori AM, Paniccia R, Giusti B, Valente S, Giglioli C, et al. (2009) Cardiovascular death and nonfatal myocardial infarction in acute coronary syndrome patients receiving coronary stenting are predicted by residual platelet reactivity to ADP detected by a point-of-care assay: a 12-month follow-up. Circulation 119: 237–242. https://doi.org/10.1161/CIRCULATIONAHA.108.812636 PMID: 19118249

23. Mikeska T, Bock C, El-Maarri O, Hubner A, Ehrentraut D, Schramm J, et al. (2007) Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. J Mol Diagn 9: 368–381. https://doi.org/10.2353/jmoldx.2007.060167 PMID: 17591937

24. Su J, Li X, Yu Q, Liu Y, Wang Y, Song H, et al. (2014) Association of P2Y12 gene promoter DNA methylation with the risk of clopidogrel resistance in coronary artery disease patients. Biomed Res Int 2014: 450814. https://doi.org/10.1155/2014/450814 PMID: 24745016

25. Jakubowski JA, Payne CD, Li YG, Brandt JT, Small DS, Farid NA, et al. (2008) The use of the VerifyNow P2Y12 point-of-care device to monitor platelet function across a range of P2Y12 inhibition levels following prasugrel and clopidogrel administration. Thromb Haemost 99: 409–415. https://doi.org/10.1160/TH07-09-0575 PMID: 18278193

26. Fojo A, Lebo R, Shimizu N, Chin JE, Roninson IB, Merlino GT, et al. (1986) Localization of multidrug resistance-associated DNA sequences to human chromosome 7. Somat Cell Mol Genet 12: 415–420. PMID: 3016920

27. Brambila-Tapia AJ (2013) MDR1 (ABCB1) polymorphisms: functional effects and clinical implications. Rev Invest Clin 65: 445–454. PMID: 24687344

28. Abuhaliema AM, Yousef AM, El-Madany NN, Bulatova NR, Awwad NM, Yousef MA, et al. (2016) Influence of Genotype and Haplotype of MDR1 (C3435T, G2677A/T, C1236T) on the Incidence of Breast Cancer—a Case-Control Study in Jordan. Asian Pac J Cancer Prev 17: 261–266. PMID: 26838221

29. Zhang Z, Li Y, Liu H, Shi J, Li X, Jiang W (2015) ABCB1 polymorphisms associated with osteonecrosis of the femeral head. Int J Clin Exp Pathol 8: 15240–15244. PMID: 26823873

30. Yin G, Xiao Z, Ni Y, Qu X, Wu H, Lu H, et al. (2016) Association of MDR1 single-nucleotide polymorphisms and haplotype variants with multiple myeloma in Chinese Jiangsu Han population. Tumour Biol.

31. Huang Z, Zhang S, Shen Y, Liu W, Long J, Zhou S (2016) Influence of MDR1 methylation on the curative effect of interventional embolism chemotherapy for cervical cancer. Ther Clin Risk Manag 12: 217–223. https://doi.org/10.2147/TCRM.S95453 PMID: 26929635

32. Spiewak M, Malek LA, Kostrzewa G, Kisiel B, Serafin A, Filipiak KJ, et al. (2009) Influence of C3435T multidrug resistance gene-1 (MDR-1) polymorphism on platelet reactivity and prognosis in patients with acute coronary syndromes. Kardiol Pol 67: 827–834. PMID: 19784880

33. Su J, Xu J, Li X, Zhang H, Hu J, Fang R, et al. (2012) ABCB1 C3435T polymorphism and response to clopidogrel treatment in coronary artery disease (CAD) patients: a meta-analysis. PLoS One 7: e46366. https://doi.org/10.1371/journal.pone.0046366 PMID: 23056288

34. Park MW, Her SH, Kim CJ, SunCho J, Park GM, Kim TS, et al. (2016) Evaluation of the incremental prognostic value of the combination of CYP2C19 poor metabolizer status and ABCB1 3435 TT polymorphism over conventional risk factors for cardiovascular events after drug-eluting stent implantation in East Asians. Genet Med 18: 833–841. https://doi.org/10.1038/gim.2015.171 PMID: 26699760

35. Malek LA, Kisiel B, Spiewak M, Grabowski M, Filipiak KJ, Kostrzewa G, et al. (2008) Coexisting polymorphisms of P2Y12 and CYP2C19 genes as a risk factor for persistent platelet activation with clopidogrel. Circ J 72: 1165–1169. PMID: 18577829

36. Chiu SN, Wu MH, Su MJ, Wang JK, Lin MT, Chang CC, et al. (2012) Coexisting mutations/polymorphisms of the long QT syndrome genes in patients with repaired Tetralogy of Fallot are associated with the risks of life-threatening events. Hum Genet 131: 1295–1304. https://doi.org/10.1007/s00439-012-1156-4 PMID: 22407026

37. Razin A, Webb C, Szyf M, Yisraeli J, Rosenthal A, Naveh-Many T, et al. (1984) Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. Proc Natl Acad Sci U S A 81: 2275–2279. PMID: 6585800

38. Morita S, Takahashi RU, Yamashita R, Toyoda A, Horii T, Kimura M, et al. (2012) Genome-wide analysis of DNA methylation and expression of microRNAs in breast cancer cells. Int J Mol Sci 13: 8259–8272. https://doi.org/10.3390/ijms13078259 PMID: 22942701

39. Legendre CR, Demeure MJ, Whitsett TG, Gooden GC, Bussey KJ, Jung S, et al. (2016) Pathway Implications of Aberrant Global Methylation in Adrenocortical Cancer. PLoS One 11: e0150629. https://doi.org/10.1371/journal.pone.0150629 PMID: 26963385

