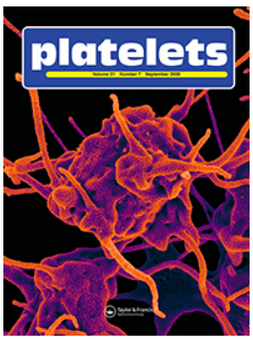

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microRNA-605 rs2043556 polymorphisms affect clopidogrel therapy through modulation of CYP2B6 and P2RY12 in acute coronary syndrome patients

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Abstract

Clopidogrel therapy reduces the occurrence of major vascular events in acute coronary syndrome (ACS) patients, but treatment efficacy is variable. The present study aims to determine the mechanisms that underlie associations between certain miRNA polymorphisms and clinical outcomes of clopidogrel therapy. Our study focused on 9 miRNA single nucleotide polymorphisms in addition to *CYP2C19*2* and *CYP2C19*3*. We found that the *miR-605* rs2043556 AG genotype significantly decreased the risk of acute myocardial infarction (odds ratio, OR = 0.13, 95%CI 0.02–0.96, $P = .045$) and that the rs2043556 GG genotype significantly decreased the risk of unstable angina (OR = 0.19, 95%CI 0.05–0.65, $P = .008$) in ACS patients receiving clopidogrel therapy for more than one year. Dual-luciferase analysis indicated that miR-605 significantly decreased the mRNA expression of CYP2B6 and P2RY12 ($P < .01$). In cells treated with miR-605-A, the protein and mRNA expression of CYP2B6 and P2RY12 were significantly lower than that of cells treated with miR-605-G ($P < .05$). The results demonstrate that miR-605 targets the mRNA of the CYP2B6 and P2RY12 genes, and that *rs2043556* A/G polymorphisms in miR-605 modulate the mRNA and protein expression of CYP2B6 and P2RY12 differently, which may impact the effect of clopidogrel in ACS patients.

Keywords

Acute coronary syndrome, clopidogrel, CYP2B6, miRNA-605, P2RY12, single nucleotide polymorphism

History

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Introduction

Clopidogrel is one of the most commonly prescribed drugs in patients suffering from acute coronary syndrome (ACS) [1,2]. The pathophysiology of ACS mainly involves platelet activation and aggregation [3,4]. Clopidogrel significantly inhibits platelet function and reduces the risk of major adverse cardiovascular events in ACS patients [1,5]. However, some recipients still experience recurrent ischemic events [6,7].

Clopidogrel is an inactive prodrug that is absorbed through ABCB1 intestinal transporters and activated to the thiol-containing metabolite primarily by CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4, and CYP3A5 in a two-step process in the liver [8,9]. The active metabolite irreversibly binds to P2RY12 receptor on the platelet membrane and effectively inhibits platelet activation. However, an early report showed that approximately 4–30% of patients receiving conventional doses of clopidogrel displayed a poor antiplatelet response [10]. Subsequently, a series of studies focused on the important role of genetic polymorphisms in *ABCB1, CYP450* and *P2RY12* in differential clopidogrel efficacies and discovered that the loss-of-function (LOF) single nucleotide polymorphisms (SNPs), *CYP2C19*2* and *CYP2C19*3*, could cause a reduction in plasma active clopidogrel metabolite levels and reduce the antiplatelet effect of clopidogrel [6,7,11,12]. However, the Pharmacogenomics of Antiplatelet Intervention study revealed that *CYP2C19* polymorphisms are only responsible for about 12% of the between-subject variability in the response to clopidogrel treatment [13]. Thus, the full scope of the underlying mechanisms for the high inter-individual variability of clopidogrel remains to be elucidated.

miRNAs are a class of endogenous small non-coding RNAs approximately 22 nucleotides in length, which have emerged as key regulators of fundamental biological processes owing to their ability to regulate over 30% of protein coding genes [14,15]. Mature miRNAs target genes by primarily binding the mRNA 3′-untranslated region (3′-UTR) and inhibiting mRNA translation or by degrading mRNAs [16]. A single miRNA can bind to 3′-UTR of many mRNAs [17]. SNPs in miRNAs potentially change miRNA function or affect miRNA-mRNA interaction [18,19]. Detailed studies have reported that several miRNAs can be used as diagnostic markers in CAD [20,21], and that miR-2355 and miR-4665 polymorphisms are significantly associated with cerebrovascular events and acute myocardial infarction (AMI) in ACS patients during clopidogrel treatment [22]. This indicates that

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/iplt.

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analysis of miRNAs and their polymorphisms could be used to assess individual responses to the antiplatelet drug, clopidogrel.

After performing *in silico* analysis to identify miRNAs targeting *ABCB1, CES1, CYP2C19, CYP2B6, CYP2C9, CYP1A2, CYP3A4, CYP1A2* and *P2RY12*, as well as checking whether miRNA variants are present in Chinese populations, in which effects of miRNA polymorphisms on clopidogrel efficacy are unknown, we hypothesized that certain miRNA polymorphisms are associated with differential clinical outcomes of clopidogrel therapy in ACS patients. Furthermore, we hypothesized that this association is based on differential miRNA polymorphisms modulation of target gene expression at the protein and mRNA levels.

Materials and Methods

Study Population

This study was conducted in two clinical centers: Xiangya Hospital of Central South University (Changsha, China) and the Shi-jing-shan Institute of Hypertension (Beijing, China). In each participating center, two cardiologists were responsible for the collection of data from all patients. miRNA target genes were predicted using TargetScan, miRBase, Pictar, and miRDB. miRNA SNPs were selected according to the National Center for Biotechchnology Information (NCBI). The minor allele frequencies (MAFs) of these SNPs were > 0.05 in the Han Chinese population in Beijing (CHB) and Southern Han Chinese population (CHS) (except *CYP2C19*3*).

