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ORIGINAL RESEARCH

Influence of *GAS5*/MicroRNA-223-3p/P2Y12 Axis on Clopidogrel Response in Coronary Artery Disease

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BACKGROUND: Dual antiplatelet therapy based on aspirin and P2Y12 receptor antagonists such as clopidogrel is currently the primary treatment for coronary artery disease (CAD). However, a percentage of patients exhibit clopidogrel resistance, in which genetic factors play vital roles. This study aimed to investigate the roles of GAS5 (growth arrest-specific 5) and its rs55829688 polymorphism in clopidogrel response in patients with CAD.

METHODS AND RESULTS: A total of 444 patients with CAD receiving dual antiplatelet therapy from 2017 to 2018 were enrolled to evaluate the effect of *GAS5* single nucleotide polymorphism rs55829688 on platelet reactivity index. Platelets from 37 patients of these patients were purified with microbeads to detect GAS5 and microRNA-223-3p (miR-223-3p) expression. Platelet-rich plasma was isolated from another 17 healthy volunteers and 46 newly diagnosed patients with CAD to detect GAS5 and miR-223-3p expression. A dual-luciferase reporter assay was performed to explore the interaction between miR-223-3p and *GAS5* or *P2Y12* 3′-UTR in (human embryonic kidney 293 cell line that expresses a mutant version of the SV40 large T antigen) HEK 293T and (megakaryoblastic cell line derived in 1983 from the bone marrow of a chronic myeloid leukemia patient with megakaryoblastic crisis) MEG-01 cells. Loss-of-function and gain-of-function experiments were performed to reveal the regulation of GAS5 toward P2Y12 via miR-223-3p in MEG-01 cells. We observed that rs55829688 CC homozygotes showed significantly decreased platelet reactivity index than TT homozygotes in *CYP2C19* poor metabolizers. Platelet GAS5 expression correlated positively with both platelet reactivity index and *P2Y12* mRNA expressions, whereas platelet miR-223-3p expression negatively correlated with platelet reactivity index. Meanwhile, a negative correlation between GAS5 and miR-223-3p expressions was observed in platelets. MiR-223-3p mimic reduced while the miR-223-3p inhibitor increased the expression of GAS5 and P2Y12 in MEG-01 cells. Knockdown of GAS5 by siRNA increased miR-223-3p expression and decreased P2Y12 expression, which could be reversed by the miR-223-3p inhibitor. Meanwhile, overexpression of GAS5 reduced miR-223-3p expression and increased P2Y12 expression, which could be reversed by miR-223-3p mimic.

CONCLUSIONS: *GAS5* rs55829688 polymorphism might affect clopidogrel response in patients with CAD with the *CYP2C19* poor metabolizer genotypes, and GAS5 regulates P2Y12 expression and clopidogrel response by acting as a competitive endogenous RNA for miR-223-3p.

Key Words: clopidogrel ■ coronary artery disease ■ *GAS5* ■ miR-223-3p ■ P2Y12 ■ rs55829688

The rapid development of bench-to-bedside projects and progress in translational medicine has dramatically improved the prognosis of coronary artery disease (CAD).[1] Despite these advances, CAD remains the primary cause of cardiovascular-related death in developed and developing countries, including China. The prevalence of CAD was 27.8‰ among people older than 60 years in China.[2,3] Percutaneous coronary intervention followed by dual antiplatelet therapy with aspirin and the P2Y12 receptor antagonist is

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CLINICAL PERSPECTIVE

What Is New?

- First, we investigated the influence of GAS5 (growth arrest-specific 5) expression and its genetic polymorphism on clopidogrel response in patients with coronary artery disease.
- We confirmed the existence of the "GAS5/miR-223-3p/P2Y12 axis" in platelets and explored its potential role in clopidogrel response in patients with coronary artery disease.

What Are the Clinical Implications?

- We confirmed the effect of *CYP2C19* genotypes on clopidogrel response, which further supported the application of *CYP2C19* genotyping in individualized clopidogrel therapy.
- Platelet GAS5 expression and *GAS5* rs55829688 polymorphism might be novel biomarkers to predict clopidogrel response.

Nonstandard Abbreviations and Acronyms

ceRNA	competitive endogenous RNA
EM	extensive metabolizer
GAS5	growth arrest-specific 5
HTPR	high on-treatment platelet reactivity
IM	intermediate metabolizer
lncRNA	long non-coding RNA
NTPR	normal on-treatment platelet reactivity
PM	poor metabolizer
PRI	platelet reactivity index
VASP	vasodilator-stimulated phosphoprotein

the major treatment for CAD.[4,5] However, about 15% to 44% of patients exhibit resistance to antiplatelet agents such as clopidogrel and suffered from increased risk of on-treatment ischemic cardiovascular events.[6–8]

Clopidogrel is a second-generation thienopyridine antiplatelet drug. Approximately 85% of clopidogrel is metabolized into the inactive metabolite SR26334 after oral administration, while only 15% is metabolized into the active metabolite through the cytochrome P450 (CYP450) system, such as CYP2C19, CYP1A2, CYP2B6, CYP3A4, and CYP2C9 in the liver.[9] The active metabolite can irreversibly bind to the purinergic receptor P2Y12 on the platelet surface and block the ADP-stimulated platelet activation and aggregation.[10,11] Many genome-wide association studies and candidate gene-based association studies have identified genetic variants associated with clopidogrel metabolism and/or platelet reactivity.[12–14] For example, loss-of-function alleles of *CYP2C19* (*CYP2C19∗2* and *CYP2C19∗3*) and the *CYP2C19* gain-of-function allele *CYP2C19∗17* were observed to clearly affect the antiplatelet efficacy of clopidogrel as well as the clinical outcome.[15,16] In our previous studies, we have reported that the *N6AMT1* single nucleotide polymorphism (SNP) rs2254638 was associated with increased risk of clopidogrel resistance in Chinese patients with CAD. In contrast, *FMO3* rs1736557 and *CRISPLD1* rs12115090 SNPs might increase the anti-platelet efficacy of clopidogrel.[17–19] Even though clopidogrel resistance could be partially explained by known genetic variations, more genetic factors should be discovered and applied to explain clopidogrel resistance in patients with CAD.

GAS5 (*growth arrest-specific 5*) is a long non-coding RNA (lncRNA), with its coding gene located on human chromosome 1q25.1. Several works have revealed that *GAS5* played pivotal roles in cell proliferation,[20] apoptosis,[21] inflammatory response,[22,23] oxidative stress,[23] and autophagy flux[24] in various cardiovascular diseases. For instance, *GAS5* expression was elevated in endothelial cells of atherosclerosis rats, and knockdown of *GAS5* enhanced the proliferation while depressing the apoptosis of endothelial cells through miR-194-3p-targeted TXNIP.[25] Recently, studies have revealed that the plasma level of *GAS5* was decreased in patients with CAD, indicating a promising biomarker of *GAS5* for CAD.[26,27] Functional polymorphisms in *GAS5* have also been observed.[28,29] For example, rs55829688 is an SNP in *GAS5* promoter and increases *GAS5* transcription by affecting the binding affinity of some transcription factors.[29] However, whether *GAS5* and *GAS5* rs55829688 polymorphism play a role in the clopidogrel response of patients with CAD remains unclear.

MicroRNA-223-3p (miR-223-3p) is one of the most abundant microRNAs released by activated platelets.[30] Shi et al. reported that the expression of miR-223-3p was decreased in 16 clopidogrel low-responders compared with that in normal responders.[31] Another study involving 62 patients with non-ST elevation acute coronary syndrome indicated that the miR-223-3p expression in plasma was also decreased in clopidogrel low-responders.[32] In addition, circulating miR-223-3p was found to be reduced in patients with CAD after antiplatelet therapy.[33] These findings suggested that the expression of platelet miR-223-3p might be a biomarker to indicate clopidogrel response in patients with CAD. There was also evidence revealing that P2Y12, the direct binding site of active clopidogrel metabolite, was a target of miR-223.[34,35] This illustrated the possible mechanism by which miR-223-3p played a role in clopidogrel response.