40. Wang Y, Zhang J, Xiao X, Liu H, Wang F, Li S, et al. (2016) The identification of age-associated cancer markers by an integrative analysis of dynamic DNA methylation changes. Sci Rep 6: 22722. https://doi.org/10.1038/srep22722 PMID: 26949191

41. Ramkaran P, Phulukdaree A, Khan S, Moodley D, Chuturgoon AA (2015) Methylenetetrahydrofolate reductase C677T polymorphism is associated with increased risk of coronary artery disease in young South African Indians. Gene 571: 28–32. https://doi.org/10.1016/j.gene.2015.06.044 PMID: 26095803

42. Matrisciano F, Panaccione I, Grayson DR, Nicoletti F, Guidotti A (2016) Metabotropic Glutamate 2/3 Receptors and Epigenetic Modifications in Psychotic Disorders: A Review. Curr Neuropharmacol 14: 41–47. https://doi.org/10.2174/1570159X13666150713174242 PMID: 26813121

43. Yang J, Zhou JS, Zhao YX, Yang ZH, Zhao HD, Zhang YD, et al. (2015) ABCB1 hypomethylation is associated with decreased antiplatelet effects of clopidogrel in Chinese ischemic stroke patients. Pharmazie 70: 97–102. PMID: 25997249

44. Spiliopoulos S, Pastromas G (2015) Current status of high on-treatment platelet reactivity in patients with coronary or peripheral arterial disease: Mechanisms, evaluation and clinical implications. World J Cardiol 7: 912–921. https://doi.org/10.4330/wjc.v7.i12.912 PMID: 26730297

45. Aradi D, Storey RF, Komocsi A, Trenk D, Gulba D, Kiss RG, et al. (2014) Expert position paper on the role of platelet function testing in patients undergoing percutaneous coronary intervention. Eur Heart J 35: 209–215. https://doi.org/10.1093/eurheartj/eht375 PMID: 24067509

46. Gremmel T, Steiner S, Seidinger D, Koppensteiner R, Panzer S, Kopp CW (2010) Adenosine diphosphate-inducible platelet reactivity shows a pronounced age dependency in the initial phase of antiplatelet therapy with clopidogrel. J Thromb Haemost 8: 37–42. https://doi.org/10.1111/j.1538-7836.2009.03644.x PMID: 19818001

47. Tan LA, Keigher KM, Munich SA, Moftakhar R, Lopes DK (2015) Thromboembolic complications with Pipeline Embolization Device placement: impact of procedure time, number of stents and pre-procedure P2Y12 reaction unit (PRU) value. J Neurointerv Surg 7: 217–221. https://doi.org/10.1136/neurintsurg-2014-011111 PMID: 24553344

48. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Sabate M, Jimenez-Quevedo P, et al. (2005) Platelet function profiles in patients with type 2 diabetes and coronary artery disease on combined aspirin and clopidogrel treatment. Diabetes 54: 2430–2435. PMID: 16046311

49. Schiffrin EL, Lipman ML, Mann JF (2007) Chronic kidney disease: effects on the cardiovascular system. Circulation 116: 85–97. https://doi.org/10.1161/CIRCULATIONAHA.106.678342 PMID: 17606856

50. Hochholzer W, Trenk D, Fromm MF, Valina CM, Stratz C, Bestehorn HP, et al. (2010) Impact of cytochrome P450 2C19 loss-of-function polymorphism and of major demographic characteristics on residual platelet function after loading and maintenance treatment with clopidogrel in patients undergoing

elective coronary stent placement. J Am Coll Cardiol 55: 2427–2434. https://doi.org/10.1016/j.jacc.2010.02.031 PMID: 20510210

51. Hermans MP, Fruchart JC (2011) Reducing vascular events risk in patients with dyslipidaemia: an update for clinicians. Ther Adv Chronic Dis 2: 307–323. https://doi.org/10.1177/2040622311413952 PMID: 23251757

52. Blazing MA, Giugliano RP, Cannon CP, Musliner TA, Tershakovec AM, White JA, et al. (2014) Evaluating cardiovascular event reduction with ezetimibe as an adjunct to simvastatin in 18,144 patients after acute coronary syndromes: final baseline characteristics of the IMPROVE-IT study population. Am Heart J 168: 205–212 e201. https://doi.org/10.1016/j.ahj.2014.05.004 PMID: 25066560

53. Su J, Xu H, Yang J, Yu Q, Yang S, Zhang J, et al. (2015) ABCB1 C3435T polymorphism and the lipid-lowering response in hypercholesterolemic patients on statins: a meta-analysis. Lipids Health Dis 14: 122. https://doi.org/10.1186/s12944-015-0114-2 PMID: 26438079

54. Fontana P, Berdague P, Castelli C, Nolli S, Barazer I, Fabbro-Peray P, et al. (2010) Clinical predictors of dual aspirin and clopidogrel poor responsiveness in stable cardiovascular patients from the ADRIE study. J Thromb Haemost 8: 2614–2623. https://doi.org/10.1111/j.1538-7836.2010.04063.x PMID: 20860677

55. Geisler T, Grass D, Bigalke B, Stellos K, Drosch T, Dietz K, et al. (2008) The Residual Platelet Aggregation after Deployment of Intracoronary Stent (PREDICT) score. J Thromb Haemost 6: 54–61. https://doi.org/10.1111/j.1538-7836.2007.02812.x PMID: 17949474

56. Teng R (2012) Pharmacokinetic, pharmacodynamic and pharmacogenetic profile of the oral antiplatelet agent ticagrelor. Clin Pharmacokinet 51: 305–318. https://doi.org/10.2165/11630960-000000000-00000 PMID: 22489610