A total of 567 ACS patients receiving clopidogrel for more than one year were recruited from the afore-mentioned two centers between January 2011 and July 2015, and clinical follow-up data were fully obtained in July 2016. The following patients were excluded from the study: (1) patients with contraindications to clopidogrel therapy, (2) patients who had been noncompliant with their clopidogrel treatment regimens for ≥12 months, (3) patients undergoing treatment with multiple anticoagulants simultaneously, and (4) patients with severe hepatic or renal dysfunction. The clinical diagnosis of ACS was based on the ACC/AHA guidelines for the diagnosis and treatment of ACS, including unstable angina (UA), acute ST-segment elevation myocardial infarction (STEMI) and acute non-ST-segment elevation myocardial infarction (NSTEMI) [23]. Clinical outcomes included UA, AMI, stent thrombosis (ST), death and bleeding events.

This study complied with the Declaration of Helsinki and was approved by the institutional review boards of the participating institutions. Informed consent was obtained in writing from the patients (age range, 27–90 years, 417 male and 150 female) or their parents or guardians, as appropriate (ChiCTR-PN -15006260).

Genotyping

Genomic DNA was extracted from 3 mL peripheral blood samples with commercially available kits (Omega BioTek, Norcross, Georgia, US) in accordance with the manufacturer's instructions. Genotyping was performed using the Sequenom MassArray. Genomic DNA of all participants was subjected to genotyping for 9 miRNA SNPs in addition to *CYP2C19*2* (rs4244285) and *CYP2C19*3* (rs4986893). The call rates of the 11 SNPs, i.e., *miR-7515* rs10192411, *miR-2053* rs10505168, *miR-3612* rs1683709, *miR-605* rs2043556, *miR-499b* rs2070960, *miR-4482* rs45596840, *miR-4268* rs4674470, *miR-7157* rs56148568, *miR-5186* rs9842591, *CYP2C19*2* rs4244285, and *CYP2C19*3* rs4986893, were greater than 97%. SNP quality control procedures were performed based on the call rate and minor allele frequency.

Cell Culture

The human hepatocyte cell line LO2 was obtained from Zhong Qiao Xin Zhou company (Shanghai, China), HEK-293T cells were purchased from the Chinese Academy of Sciences (Shanghai, China) and human aortic smooth muscle cells (HASMC) were obtained from Bei Na Institute (Beijing, China). Cells were cultured in complete growth medium, which is composed of Dulbecco′s Modied Eagle′s Medium (DMEM, Gibico, Waltham, Massachusetts, USA) supplemented with 10% Fetal Bovine Serum (FBS,Zhejiang Tianhang Biotechnology, Hangzhou, China) and 1% Penicillin/Streptomycin (Solarbio Science and Technology company, Beijing, China). All cell lines were kept in a 37°C incubator with 5% CO_2. 5×10^4 HEK293T cells were seeded in 24-well plates.

Dual Luciferase Reporter Gene Assay

The direct targets of miR-605 were predicted using the biological prediction websites TargetScan, miRbase, Pictar, and miRDB. The relationships between miR-605 and CYP2B6, ABCB1, and P2RY12 were further verified using dual luciferase reporter gene analysis. The mRNA 3′-untranslated region (3′-UTR) of CYP2B6, ABCB1 and P2RY12 were subcloned into SpeI/HindIII sites of pMIR-REPORT (Omega, Norcross, Georgia, USA). HEK293T cells were cultured in 24-well plates at a density of 10^6 cells/well overnight. Each group was treated in triplicate. Each tube was transfected with 200 ng of a recombinant plasmid (miR-605 mimic and pMIR-REPORT), or with 80 ng of the transfected inner reference plasmid pRL-TK (Promega, USA). Lastly, 80 nM miRNA mimic negative control was transfected (miRNA mimic NC) by Attractene Transfection Reagent (Invitrogen, Waltham, Massachusetts, USA). Luciferase activity was measured using the dual luciferase reporter assay system (Omega, Norcross, Georgia, USA) 24 h after transfection. In the darkroom, 10 μL of lysate was taken, and 50 μL of Deual-Glo-luciferase Reagent and Dual-Glo-stop & Glo Regent were added separately. After 24 h, the inhibition of miR-605 was quantified as the ratio of firefly luciferase activity to renilla luciferase activity in each well using a GloMax 20/20 fluorescence detector. The ratio (fluorescent expression) represents the binding activity of miRNA to a given 3′-UTR.

Quantitative Real-Time PCR (qRT-PCR)

According to the manufacturer's guidelines, total RNA was extracted from cultured cells using Trizol reagent (Thermo Fisher, Waltham, Massachusetts, USA). RNA purity was evaluated based on the A260/A280 ratio. Then, RNA was reverse transcribed using the All-in-one™ First-Strand cDNA Synthesis Kit (Guangzhou, China). Expression of mRNAs was normalized in each sample to GAPDH, while the expression of miRNAs was normalized in each sample to U6. Amplification was performed with the SYBR green quantitative Real-Time PCR Mix (Guangzhou, China) on a Real-Time PCR Detection System (Applied, USA). Data were collected and analyzed using Bio-Rad software and the $2^{-\triangle\triangle Ct}$ method for qualification of the relative expression levels of miRNAs and mRNAs (CYP2B6 and P2RY12). The primers used in this study were as follows:

Western Blotting

RIPA lysis buffer (Beyotime, Shanghai, China) was used to extract the whole protein from LO2 and HASMC cells. Proteins were quantified using the BCA Protein Assay Kit (Beyotime,

Shanghai, China). Next, 20 μg protein was separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein was transferred to a PVDF membrane (Millipore, USA) followed by blocking with 5% skim milk. Then the membrane was incubated with the primary antibody at 4°C overnight. After washing three times (10 min each), the membrane was incubated with secondary antibody for 1 h at room temperature. After washing three times with 1 × TBST (10 min each), the proteins were visualized using the ECL Plus detection system (Geneview, USA). The protein signal was quantified using Image Lab™ Software (Bio-Rad, USA).