Interestingly, studies by dual-luciferase assay and RNA immunoprecipitation experiments have indicated that *GAS5* might also be a target of miR-223.[36–38]

Therefore, we assumed that *GAS5* might act as a competitive endogenous RNA (ceRNA) of miR-223-3p to regulate the expression of P2Y12 in platelets, further affecting the clopidogrel response.

In this study, we tried to explore the influence of platelet *GAS5* expression and the rs55829688 polymorphism on clopidogrel response. We discovered the ceRNA mechanism of *GAS5* on clopidogrel response through regulating miR-223-3p/P2Y12 expression in platelets.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

A total of 444 patients diagnosed as CAD according to the guidelines of American College of Cardiology/American Heart Association[39,40] were recruited from Xiangya hospital from January 2017 to April 2018, and all of these patients had undergone percutaneous coronary intervention and received dual antiplatelet therapy with clopidogrel and aspirin. Another 17 healthy volunteers and 46 newly diagnosed patients with CAD were recruited from the same hospital in 2020. The inclusion criteria for CAD cases were as follows: (1) Clinically diagnosed with coronary artery disease; (2) Age between 18 to 80 years; and (3) Administration of a loading dose (300 mg) followed by a maintenance dose (75 mg/day, ≥5 days) of clopidogrel or only the maintenance dose without a loading dose. The exclusion criteria of patients were as follows: (1) A history of systemic bleeding or coagulopathy; (2) Anemia (hemoglobin <100 g/L); (3) Liver and kidney dysfunction; (4) Malignant tumors or other fatal diseases; (5) A history of food or drug allergic diseases; (6) Drug abuse or mental illness; and (7) Receiving platelet glycoprotein IIb/IIIa receptor antagonist before enrollment. All subjects signed the informed consent upon admission. The study was approved by the Ethics Committee of Central South University, Changsha (CTXY-140002-13) and registered on the Chinese Clinical Trial Registry (ChiCTR-OPN-15006260). All protocols were performed according to the Declaration of Helsinki.

Determination of Platelet Vasodilator-Stimulated Phosphoprotein Phosphorylation and Calculation of Platelet Reactivity Index

Blood samples were collected from patients with CAD 12 to 24 hours after receiving the loading dose of clopidogrel or 24 hours after taking the maintenance dose of clopidogrel for at least 5 days. The blood samples were drawn into 3.8% sodium citrate anti-coagulated vacutainer tubes and were immediately treated with the PLT VASP/P2Y12 kit (Biocytex, Stago, Asnières-sur-Seine, France) to detect the platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation level according to the manufacturer's instructions. Platelet reactivity index (PRI) indicating the residual platelet activity was calculated based on the phosphorylation level of VASP detected by Beckman FC500 flow cytometry (Beckman Coulter Inc., CA, USA) as described previously.[18]

Crude Extraction of Platelet From Platelet-Rich Plasma

The blood samples collected into sodium citrate blood collection tubes were mixed thoroughly and then the samples were immediately centrifuged at 244*g* for 15 minutes at room temperature. The supernatant was then gently mixed with Tyrode's solution in a volume ratio of 1:1 and centrifuged at 1360*g* for 5 minutes at room temperature.[41] The precipitation was collected for further analysis.

Purification of Platelets With Microbeads

About 10 to 15 mL of venous blood from patients with CAD was collected into sodium citrate blood collection tubes and centrifuged at 180*g* for 15 minutes at 4 °C. The upper layer of plasma was collected, and an appropriate volume of 0.5 mol/L EDTA was added to make sure the final concentration of EDTA was 2 mmol/L. The mixture was then centrifuged at 1000*g* for 10 minutes at 4 °C, and the precipitation was collected. The obtained precipitation was then resuspended in microbeads buffer mixed with CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated at 2 to 8 °C for 15 to 30 minutes by softly shaking the solution every 15 minutes. For magnetic separation, the LS column (Miltenyi Biotec, Bergisch Gladbach, Germany) was placed in the magnetic field of a midi-MACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany) and rinsed 3 times with running buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). The incubated cell suspension was then transferred to the LS column and washed 3 times. The effluent was collected and centrifuged at 2000*g* for 10 minutes to collect the precipitation for further analysis.

DNA Extraction and SNPs Genotyping

The venous blood of all participants was collected into EDTA anticoagulant tubes and stored at –70 °C until the extraction of DNA. Genomic DNA was extracted according to the standard procedures of a commercial DNA extraction kit (Promega, Madison, USA). A NanoDrop 2000 microvolume spectrophotometer (NanoDrop Technologies, Wilmington, USA) was used to determine DNA concentration, further diluted into

50 ng/μL by buffer EB. *CYP2C19*2*, *CYP2C19*3*[42] and *GAS5* rs55829688 were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism. The genotyping assays were verified by Sanger Sequencing (Sangon Biotech, Shanghai, China) for a subset of these samples, yielding a 100% concordance. The forward and reverse primers of PCR were synthesized by Sangon Biotech (Shanghai, China), and the primer sequences are listed in Table S1.

Cell Culture

(Human embryonic kidney 293 cell line that expresses a mutant version of the SV40 large T antigen) HEK 293T cells were cultured in DMEM (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (Thermo Fisher Scientific, MA, USA) at 37°C in a humidified chamber supplemented with 5% CO_2. Then, 0.05% trypsin (Thermo Fisher HyClone, Utah, USA) was used to digest cells in the logarithmic growth phase. Human megakaryoblastic leukemia cell line (megakaryoblastic cell line derived in 1983 from the bone marrow of a chronic myeloid leukemia patient with megakaryoblastic crisis) MEG-01, purchased from National Biomedical Laboratory Cell Resource Bank (Beijing, China), was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, MA, USA) supplemented with 20% fetal bovine serum and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in the air. Both HEK 293T and MEG-01 cells were split into 1:3 every 2 to 3 days for passage.

Vector Construction

To construct vectors for ceRNA study, public online tools including ENCORI (http://starbase.sysu.edu.cn/index.php)[43] and TargetScan (http://www.targetscan.org/vert_72/)[44] were applied to analyze the recognizing sequences of miR-223-3p in *GAS5* and *P2Y12*. The sequences of the *GAS5* and *P2Y12* 3'-untranslated region (3'-UTR) containing the predicted miR-223-3p binding sequences were amplified by PCR with a DNA template from a healthy volunteer. The PCR products were purified from agarose gel. They were then subcloned into the *Nhe*I (Thermo Fisher Scientific, MA, USA) and *Xba*I (Thermo Fisher Scientific, MA, USA) restriction sites downstream the firefly luciferase (*Luc*) gene of the pmirGLO vector (YouBio, Changsha, China). These wild-type plasmids were designed as pmirGLO-GAS5-wt and pmirGLO-P2Y12-wt. Five base pair of the predicted recognizing sequences of miR-223-3p in *GAS5* and *P2Y12* 3'-UTR were mutated to generate the corresponding mutant plasmids, which were named as pmirGLO-GAS5-mut and pmirGLO-P2Y12-mut, respectively. The PCR primers were synthesized by Sangon Biotech (Shanghai, China), and the detailed sequences information is listed in Table S1.

Dual Luciferase Reporter Assay

After HEK 293T cells (5×10^4/well) and MEG-01 cells (1×10^5/well) were seeded into 24-well plates, co-transfections were conducted immediately for MEG-01 cells, while for HEK 293T cells, co-transfections were performed upon reaching 60% to 70% confluence. Cells were co-transfected with pmirGLO-GAS5-wt/pmirGLO-GAS5-mut, miR-223-3p mimic or inhibitor using Lipofectamine 3000 reagent (Thermo Fisher Scientific, MA, USA) to examine the interaction between GAS5 and miR-223-3p. To investigate the interaction between P2Y12 and miR-223-3p, cells were co-transfected with pmirGLO-P2Y12-wt/pmirGLO-P2Y12-mut and miR-223-3p mimic or inhibitor. According to the manufacturer's protocol of the Dual- Luciferase Reporter Assay System (Promega, Madison, USA), cell lysates were harvested 36 hour after transfection. Luciferase activity was measured in at least triplicate using the Single Tube Luminometer (Berthold, Bad Wildbad, Germany) following the manufacturer's instruction.

Oligonucleotides and Vectors Transfection

Oligonucleotides of 3 *GAS5*-specific siRNAs (GAS5 si-1, GAS5 si-2, and GAS5 si-3) were used to knock down *GAS5* referring to the study by Houqi Liu et al.,[45] and the non-silencing control siRNA oligonucleotide was used as a negative control (GenePharma, Shanghai, China). *GAS5* cDNA was amplified by PCR and subcloned into GV219 to generate the overexpression vector named GV219-GAS5 (Genechem, Shanghai, China), with the empty GV219 vector (GV219-NC) as a control. The miR-223-3p mimic, inhibitor, and the corresponding negative controls were synthesized by Ribobio (Guangzhou, China); 4×10^5/well of MEG-01 cells were plated into a 6-well plate, and plasmids were transfected into the cells using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA) following the manufacturer's protocol. Lipofectamine RNAiMAX (Thermo Fisher Scientific, MA, USA) was used to transfect GAS5 siRNAs and the miR-223-3p mimic/inhibitor following the manufacturer's instructions. The cells were harvested 48 hours after transfection for further analysis. GAS5-specific siRNA sequences and miR-223-3p mimic or inhibitor sequences are listed in Table S1.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from platelets and cultured cells with RNAiso reagent (Takara, Kyoto, Japan) following the manufacturer's instructions. The extracted total RNA was reversely transcribed using PrimeScript

RT reagent Kit with gDNA Eraser (Takara, Kyoto, Japan) for *GAS5* and *P2Y12*, and PrimeScript RT reagent Kit for miR-223-3p (Takara, Kyoto, Japan). The expressions of *GAS5*, *P2Y12*, and miR-223-3p were semi-quantified by real-time quantitative PCR using TB Green real-time qPCR Kit (Takara, Kyoto, Japan) on a LightCycler480 (Roche, Basel, Switzerland). The expressions of *GAS5*, *P2Y12*, and miR-223-3p were normalized to that of *GAPDH* and *U6*, respectively, with $2^{-\Delta\Delta Ct}$ calculated to indicate the expression level. The forward and reverse primers for miR-223-3p and U6 were purchased from Ribobio (Guangzhou, China), and primers for *GAS5*, *P2Y12*, and *GAPDH* were synthesized by Sangon Biotech (Shanghai, China). Details of the primer sequences are listed in Table S1.

Western Blot

Cells were collected and rinsed in pre-cooled PBS before lysed with RIPA buffer (Beyotime, Shanghai, China), and a BCA Protein Assay kit (Beyotime, Shanghai, China) was used to determine protein concentrations. Total protein was separated on 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes (Merck Millpore, Darmstadt, Germany). Next, the membranes were blocked with 5% (m/v) non-fat milk for 1 to 2 hours at room temperature and incubated with the primary antibody to P2Y12 (Proteintech, Wuhan, China) or GAPDH (Proteintech, Wuhan, China) overnight at 4 °C. After being washed 3 times with Tris buffered saline with Tween 20, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 2 hours. The protein bands were visualized with the aid of enhanced chemiluminescence (GE Healthcare, Chicago, USA), and images were captured by Image Lab software (Bio Rad, CA, USA).

Statistical Analysis

A Shapiro–Wilk test was performed to evaluate the assumption of normality for continuous variables and an *F* test was performed to test the assumption of homogeneity of variance. If the data followed a normal distribution, they were presented as mean±SD, and a Student *t*-test or Welch *t*-test was performed to determine differences between 2 groups. One-way ANOVA was performed to determine differences among >2 groups and was corrected by Bonferroni multiple comparisons test. If the data were not normally distributed, they were presented as a median with interquartile range (IQR). A Mann-Whitney *U* test was performed to determine differences between 2 groups. The Kruskal–Wallis *H* test was performed to determine differences among >2 groups and was corrected by Dunn test. Categorical variables were expressed as frequencies with percentages and Pearson Chi-squared test, and Yates correction for continuity or Fisher exact test was performed to compare them, as appropriate. *Spearman* rank correlation analysis was conducted to determine the relationships between multiple genes expressions or between gene expression and PRI. All statistical analyses were performed using SPSS software (version 24.0, IBM Corporation, Armonk, USA) and GraphPad Prism software (version 8.3, GraphPad Software Inc., CA, USA). A 2-tailed $P<0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics of the Patients With CAD

A total of 444 patients with determined platelet VASP phosphorylation levels were enrolled in this study, and the baseline characteristics are shown in Table 1. The median (IQR) age was 62 years (IQR, 53–69 years) and 289 (65.1%) of these patients were men. High on-treatment platelet reactivity (HTPR) was defined as PRI >50%, while normal on-treatment platelet reactivity (NTPR) was defined as PRI ≤ 50%, as described elsewhere.[46,47] According to these criteria, 295 (66.4%) patients were assigned to the HTPR group, while 149 (33.6%) patients were assigned to the NTPR group. The median (IQR) PRIs in the HTPR group and NTPR group were 70.84% (IQR, 59.78%–78.6%) and 36.08% (IQR, 23.32%–43.69%), respectively. The median (IQR) serum triglyceride was significantly higher in the HTPR group (1.745 mmol/L [IQR, 1.23–2.43 mmol/L] versus 1.47 mmol/L [IQR, 1.04–2.15 mmol/L], $P=0.0095$). The median (IQR) low-density lipoprotein cholesterol was also increased in the HTPR group (2.71 mmol/L [IQR, 2.115–3.245 mmol/L] versus 2.39 mmol/L [IQR, 1.9–3.01 mmol/L], $P=0.0184$). More patients used statins concomitantly (54.4% versus 44.4%, $P=0.047$). Fewer patients received the 300 mg loading dose of clopidogrel (53.7% versus 70.8%, $P<0.0001$) in the NTPR group, which was consistent with the previous report that a continuous administration of clopidogrel at 75 mg/day for at least 5 days was more effective than a loading dose of 300 mg clopidogrel in 12 to 24 hours.[48] No significant differences between NTPR and HTPR groups in age, sex, hypertension, dyslipidemia, diabetes, smoking, drinking, and other clinical characteristics were observed (Table 1).

Influence of *CYP2C19* Metabolic Type and *GAS5* rs55829688 Polymorphism on Clopidogrel Response in Patients With CAD

According to the carrying status of *CYP2C19*2* and **3* variants, the patients were divided into *CYP2C19* extensive

Table 1. Clinical Characteristics of the Study Population

	Entire cohort (N=444)	HTPR cohort (n=295)	NTPR cohort (n=149)	*P* value
Age, y	62 (53–69)	61 (53–68)	63 (54–71.75)	0.0818
Men, n (%)	289 (65.1)	191 (64.75)	98 (65.8)	0.830
Hypertension, n (%)	232 (52.3)	145 (49.2)	87 (58.4)	0.066
Dyslipidemia, n (%)	70 (15.8)	47 (15.9)	23 (15.4)	0.892
Diabetes, n (%)	83 (18.7)	54 (18.3)	29 (19.5)	0.768
Smoking, n (%)	149 (33.6)	99 (33.6)	50 (33.6)	1.000
Drinking, n (%)	101 (22.7)	65 (22.0)	36 (24.2)	0.614
TC, mmol/L	4.21 (3.41–5.00)	4.31 (3.51–5.025)	4.09 (3.33–4.91)	0.1305
Triglycerides, mmol/L	1.67 (1.17–2.31)	1.745 (1.23–2.43)	1.47 (1.04–2.15)	0.0095*
HDL-C, mmol/L	1.03 (0.89–1.29)	1.04 (0.89–1.285)	1.02 (0.91–1.32)	0.8160
LDL-C, mmol/L	2.61 (1.95–3.20)	2.71 (2.115–3.245)	2.39 (1.9–3.01)	0.0184*
Comedications				
PPIs, n (%)	198 (44.6)	126 (42.7)	72 (48.3)	0.261
CCBs, n (%)	110 (24.8)	67 (22.7)	43 (28.9)	0.157
Statins, n (%)	212 (47.7)	131 (44.4)	81 (54.4)	0.047*
Morphine, n (%)	52 (11.7)	29 (9.8)	23 (15.4)	0.083
Tirofiban, n (%)	8 (1.8)	7 (2.4)	1 (0.7)	0.371
Platelet count (10^9/L)	197 (161–241)	199 (159.5–241.5)	197 (163–240.8)	0.8421
300 mg of clopidogrel, n (%)	289 (65.1)	209 (70.8)	80 (53.7)	<0.0001*
MPV (fL)	10.35 (9.2–11.6)	10.5 (9.085–11.7)	10.3 (9.3–11.2)	0.5752
PRI (%)	59.78 (43.6–75.38)	70.84 (59.78–78.6)	36.08 (23.32–43.69)	—

Data were presented as median (interquartile range), or frequency (%) as appropriate.