Statistical Analysis

Continuous data are described as mean ± standard deviation (SD), whereas discrete data are expressed as percentages. Five clinical outcomes including UA, AMI, stent thrombosis (STs), death, and bleeding events were considered to evaluate the response of clopidogrel. Chi-squared/Fisher's exact tests and Student's *t*-tests were performed to assess differences in the distributions of various clinical variables and associations among various genotypes or alleles for the afore-mentioned five clinical outcomes. The associations between *miR-605*

Table I. Clinical characteristics and outcomes of Chinese acute coronary syndrome patients.

Characteristics no. (%)	AMI	Stent thrombosis	UA	Bleeding events	Death
Gender					
Male (417)	26 (6.2)	11 (2.6)	186 (44.6)	38 (9.1)	5 (1.2)
Female (150)	2 (1.3)	3 (2.0)	71 (47.3)	7 (4.7)	4 (2.7)
P	0.015*	1	0.565	0.084	0.254
Age (years)					
Mean±SEM	65.00 ± 12.04	62.50 ± 10.24	64.33 ± 11.98	60.20 ± 11.72	74.89 ± 8.02
<75 (463)	21 (4.5)	12 (2.6)	197 (42.5)	36 (7.8)	3 (0.6)
≥75 (104)	7 (6.7)	2 (1.9)	60 (57.7)	9 (8.7)	6 (5.8)
P	0.350	1	0.005*	0.765	0.002*
Smoking					
Yes (281)	13 (4.6)	9 (3.2)	116 (41.3)	24 (8.5)	3 (1.1)
No (286)	15 (5.2)	5 (1.7)	141 (49.3)	23 (7.3)	6 (2.1)
P	0.734	0.264	0.055	0.598	0.504
Hypertension					
Yes (405)	21 (5.2)	9 (2.2)	195 (48.1)	28 (6.9)	5 (1.2)
No (162)	7 (4.3)	5 (3.1)	62 (38.3)	17 (10.5)	4 (2.5)
P	0.668	0.549	0.033*	0.154	0.284
Diabetes mellitus					
Yes (183)	9 (4.9)	3 (1.6)	80 (43.7)	16 (8.7)	3 (1.6)
No (384)	19 (4.9)	11 (2.9)	177 (46.1)	29 (7.6)	6 (1.6)
P	0.988	0.564	0.595	0.624	1
Hyperlipidemia					
Yes (304)	19 (8.2)	5 (2.2)	128 (42.1)	18 (7.8)	3 (1.3)
No (263)	8 (3.0)	9 (2.7)	129 (49.0)	27 (8.0)	6 (1.8)
P	0.053	0.698	0.098	0.726	0.648
PCI					
Yes (522)	27 (5.2)	14 (2.7)	232 (44.4)	41 (7.9)	6 (1.1)
No (45)	1 (2.2)	0 (0)	25 (55.6)	4 (8.9)	3 (6.7)
P	0.716	0.617	0.151	0.773	0.028*
Statin—no. (%)					
Yes (540)	26 (4.8)	12 (2.2)	245 (45.4)	42 (7.8)	9 (1.7)
No (27)	2 (7.4)	2 (7.4)	12 (44.4)	3 (11.1)	0 (0)
P value	0.637	0.140	0.925	0.465	1
Beta-blocker—no. (%)					
Yes (470)	26 (5.5)	10 (2.1)	212 (45.1)	34 (7.2)	7 (1.5)
No (97)	2 (2.1)	4 (4.1)	45 (46.4)	11 (11.3)	2 (2.1)
P value	0.200	0.275	0.824	0.173	0.655
ACEI or ARB—no. (%)					
Yes (357)	22 (6.2)	9 (2.5)	165 (46.2)	14 (3.9)	4 (1.1)
No (210)	6 (2.9)	5 (2.4)	92 (43.8)	31 (14.8)	5 (2.4)
P value	0.079	0.917	0.578	<0.001*	0.302
CCB—no. (%)					
Yes (251)	13 (5.2)	3 (1.2)	126 (50.2)	19 (7.6)	1 (0.4)
No (316)	15 (4.7)	11 (3.5)	131 (41.5)	26 (8.2)	8 (2.5)
P value	0.813	0.104	0.038*	0.773	0.049*
PPI—no. (%)					
Yes (68)	2 (2.9)	4 (5.9)	33 (48.5)	8 (11.8)	1 (1.5)
No (499)	26 (5.2)	10 (2.0)	224 (44.9)	37 (7.4)	8 (1.6)
P value	0.561	0.075	0.572	0.213	1

*: $P < 0.05$

AMI: acute myocardial infarction; UA: unstable angina; ARB: angiotensin-receptor blocker; ACEI: angiotensin-converting enzyme inhibitor; PPI: proton-pump inhibitor; CCB: calcium channel blocker. PCI: percutaneous coronary intervention.

Table II. Distributions of specific miRNA genotypes among patients experiencing different clinical outcomes after one year of clopidogrel treatment.