CCBs indicates calcium channel blockers; HDL-C, high-density lipoprotein cholesterol; HTPR, high on-treatment platelet reactivity; LDL-C, low-density lipoprotein cholesterol; MPV, mean platelet volume; NTPR, normal on-treatment platelet reactivity; PPIs, proton pump inhibitors; PRI, platelet reactivity index; and TC, total cholesterol.

*Bold *P* value means $P<0.05$. Mann-Whitney *U* test was performed for continuous variables. Pearson's chi-squared test, Yates correction for continuity or Fisher exact test was performed for categorical variables, as appropriate.

metabolizers (EMs, *CYP2C19*1/*1*), intermediate metabolizers (IMs, *CYP2C19*1/*2*+*CYP2C19*1/*3*), and poor metabolizers (PMs, *CYP2C19*2/*2*+*CYP2C19*2/*3* + *CYP2C19*3/*3*).[49] PRI values of *CYP2C19* IMs (n=183) were significantly higher than those of *CYP2C19* EMs (n=183) (62.76% [IQR, 47.95%–76.58%] versus 51.74% [IQR, 36.55%–67.45%], $P<0.0001$), while significantly lower than those of the PMs (n=56) (62.76% [IQR, 47.95%–76.58%] versus 75.30% [IQR, 60.09%–79.97%], $P=0.0148$, Kruskal-Wallis *H* test followed by Dunn test), which was consistent with the previous reports.[50–52] No difference in PRI among *GAS5* rs55829688 genotypes was observed in the overall patients (Figure 1A). When the patients were stratified by *CYP2C19* metabolic type, no difference in PRI among *GAS5* rs55829688 genotypes was observed in the EMs and IMs, whereas carriers of rs55829688 CC genotype exhibited a tendency of increased PRI in the PMs (Figure 1B). However, a marginally significant difference in PRI ($P=0.0883$, Figure 1B) was observed in PMs. We further compared the differences in PRI based on the genetic models and found that in the additive genetic model (TT versus CC) among *CYP2C19* PMs, rs55829688 CC homozygotes exhibited significantly lower PRI than TT homozygotes ($P=0.0374$, Figure 1B). No difference in *GAS5* rs55829688 genotype distribution between the NTPR and HTPR groups was observed, regardless of whether the *CYP2C19* metabolic type was considered (Table 2).

Relationship of Platelet GAS5 and miR-223-3p Expression With Clopidogrel Response in Patients With CAD

Platelets of 37 patients out of the 444 patients with CAD were purified with microbeads to detect GAS5 and miR-223-3p expressions. Correlation analysis revealed that PRI values were positively correlated with platelet GAS5 expression (*Spearman r*=0.3622, $P=0.0276$, Figure 2A), while negatively correlated with platelet miR-223-3p expression (*Spearman r*=−0.3265, $P=0.0486$, Figure 2B). Since P2Y12 on platelets is a direct target of clopidogrel active metabolite, the expression of platelet P2Y12 is critical to clopidogrel response.[53] Interestingly, we observed that mRNA expression of *P2Y12* was also correlated positively with

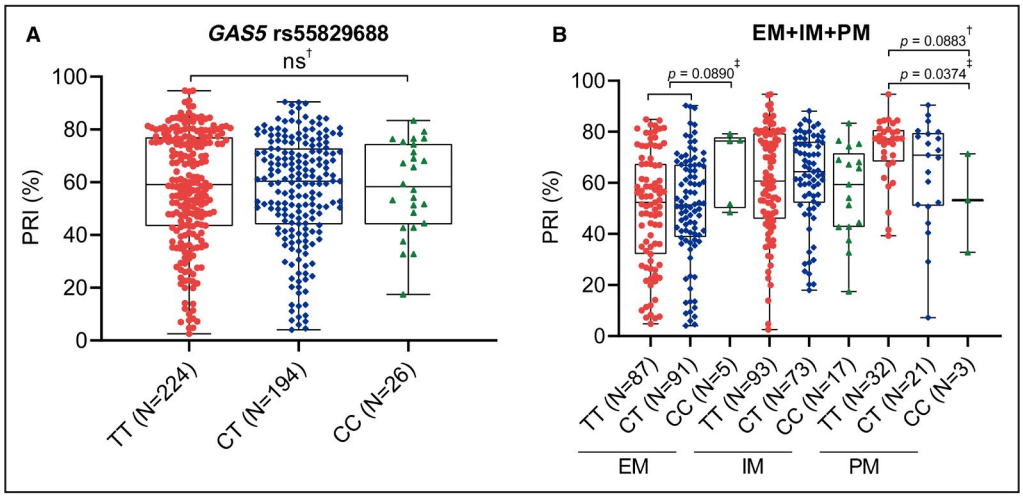

Figure 1. Influence of *GAS5* (growth arrest-specific 5) rs55829688 polymorphism on clopidogrel response in patients with coronary artery disease.
(**A** and **B**) The influence of *GAS5* rs55829688 on platelet reactivity index in the entire cohort (**A**) and in the different CYP2C19 metabolic type (**B**). Data were presented as median (interquartile range). EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer. ns, not significant. †*P* values by Kruskal–Wallis *H* test (**A** and **B**), ‡*P* value by Mann-Whitney *U* test in the recessive model (TT+CT vs CC) or additive model (TT vs CC) (**B**).

GAS5 expression in platelets from patients with CAD (*Spearman r*=0.6052, *P*<0.0001, Figure 2C). However, no similar correlation was found between the expression of GAS5 and the expression of other genes in the platelet signaling pathway, including *VASP* and integrin subunit beta 3 (ITGB3) (Figure S1).

GAS5 Acted as a ceRNA for miR-223-3p

To determine the mechanism by which GAS5 and miR-223-3p affected clopidogrel response, we first analyzed the correlation between the *GAS5* expression and miR-223-3p expression in platelets of patients with CAD. For both crudely extracted platelets (n=46) and microbeads purified platelets (n=37), significant negative correlation between *GAS5* expression and miR-223-3p expression was observed (crudely extracted platelets: *Spearman r*=−0.5295, *P*=0.0002; microbeads purified platelets: *Spearman r*=−0.5655, *P*=0.0003, Figure 3A and 3B). A similar negative correlation between *GAS5* expression and miR-223-3p expression in crudely extracted platelets of the 17 healthy volunteers or the combined subjects (17 healthy volunteers and 46 patients with CAD) was also observed (Figure S2). Considering that *GAS5* has been reported to be predominantly localized in the cell cytoplasm,[54] we assumed that *GAS5*, like other lncRNAs,[55–58] might serve as a ceRNA in regulating platelet miR-223 expression.