Genes	AMI	Stent thrombosis	UA	Bleeding events	Death
miR-605 rs2043556 -no. (%)					
AA (428)	26 (6.1)	9 (2.1)	209 (48.8)	31 (7.3)	6 (1.4)
AG (109)	1 (0.9)	5 (4.6)	42 (38.5)	12 (11.0)	3 (2.8)
GG (20)	0 (0)	0 (0)	3 (15.0)	2 (10.0)	0 (0)
$P_{genotype}$	0.043*	0.288	0.003*	0.311	0.567
A (965)	53 (5.5)	23 (2.4)	460 (47.7)	74 (7.7)	15 (1.6)
G (149)	1 (0.7)	5 (3.4)	48 (32.2)	16 (10.7)	3 (2.0)
P_{allele}	0.010*	0.483	<0.001*	0.203	0.703
*CYP2C19*2*(rs4244285) -no. (%)					
GG (278)	10 (3.6)	7 (2.5)	127 (45.7)	19 (6.8)	3 (1.1)
GA (234)	12 (5.1)	5 (2.1)	101 (43.2)	21 (9.0)	6 (2.6)
AA (48)	6 (12.5)	2 (4.2)	25 (52.1)	4 (8.3)	0 (0)
$P_{genotype}$	0.033*	0.657	0.513	0.610	0.372
G (790)	32 (4.1)	19 (2.4)	355 (44.9)	59 (7.5)	12 (1.5)
A (330)	24 (7.3)	9 (2.7)	151 (45.8)	29 (8.8)	6 (1.8)
P_{allele}	0.043*	0.764	0.725	0.471	0.726
*CYP2C19*3*(rs4986893) -no. (%)					
GG (525)	26 (5.0)	10 (1.9)	236 (45)	40 (7.6)	9 (1.7)
GA (41)	2 (4.9)	3 (7.3)	20 (48.8)	5 (12.2)	0 (0)
AA (1)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
$P_{genotype}$	1.00	0.002*	0.619	0.411	1.00
G (1091)	54 (4.9)	23 (2.1)	492 (45.1)	85 (7.8)	18 (1.6)
A (43)	2 (4.7)	5 (11.6)	22 (51.2)	5 (11.6)	0 (0)
P_{allele}	0.262	<0.001*	0.433	0.361	0.996

*: $P < 0.05$
AMI: acute myocardial infarction; UA: unstable angina.

rs2043556, *CYP2C19*2*, and *CYP2C19*3* polymorphisms and clinical outcomes were investigated through multivariable logistic regression analysis by adjusting the baseline statistically significant characteristics. Multiple comparisons over two groups were performed using a one-way ANOVA followed by a Tukey post hoc test or Bonferroni post hoc analysis. Statistical analysis was performed using the Statistical Package for Social Sciences, version 22.0 (SPSS, Inc., Somers, NY, USA). A two-tailed *P* value <.05 was considered significant.

Results

Clinical Characteristics and Outcomes

The demographic and clinical characteristics of the genotyped patients are presented in Table I. A total of 567 patients (417 [73.5%] male, 150 [26.5%] female; median age, 63 years [range, 27–90 years]) were included. Of the total, 92.1% underwent percutaneous coronary intervention (PCI) with stent placement. Most patients enrolled herein received long-term treatment with β-blockers, statins, and angiotensin converting enzyme inhibitors or angiotensin II receptor blockers. A total of 44.3% patients received calcium channel blockers and approximately 12% of patients received proton-pump inhibitors intermittently.

In total, 28 patients (4.9%) presented with AMI, 14 (2.3%) developed ST, 257 (45.3%) had UA, 45 (7.9%) experienced bleeding events, and 9 (1.6%) died.

Distribution and Frequency of Genotyping

Eleven SNPs were genotyped by using Sequenom's MassARRAY system. Among them, 8 miRNA SNPs were not significantly correlated with clopidogrel efficacy (i.e., mitigation of UA, AMI, stent thrombosis (ST), death and bleeding events) in ACS patients. Minor allele frequencies for *miR-605* rs2043556 G, *CYP2C19*2* rs4244285 A, and *CYP2C19*3* rs4986893 A were 0.134, 0.295, and 0.038, respectively.

As shown in Table II, *miR-605* rs2043556 was significantly associated with AMI in the study at the genotypic ($P = .043$) and allelic ($P = .010$) levels. *CYP2C19*2* rs4244285 was also significantly associated with AMI at the genotypic ($P = .033$) and allelic levels ($P = .043$). *CYP2C19*3* rs4986893 was significantly associated with ST at the genotypic ($P = .002$) and allelic ($P < .001$) levels.

To further investigate the association between the three SNPs (*miR-605* rs2043556, *CYP2C19*2* rs4244285, *CYP2C19*3* rs4986893) and four clinical outcomes in patients receiving one year of clopidogrel therapy, we performed multivariable logistic regression analysis. As shown in Table III, no patients with *miR-605* rs2043556 GG genotype suffered from AMI and no patients with the *CYP2C19*3* rs4986893 AA genotype suffered from any of the clinical outcomes assessed in this study. The *miR-605* rs2043556 AG genotype significantly decreased the risk of AMI (odds ratio (OR) = 0.13, 95% confidence interval (CI) 0.02–0.96, $P = .045$; *miR-605* rs2043556 GG genotype, UA (OR = 0.19, 95%CI 0.05–0.65, $P = .008$). Furthermore, the *CYP2C19*2* rs4244285 AA genotype significantly increased the risk of AMI (OR = 3.69, 95%CI 1.26–10.79, $P = .017$). The *CYP2C19*3* rs4986893 GA genotype increased the risk of ST (OR = 4.07, 95% CI 1.07–15.40, $P = .039$). Among patients bearing the two *CYP2C19* LOF alleles, the incidence of ST was significantly increased compared to those bearing only one LOF allele and two non-carriers (7.7% *vs*. 1.6% *vs*. 2.0%, respectively; $P = .033$) during one year of clopidogrel treatment (Table IV). The risk of AMI (OR = 3.27; 95%CI 1.16–9.22; $P = .025$) and STs (OR = 4.12; 95%CI 1.16–14.68; $P = .029$) were significantly increased in patients bearing the two LOF alleles.

The 3′-UTRs of CYP2B6 and P2RY12 mRNA are Targets of miR-605

As indicated in Figure 1(a,d), TargetScan predicted that miR-605-5p targets the 3′-UTR of *P2RY12* mRNA, *ABCB1* mRNA, and *CYP2B6* mRNA, while miR-605-3p targets the 3′-UTR of

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Table III. Associations between *miR-605* rs2043556, *CYP2C19*2* and *CYP2C19*3* polymorphisms and clinical outcomes after one year of clopidogrel treatment.

		AMI			Stent thrombosis			UA			Bleeding event		
Gene	Genotype	OR (95% CI)	*P*	*P'*	OR(95% CI)	*P*	*P'*	OR (95% CI)	*P*	*P'*	OR (95% CI)	*P*	*P'*
miR-605	AA		0.135	0.165		0.366	0.366		0.012	0.006		0.835	0.419
rs2043556	AG	0.13 (0.02–0.96)	0.045*	0.058	2.24 (0.74–6.8)	0.156	0.156	0.70 (0.45–1.10)	0.104	0.055	1.25(0.61–2.6)	0.552	0.552
	GG	-	-	-	-	-	-	0.19 (0.05–0.66)	0.009*	0.008*	1.13 (0.24–5.3)	0.873	0.873
*CYP2C19*2*	GG		0.052	0.045		0.720	0.720		0.611	0.514		0.559	0.665
rs4244285	GA	1.71 (0.70–4.17)	0.240	0.397	0.85 (0.27–2.70)	0.777	0.777	0.93 (0.65–1.34)	0.706	0.568	1.42 (0.73–2.7)	0.303	0.370
	AA	3.92 (1.30–11.81)	0.015*	0.013*	1.68 (0.34–8.36)	0.524	0.524	1.29 (0.69–2.41)	0.429	0.413	1.44 (0.46–4.5)	0.542	0.709
*CYP2C19*3*	GG		0.872	0.998		0.119	0.119		0.896	0.894		0.851	0.587
rs4986893	GA	1.51 (0.33–6.93)	0.600	0.951	4.07 (1.07–15.40)	0.039*	0.039*	1.167(0.61–2.24)	0.639	0.636	1.34 (0.49–3.7)	0.569	0.302
	AA	-	-	-	-	-	-	-	-	-	-	-	-

Analysis of the correlation of rs4244285 with AMI needing adjustment for gender and rs2043556, analysis of the correlation of rs2043556 with AMI needing adjustment for gender and rs4244285, and rs2043556 with UA needing adjustment for age, hypertension and CCB drug.
*: *P* or *P'* < 0.05; *P* Adjusted value, *P'* nominal value.

Table IV. Associations between *CYP2C19* loss-of-function alleles and clinical outcomes after one year of clopidogrel treatment.

CYP2C19 LOF alleles	Clinical outcomes	Occurrence no. (%)	*P*	OR	95% CI	*P'*
0 LOF	AMI	9 (3.6)	0.060			0.075
1 LOF		12 (4.9)		1.39	0.57–3.36	0.471
2 LOF		7 (10.8)		3.27	1.16–9.22	0.025*
0 LOF	Stent thrombosis	5 (2.0)	0.033*			0.032*
1 LOF		4 (1.6)		0.83	0.22–3.12	0.779
2 LOF		5 (7.7)		4.12	1.16–14.68	0.029*
0 LOF	UA	116 (46.0)	0.129			0.113
1 LOF		101 (41.6)		0.84	0.59–1.21	0.355
2 LOF		36 (55.4)		1.52	0.87–2.65	0.137
0 LOF	Bleeding events	18 (7.1)	0.364			0.375
1 LOF		18 (7.4)		1.03	0.52–2.04	0.929
2 LOF		8 (12.3)		1.82	0.75–4.40	0.186
0 LOF	Death	3 (1.2)	0.388			0.608
1 LOF		6 (2.5)		2.07	0.50–8.70	0.318
2 LOF		0		-	-	0.997

* *P* values were measured by Chi-squared/Fisher's exact tests; *P'* values were measured by the multivariable logistic regression analysis.
* 0 LOF (*CYP2C19**1/*1); 1 LOF (*CYP2C19**1/*2 or *1/*3); 2 LOF (*CYP2C19**2/*2 or *2/*3 or *3/*3).
* Analysis of the correlation of *CYP2C19* LOF alleles with AMI needing adjustment for gender and with death needing adjustment for age and PCI, as well as for UA needing adjustment for age, hypertension and CCB.
LOF: loss-of-function