The luciferase reporter vectors containing the wild or mutant *GAS5* binding sites for miR-223-3p were successfully constructed (Figure 3C). A dual-luciferase reporter assay was carried out to confirm the interaction of *GAS5* with miR-223-3p. The relative luciferase activities of the pmirGLO-GAS5-wt were significantly decreased in HEK 293T cells and MEG-01 cells when co-transfected with miR-223-3p mimic compared with miR-223-3p mimic control. However, no significant difference in relative luciferase activities of pmirGLO-GAS5-mut was observed between miR-223-3p mimic, and miR-223-3p mimic control-treated groups (Figure 3D). On the contrary, the relative luciferase activities of the pmirGLO-GAS5-wt were significantly increased in HEK 293T cells and MEG-01 cells when co-transfected with miR-223-3p inhibitor. In contrast, the relative luciferase activity of pmirGLO-GAS5-mut was not affected when co-transfected with the miR-223-3p inhibitor (Figure 3E). Furthermore, *GAS5* knockdown enhanced miR-223-3p expression in MEG-01 cells, while *GAS5* overexpression with GV219-GAS5 plasmid diminished miR-223-3p expression substantially (Figure 3F). Similarly, overexpression of miR-223-3p or inhibition of miR-223-3p dramatically reduced or increased *GAS5* expression, respectively (Figure 3G). The overexpression and interference efficiency of *GAS5* and miR-223-3p in the MEG-01 cells are shown in Figure S3.

GAS5 Regulated P2Y12 Expression by Sponging miR-223-3p

P2Y12 has been identified as a target gene of miR-223 through luciferase assay and argonaute RISC catalytic component 2 (Ago2) immunoprecipitant assay.[34] In

Table 2. Distribution of *GAS5* rs55829688 Genotypes in the Patients With NTPR and HTPR and Stratified by *CYP2C19* Genotypes

Group	*GAS5* rs55829688 genotypes	HTPR	NTPR	*P* value
Overall	No. of patients with data	295	149	
	TT, n (%)	146 (49.5)	78 (52.3)	
	CT, n (%)	131 (44.4)	63 (42.3)	0.613
	CC, n (%)	18 (6.1)	8 (5.4)	0.681
EMs	No. of patients with data	101	82	
	TT, n (%)	48 (47.5)	39 (47.6)	
	CT, n (%)	49 (48.5)	42 (51.2)	0.859
	CC, n (%)	4 (4.0)	1 (1.2)	0.532
IMs	No. of patients with data	133	50	
	TT, n (%)	64 (48.1)	29 (58.0)	
	CT, n (%)	58 (43.6)	15 (30.0)	0.123
	CC, n (%)	11 (8.3)	6 (12.0)	0.738
PMs	No. of patients with data	48	8	
	TT, n (%)	29 (59.2)	3 (37.5)	
	CT, n (%)	17 (34.7)	4 (50.0)	0.547
	CC, n (%)	2 (4.1)	1 (12.5)	0.313

EMs indicates extensive metabolizers; *GAS5*, growth arrest-specific 5; HTPR, high on-treatment platelet reactivity; IMs, intermediate metabolizers; NTPR, normal on-treatment platelet reactivity; and PMs, poor metabolizers.

Data were presented as frequency (%). Pearson Chi-squared test, Yates correction for continuity or Fisher exact test was performed as appropriate.

our study, we repeated the luciferase reporter assay. We demonstrated that miR-223-3p reduced the luciferase activity of the pmirGLO-P2Y12-wt but not that of the pmirGLO-P2Y12-mut (Figure 4A). At the same time, the miR-223-3p inhibitor increased the luciferase activity of pmirGLO-P2Y12-wt in both the HEK 293T and the MEG-01 cells (Figure 4B). In cultured MEG-01 cells, overexpression of miR-223-3p significantly decreased both mRNA and protein levels of P2Y12 (Figure 4C). Conversely, miR-223-3p inhibitor increased both mRNA and protein levels of P2Y12 significantly (Figure 4D).

To determine whether *GAS5* could regulate P2Y12 expression via the ceRNA mechanism, we detected P2Y12 mRNA and protein levels after *GAS5* and miR-223-3p intervention. We observed that both P2Y12 mRNA and protein levels were substantially reduced by *GAS5* siRNA in MEG-01 cells (Figure 4E), and the *P2Y12* mRNA level could be reversed by co-transfection with the miR-223-3p inhibitor (Figure 4F). On the contrary, *GAS5* overexpression increased P2Y12 mRNA and protein expression (Figure 4G), while *P2Y12* mRNA level was reversed by miR-223-3p mimic (Figure 4H).

DISCUSSION

Previous studies concerning the clopidogrel response have mainly focused on genetic variations on clopidogrel metabolic genes, such as *CES1* G143E,[59] *ABCB1* 3435C→T,[60] and *CYP2C19*2/*3*.[15,16] However, a few studies have explored the effect and mechanism of lncRNA on those genes. Within this study, we illustrated for the first time the potential role of the platelet *GAS5* in clopidogrel response, in which the mechanism acts as a ceRNA for miR-223-3p to regulate P2Y12 expression.

Here, we analyzed the association between clopidogrel response reflected by phosphorylation level of VASP and different *CYP2C19* metabolic types in patients with CAD. We found that the clopidogrel response in *CYP2C19* PMs and IMs was significantly decreased compared with *CYP2C19* EMs, which was consistent with the previous reports.[15,16] LncRNAs are a group of functional RNAs without protein-coding ability and participate in the pathogenesis and development of many

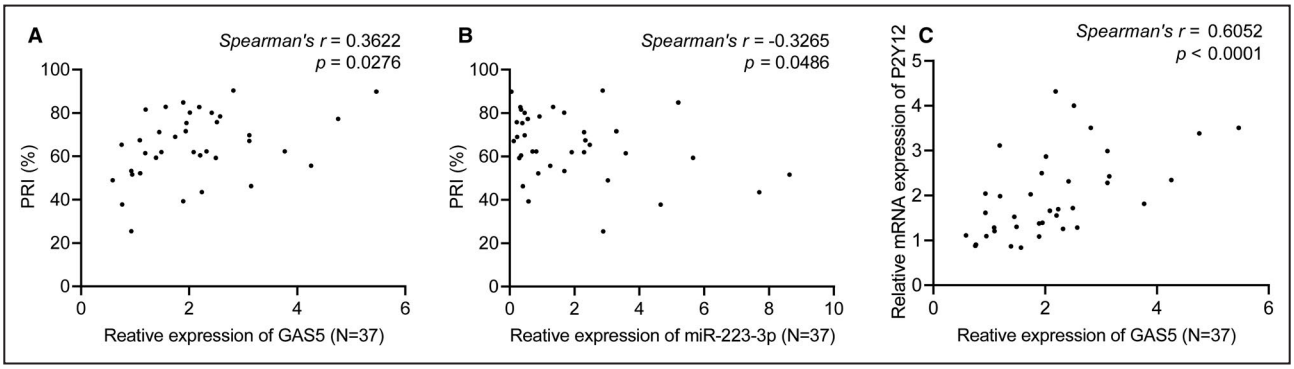

Figure 2. Relationship of platelets *GAS5* (growth arrest-specific 5) expression and microRNA-223-3p expression with clopidogrel response in patients with coronary artery disease (n=37).

(**A** and **B**) Correlation of platelet reactivity index with *GAS5* expression (**A**) and microRNA-223-3p expression (**B**) in platelets. (**C**) Correlation of *P2Y12* mRNA expression with *GAS5* expression in platelets. *P* values based on *Spearman* rank correlation test.