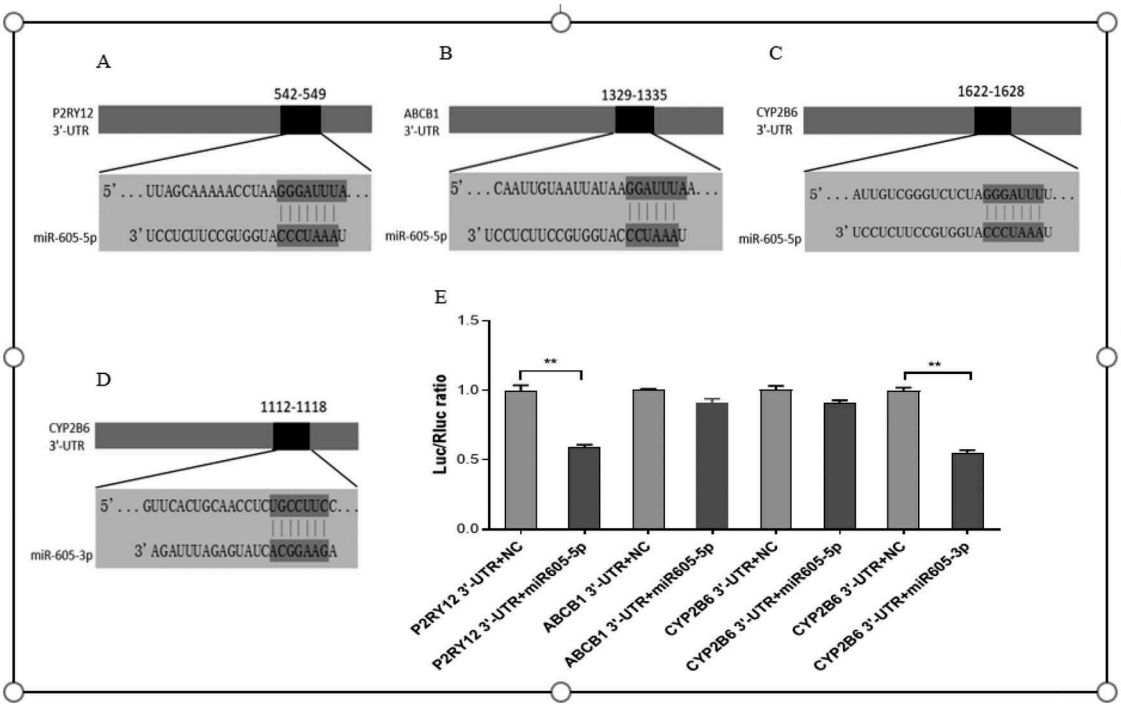

Figure 1. The 3′-UTRs of CYP2B6 and P2RY12 mRNA are targets of miR-605. (a–c), TargetScan prediction of miR-605-5p targeting the 3′-UTR of *P2RY12* mRNA, *ABCB1* mRNA, and *CYP2B6* mRNA. (d), TargetScan prediction of miR-605-3p targeting the 3′-UTR of *CYP2B6* mRNA. (e), Luciferase reporters containing wild type 3′-UTR of *CYP2B6, P2RY12* and *ABCB1* were generated, and HEK293T cells were cotransfected with miR-605-5p mimic or miR-605-3p mimic or mimic negative control (NC). The luciferase activity in the cells was assayed. n = 3 per group, and the numerical expression is mean ± SD, ** $P < .01$ *vs* NC.

CYP2B6 mRNA. We cloned luciferase reporter plasmids with the wild-type P2RY12, ABCB1, and CYP2B6 3′-UTR (WT-3′-UTR) and performed reporter analysis in HEK293 cells. As presented in Figure 1(e), Duel luciferase reporter gene analysis indicated that the activity of the P2RY12 3′-UTR reporter gene was significantly decreased by miR-605-5p and that the activity of the CYP2B6 3′-UTR reporter gene was significantly decreased by miR-605-3p.

The miR-605 rs2043556 SNP Affects the Processing Efficiency of pre-miR-605

miRNA plasmids containing pre-miR-605-A and pre-miR-605-G were transiently transfected into LO2 and HASMC cells. Peak maps of miR-605 plasmid sequencing are shown in Figure 2(a). The expression levels of mature miR-605 were tested with qRT-PCR measurement. The processing efficiency of the pre-miR -605-G plasmid was 52.42% and 77.64% lower than that of the pre-miR-605-A plasmid in LO2 and HASMC cells, respectively (Figure 2(b,c)) ($P < .01$),which is consistent with the study by Sait et al. [24]

miR-605 Rs2043556 Polymorphism Regulates the mRNA and Protein Expression of CYP2B6 and P2RY12 in LO2 and HASMC Cells

Pre-miR-605-A, pre-miR-605-G or empty vector plasmids were generated and used to transfect LO2 and HASMC cells for 24 hours. Expression of CYP2B6 protein and mRNA in LO2 cells treated with miR-605-A was noticeably decreased compared with those treated with miR-605-G, empty vector, or blank control ($P < .05$). The protein and mRNA expression of CYP2B6 in the miR-605-G group was not significantly changed when compared with that of the empty vector group or the blank control group ($P > .05$) (Figure 2(d)). The expression of P2RY12 protein and mRNA in HASMC cells treated with miR-605-A was significantly decreased compared to that of HASMC cells treated with the miR-605-G, empty vector or blank control ($P < .05$). The protein and mRNA expression of CYP2B6 in the miR-605-G group was not significantly changed when compared with the empty vector group or blank control group ($P > .05$) (Figure 2(e)).

Discussion

In the present study, we found that ACS patients bearing the *miR-605* rs2043556 G allelic variant are less likely to experience AMI and UA during one year of clopidogrel treatment. We also verified that the mRNA 3′-UTRs of CYP2B6 and P2RY12 are the targets of miR-605 by dual luciferase reporter gene analysis, and that the miR-605 rs2043556 A/G polymorphism shows a significantly different effect on the expression of CYP2B6 and P2RY12 mRNA and protein in the LO2 and HASMC cell lines.