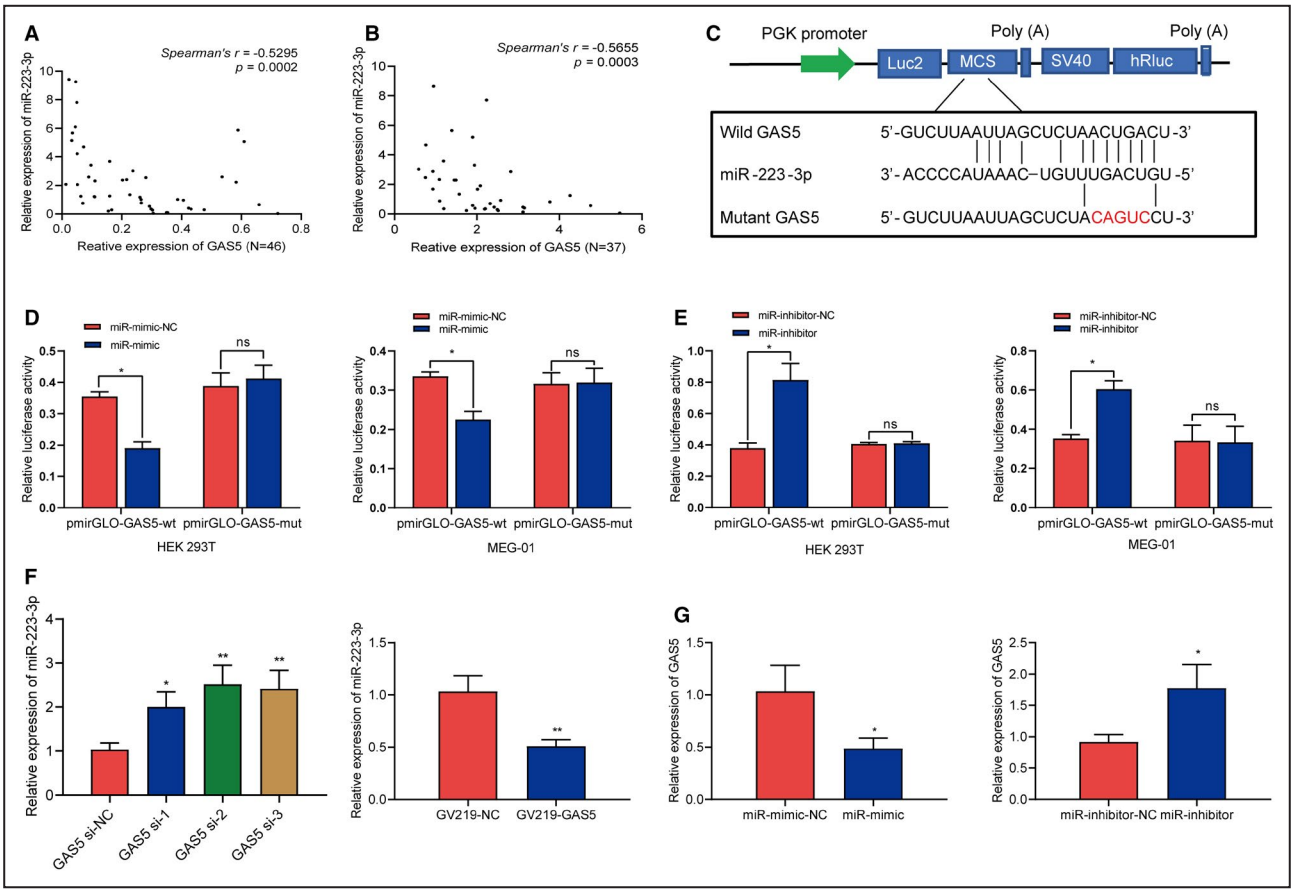

Figure 3. Long non-coding RNA GAS5 (growth arrest-specific 5) acted as a competitive endogenous RNA for microRNA-223-3p (miR-223-3p).

(**A** and **B**) Correlation of miR-223-3p expression with GAS5 expression in crudely extracted platelets (n=46) (**A**) and microbeads purified platelets (n=37) (**B**). (**C**) The potential binding sites of GAS5 and miR-223-3p. (**D**) Relative luciferase activity detected in HEK 293T (left) and MEG-01 (right) cells after co-transfection with miR-223-3p mimic or control and pmirGLO-GAS5-wt or pmirGLO-GAS5-mut constructs, respectively. (**E**) Relative luciferase activity detected in HEK 293T (left) and MEG-01 (right) cells after co-transfection with miR-223-3p inhibitor or control and pmirGLO-GAS5-wt or pmirGLO-GAS5-mut constructs, respectively. (**F**) Expression of miR-223-3p after knockdown (left) or overexpression (right) of GAS5 in MEG-01 cells. (**G**) Expression of GAS5 after overexpression (left) or knockdown (right) of miR-223-3p in MEG-01 cells. Data were presented as median (interquartile range). miR indicates microRNA; GAS5, growth arrest specific 5; HEK, human embryonic kidney cells; MEG, megakaryoblastic leukemia cell line; miR-mimic-NC, miR-223-3p-mimic-NC; miR-mimic, miR-223-3p-mimic; miR-inhibitor-NC, miR-223-3p-inhibitor-NC; miR-inhibitor, miR223-3p-inhibitor; NC, negative control; ns, not significant; pmirGLO-GAS5-wt, pmirGLO-GAS5-wild type; si-NC, non-silencing control siRNA; si-1, small interfering RNA-1; si-2, small interfering RNA-2; si-3, small interfering RNA-3, *P<0.05, **P<0.01. P values based on *Spearman* rank correlation test (**A** and **B**) or Student t-test (**D** through **G**).

diseases by controlling transcription, chromatin modification, splicing, and other biological processes.[61] *GAS5* is a typical lncRNA found to be involved in the development and prognosis of a variety of diseases. For instance, *GAS5* expression was significantly lower and exhibited predictive values for the occurrence and recurrence in chronic heart failure patients.[36] Meng et al. reported that *GAS5* expression was significantly higher in (a human monocytic cell line derived from the peripheral blood of a childhood case of acute monocytic leukemia) THP-1 macrophage-derived foam cells and that the knockdown of *GAS5* could decrease enhancer of zeste homolog 2 (EZH2)-mediated transcriptional regulation of ATP binding cassette subfamily A member 1 (ABCA1) via histone methylation to prevent the development of atherosclerosis.[62] Genetic variations of lncRNAs, such as SNPs, have drawn significant attention in recent years. For example, prostate cancer associated transcript 19 (PCAT19) rs11672691 was associated with aggressive prostate cancer and the rs11672691 region was bifunctional with both promoter and enhancer.[63,64] In our previous study, we found that patients with acute myeloid leukemia with rs55829688 CC genotypes exhibited higher expression of *GAS5* in peripheral blood mononuclear cells and harbored longer recovery times than T allele carriers.[29] However, the roles of *GAS5* and