Investigation of miRNAs has revealed a new field related to the molecular mechanism of high inter-individual variability in the response to drug treatment. First predicted through *in silico* analysis, we investigated the association between novel miRNA polymorphisms and clinical outcomes following one year of clopidogrel treatment in Chinese ACS patients. Interestingly, we revealed that *miR-605* rs2043556 A/G polymorphisms are significantly associated with UA and AMI. This potentially functional polymorphism within miR-605 has been reported to be associated with the risk of certain types of cancers in Chinese individuals [25–27]. We also demonstrated that the expression of mature miR-605 in cells transfected with the miR-605 rs2043556 G group is lower than that in cells transfected with rs2043556 A, which is consistent with studies of miR-605 in Li-Fraumeni syndrome [24]. Functionally, the G allele of miR-605 rs2043556

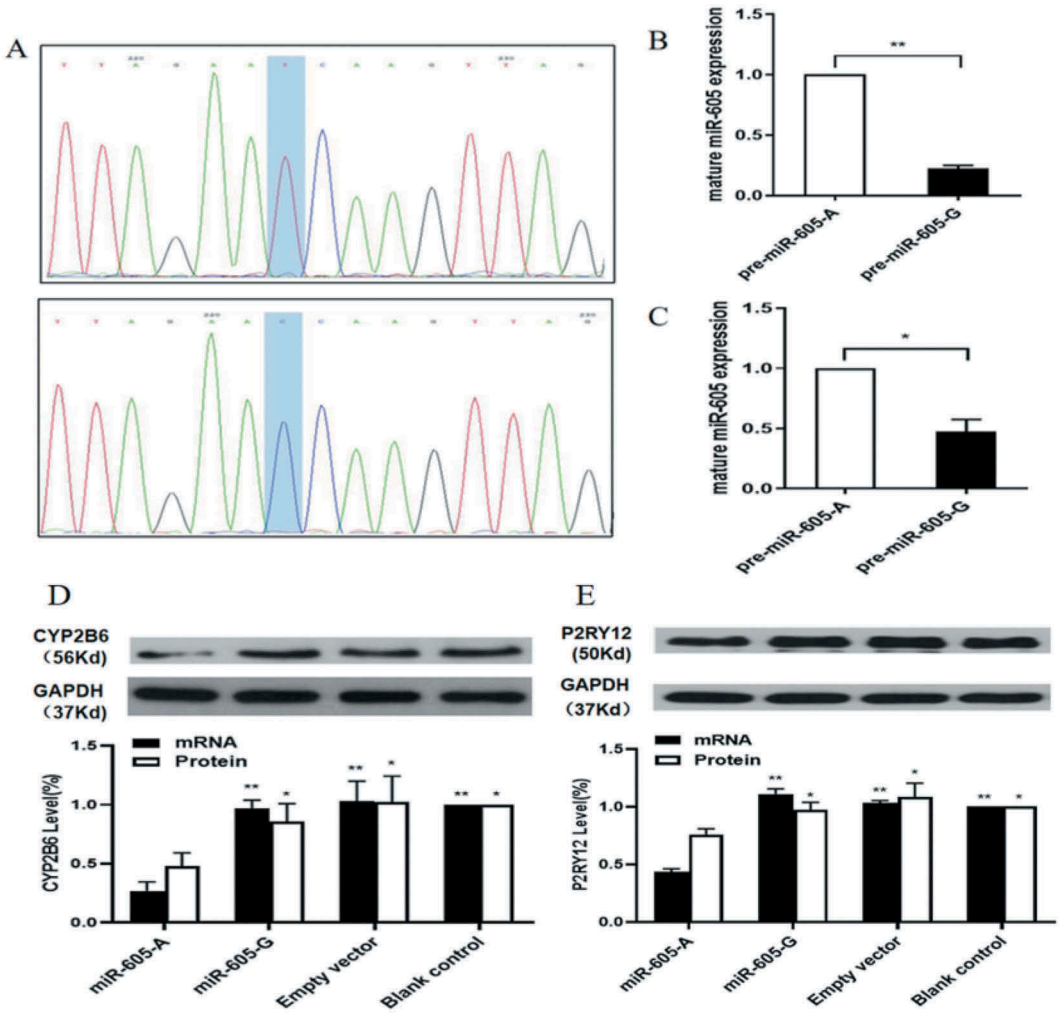

Figure 2. Sanger sequencing of the pre-miR-605-A and pre-miR-605-G vectors, expression level of mature miR-605 after transfection of different alleles of pre-miR-605 rs2043556, and protein and mRNA expression of CYP2B6 in LO2 cells and P2RY12 in HASMC cells transfected with different plasmids. (a), Sanger sequencing of the pre-miR-605-A and pre-miR-605-G vectors. (b), Impact of the miR-605 rs2043556 SNP on mature miR-605 levels. The miR-605-A and miR-605-G plasmids were transiently transfected into LO2 cells by nucleofection. $^{**}P < .01$ *vs* miR-605-A. (c), Impact of the miR-605 rs2043556 SNP on mature miR-605 levels. The miR-605-A and miR-605-G plasmids were transiently transfected into HASMC cells by nucleofection. $^{**}P < .01$ vs miR-605-A. (d), Protein and mRNA levels of CYP2B6 were determined by western blot and RT-PCR. n = 3 per group. *: $P < .05$ or **: $P < .01$ versus miR-605-A. (e), Protein and mRNA levels of P2RY12 were determined by western blot and RT-PCR. n = 3 per group. *: $P < .05$ or **: $P < .01$ versus miR-605-A.

causes a defect in the processing efficiency of its host miRNA, which indicates that the acquisition of this polymorphism may be a prominent mechanism underlying clopidogrel resistance.

Through dual luciferase reporter gene analysis, we verified that miR-605 targets the mRNA 3′-UTR of CYP2B6. CYP2B6 is recognized as a hepatic enzyme of potential importance for the metabolism of clinical drugs and environmental or abused toxicants [28,29]. CYP2B6 mediates two successive oxidation reactions of clopidogrel in the liver, as does CYP2C19 [9]. However, no clear association was previously found between genetic polymorphisms of *CYP2B6* and long-term pharmacodynamics or clinical outcomes of clopidogrel therapy [30–33]. Since CYP2B6 exhibits up to a 250-fold difference in expression variability between individuals [34], its expression level may affect clopidogrel clinical treatment efficacy. Presently, *in silico* and *in vitro* analysis have reported that the miR-25-3p downregulates CYP2B6 in human liver cells via an epigenetic mechanism [35]. We further found that in cultured LO2 cells transfected with two different miR-605 rs2043556 plasmids, those transfected with the rs2043556 G variant showed normal expression of CYP2B6 protein, while those transfected with the rs2043556 A variant showed significantly decreased CYP2B6 protein expression. According to enzyme kinetics studies *in vitro*, CYP2B6 is important for the formation of the active metabolite of clopidogrel [9]. Higher CYP2B6 levels in the liver could increase clopidogrel metabolite levels and improve the treatment effect in patients suffering from ACS.

The P2RY12 receptor plays a key role in the clopidogrel antiplatelet process [36]. The presence of polymorphisms of P2RY12 has failed previously to predict clinical outcomes of clopidogrel therapy [6,32,33]. We verified that mRNA 3′-UTR of P2RY12 is the target of miR-605 through dual luciferase reporter gene analysis. The P2RY12 receptor was originally identified in platelets, and then found to be expressed in vascular smooth cells [37]. Anucleate platelets contain messenger RNA (mRNA) and can synthesize protein [38–40]. Further studies discovered that platelets contain Dicer and Argonaute 2 (Ago2) complexes that are functional in that they can process exogenously supplied miRNA precursor (pre-miRNA). P2RY12 mRNA expression may be subject to miRNA control in human platelets [41]. In HASMC we found that rs2043556 G treatment led to normal expression of P2RY12 protein, while miR-605 rs2043556

A decreased the protein expression of P2RY12 significantly ($P < .05$). Thus, miR-605 rs2043556 G could induce a normal number of ADP receptors to combine with the active clopidogrel product. In patients with miR-605 rs2043556 G, clopidogrel could effectively execute its antiplatelet function, which may decrease the incidence of certain major cardiovascular events in ACS patients.

With respect to *miRNA* SNPs, our research focused on common and functionally relevant polymorphisms within the *CYP2C19* gene. The present results also indicated that after one year of clopidogrel treatment in ACS patients, the *CYP2C19*2* polymorphism increased the risk of AMI, while the *CYP2C19*3* polymorphism increased the risk of STs. Moreover, patients bearing two LOF alleles had an increased incidence of AMI and STs compared with those bearing one LOF allele. The *CYP2C19*2* and *CYP2C19*3* LOF polymorphisms result in the loss of CYP2C19 enzyme activity and reduced conversion of clopidogrel to its active metabolite [42,43]. A series of clinical studies have also discovered that the OR for major cardiovascular events is much higher among carriers such as CYP2C19 LOF alleles (*CYP2C19*2* and *CYP2C19*3*) than among non-carriers [6,7,13,32,33].

In the current study, we showed for the first time that miR-605 targets the mRNA 3′-UTRs of CYP2B6 and P2RY12 by dual luciferase reporter gene analysis. In transfected cell lines (LO2 and HASMC), miR-605 rs2043556 A/G polymorphisms affected the amount of mature miR-605 as well as the protein and mRNA expression of CYP2B6 and P2RY12 significantly ($P < .05$). Polymorphisms of miR-605 rs2043556 A > G are associated with a decreased risk of UA and AMI in ACS patients taking clopidogrel for more than one year.

Although our results are promising, the present study has the following limitations. First, only 567 Chinese ACS patients were assessed; thus, our findings regarding the association between the *miR-605* rs2043556 polymorphism and clinical outcomes of clopidogrel treatment should be validated in further studies with larger samples, particularly to detect platelet function during treatment. Secondly, since this study involved exclusively Chinese ACS patients, the results may not be extrapolated to the Caucasian population. Furthermore, we should detect the concentration of clopidogrel and its active metabolite to assess CYP2B6 activity. Since several polymorphisms impacted the clinical outcomes of clopidogrel therapy in the present study, a strategy utilizing a pharmacogenomic polygenic response score is needed to predict cardiovascular events in the future [44].

Conclusions

In summary, this study indicates that genetic polymorphisms of *miR-605* rs2043556 G may decrease the risk of deleterious outcomes in ACS patients taking clopidogrel for more than one year. miR-605 targets the mRNA 3′-UTRs of CYP2B6 and P2RY12. miR-605 rs2043556 A/G polymorphisms affect the amount of mature miR-605 as well as the protein and mRNA expression of CYP2B6 and P2RY12. These results suggest that *miR-605* rs2043556 polymorphisms may be a potential biomarker for the risk of clinical cardiovascular events during long-term clopidogrel therapy.

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Author Contributions

This study was a collaborative effort of all authors.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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