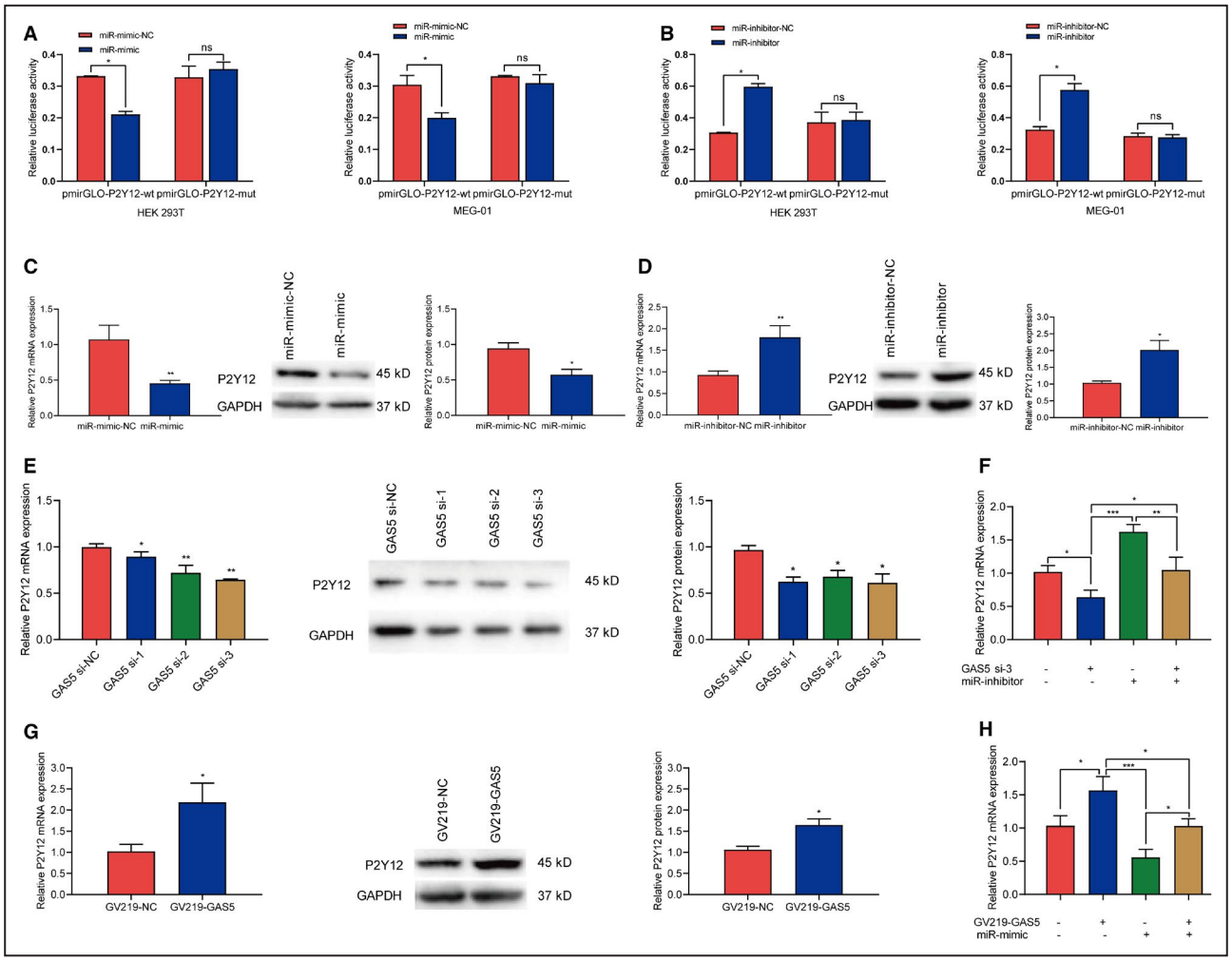

Figure 4. GAS5 (growth arrest-specific 5) regulated the expression of P2Y12 by sponging microRNA-223-3p (miR-223-3p). (**A**) Relative luciferase activity detected in HEK 293T (left) and MEG-01 (right) cells after co-transfection with miR-223-3p mimic or control and pmirGLO-P2Y12-wt or pmirGLO-P2Y12-mut constructs, respectively. (**B**) Relative luciferase activity detected in HEK 293T (left) and MEG-01 (right) cells after co-transfection with miR-223-3p inhibitor or control and pmirGLO-P2Y12-wt or pmirGLO-P2Y12-mut constructs, respectively. (**C**) Expression of P2Y12 mRNA (left) and protein (middle and right) levels after overexpression of miR-223-3p in MEG-01 cells. (**D**) Expression of P2Y12 mRNA (left) and protein (middle and right) levels after knockdown of miR-223-3p in MEG-01 cells. (**E**) mRNA (left) and protein (middle and right) levels of P2Y12 after knockdown of GAS5 in MEG-01 cells. (**F**) *P2Y12* mRNA expression level in MEG-01 cells after co-transfection with GAS5 siRNA and miR-223-3p inhibitor. (**G**) mRNA (left) and protein (middle and right) levels of P2Y12 in MEG-01 cells after GAS5 overexpression. (**H**) *P2Y12* mRNA expression level in MEG-01 cells after co-transfection with GV219-GAS5 and miR-223-3p mimic. Data were presented as means±SD. miR indicates microRNA; HEK, Human Embryonic Kidney cells; MEG, megakaryoblastic leukemia cell line; miR-mimic-NC, miR-223-3p-mimic-NC; miR-mimic, miR-223-3p-mimic; miR-inhibitor-NC, miR-223-3p-inhibitor-NC; miR-inhibitor, miR-223-3p-inhibitor; NC, negative control; ns, not significant; pmirGLO-P2Y12-wt, pmirGLO-P2Y12-wild type; P2Y12, purinergic receptor P2Y12; si-NC, non-silencing control siRNA; si-1, small interfering RNA-1; si-2, small interfering RNA-2; si-3, small interfering RNA-3, **P*<0.05, ***P*<0.01 and ****P*<0.001. *P* values based on Student *t*-test (**A** through **E** and **G**) and 1-way ANOVA corrected by Bonferroni multiple comparisons test (**F** and **H**).

rs55829688 in clopidogrel response remain unclear. Herein, we identified no difference exhibited in PRI of different rs55829688 genotypes. However, the clopidogrel response might be increased in the *GAS5* rs55829688 CC homozygotes compared with TT homozygotes in *CYP2C19* PMs, which should be further clarified in larger cohorts.

Platelets are a component of blood that can respond to abnormalities on the vessel wall and may result in inappropriate platelet adhesion/activation and thrombosis. Despite anucleate status, platelets still possess a rich repertoire of RNAs, including mRNAs, lncRNAs, and miRNAs.[65,66] Recent studies have shown some platelet miRNAs levels, such as miR-265-3p, correlate with HTPR in patients with CAD,[67] which indicates that gene expression in platelets might be associated with platelet activity. This study found that the platelet *GAS5* expression positively correlated with PRI and

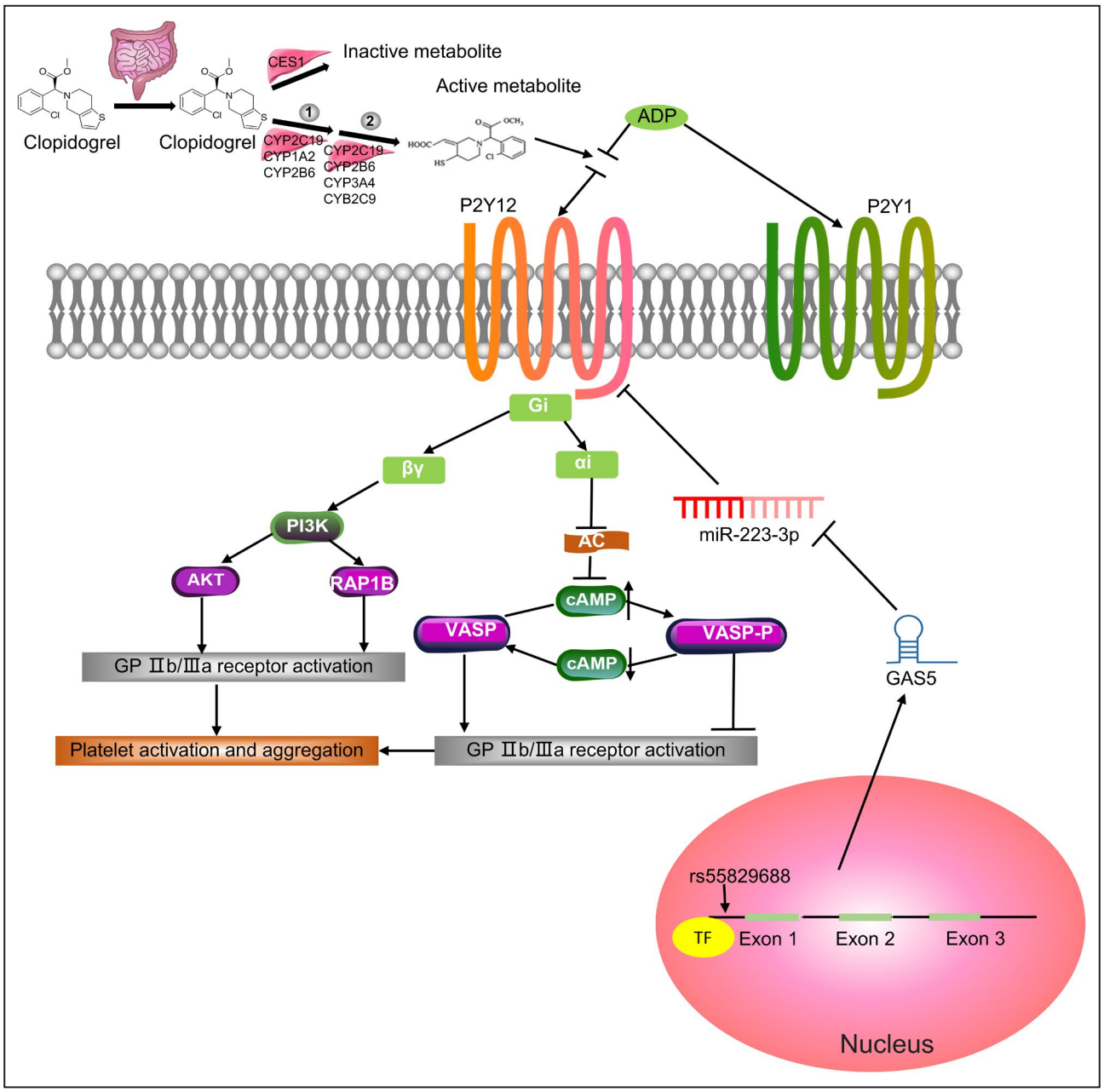

Figure 5. Hypothetical model illustrates *GAS5* (growth arrest-specific 5) affects clopidogrel response through regulating the expression of P2Y12 by sponging microRNA-223-3p.
AC indicates adenylyl cyclase; ADP, adenosine diphosphate; AKT, Serine/threonine protein kinase B; cAMP, cyclic adenosine monophosphate; CES1, carboxylesterase 1; GAS5, growth arrest-specific 5; Gi, inhibitory G protein; GP IIb/IIIa, glycoprotein IIb/IIIa; P2Y1, purinergic receptor P2Y1; P2Y12, purinergic receptor P2Y12; PI3K, phosphatidylinositol 3-kinase; RAP1B, member of RAS oncogene family; TF, transcription factor; VASP, vasodilator-stimulated phosphoprotein; VASP-P, vasodilator-stimulated phosphoprotein phosphorylation; αi, Gi protein subunit αi; and βγ, Gi protein subunit βγ.

mRNA expression of *P2Y12*, which is the direct target of active clopidogrel metabolite in patients with CAD. For the first time, these findings suggest that *GAS5* rs55829688 polymorphism and its expression level might serve as potential clopidogrel response predictors in patients with CAD.

To further illustrate the possible mechanism of *GAS5* in clopidogrel response, multiple analyses were conducted to elucidate the *GAS5* pathway in platelets. Online predicting tool ENCORI was applied to predict the possibility that miRNAs possibly bind with *GAS5*. Among all the predicted miRNAs, miR-223-3p was reported to target P2Y12[34,35] and be one of the most abundant miRNAs released by activated platelets,[30] which has drawn our attention. We revealed that the miR-223-3p expression correlated negatively with PRI, consistent with the

previous reports that increased expression of platelet miR-223-3p and circulating miR-223-3p of patients with CAD were associated with higher sensitivity to clopidogrel.[32,68] Further analyses revealed that *GAS5* expression was negatively correlated with miR-223-3p expression in platelets. In this case, we assumed that inner interactions among *GAS5*, miR-223-3p, and P2Y12 might exist to regulate clopidogrel response.

Numerous studies have shown that *GAS5* is involved in multiple biological processes by acting as a competitive endogenous RNA (ceRNA) for miRNAs to regulate downstream target gene expression. For example, *GAS5* suppresses the invasion and EMT of uveal melanoma by sponging miR-21,[69] and inhibits the migration and invasion of colorectal cancer by targeting miR-222-3p.[70] Previous studies also showed that *GAS5* could serve as a ceRNA for miR-223-3p in myocardial cells[36] and endothelial progenitor cells.[38] However, whether *GAS5* plays a similar role in megakaryocytic cells MEG-01 is still unknown. This study confirmed the interaction between *GAS5* and miR-223-3p by dual-luciferase reporter assay in HEK 293T cells and MEG-01 cells. Our gain-of-function and loss-of-function studies demonstrated that *GAS5* expression could be regulated by miR-223-3p, while miR-223-3p expression could also be regulated by *GAS5* in MEG-01 cells, which suggest that *GAS5* acts as a ceRNA for miR-223-3p in MEG-01 cells.

P2Y12 is a receptor for ADP and is coupled to a Gi (inhibitory G) protein, and the activation of P2Y12 by ADP leads to the inhibition of adenylyl cyclase and activation of phosphoinositide-3-kinase. Inhibition of adenylyl cyclase reduces the level of cAMP and then decreases the phosphorylation level of VASP, promoting the activation of glycoprotein IIb/IIIa. Activation of phosphoinositide-3-kinase stimulates PKB/AKT (serine/threonine protein kinase B) and Rap1b GTP-binding proteins, promoting the activation of glycoprotein IIb/IIIa lead to the platelet activation and aggregation.[11] The active metabolite of clopidogrel is an antagonist of P2Y12 which leads to the inhibition of platelet activation and aggregation.[10] Recently, studies have revealed that P2Y12 is a target of miR-223-3p.[34,35] However, the regulatory effect of miR-223-3p to P2Y12 in MEG-01 cells is unclear. In this study, we repeated the interaction between miR-223-3p and P2Y12 through dual-luciferase reporter assay and verified that the P2Y12 expression could be regulated by miR-223-3p in MEG-01 cells. Furthermore, we discovered that *GAS5* could upregulate P2Y12 expression, which was reversed by miR-223-3p, indicating the regulatory effect of *GAS5* to P2Y12 by relieving the posttranscriptional suppression of miR-223-3p. Therefore, we clarified a new potential mechanism by which *GAS5* acts as a ceRNA for miR-223-3p to regulate P2Y12 expression and clopidogrel response in CAD.

Taken together, our findings suggest that the *GAS5* rs55829688 polymorphism may affect the antiplatelet potency of clopidogrel in *CYP2C19* poor metabolizers and that *GAS5* regulates P2Y12 expression and clopidogrel response by acting as a ceRNA for miR-223-3p (Figure 5). However, there are also several limitations to our study. First, we only used PRI to indicate the role of *GAS5* in clopidogrel-induced inhibition of platelet activity. An animal model should be used to detect the effect of *GAS5* on platelet activation and aggregation more directly in the future. Second, the calculation of power test in poor metabolic cohort indicated that the power (1-β) was 0.6854 in comparison between TT and CC group, which is less than 0.8 and indicated there might be a false negative result attributable to small sample size. Hence, the observed possible effect of the *GAS5* rs55829688 polymorphism on clopidogrel response in *CYP2C19* poor metabolizers requires further verification in larger cohorts of patients with CAD.

CONCLUSIONS

Our study demonstrates that the *GAS5* rs55829688 polymorphism may affect the antiplatelet efficacy of clopidogrel in *CYP2C19* poor metabolic patients with CAD in China, and *GAS5* may regulate P2Y12 expression and affect clopidogrel response by functioning as a ceRNA for miR-223-3p. Meanwhile, our findings provide a novel viewpoint that particular lncRNA expression and genetic polymorphism may serve as biomarkers for clopidogrel response, and they establish a new relationship among lncRNA, miRNA and proteins in the regulation of platelet reactivity.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Table S1
Figure S1–S3

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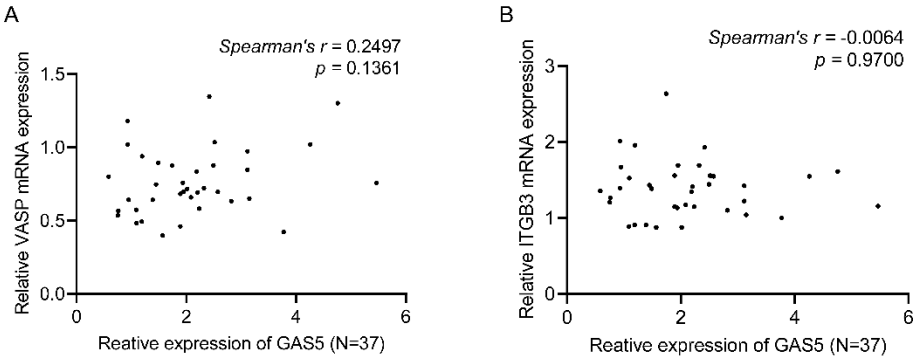
SUPPLEMENTAL MATERIAL

Table S1. Oligonucleotide Sequences used in this study.

Gene		Sequence
PCR-RFLP primers		
*CYP2C19*2*	Sense	5'-TCAGAGGCTGCTTGATAGAAATC-3'
	Antisense	5'-CCTTGACCTGTTAAACATCCGTA-3'
*CYP2C19*3*	Sense	5'-CTTCACCCTGTGATCCCACT-3'
	Antisense	5'-AAACATGCCAATTCAGCACA-3'
GAS5 rs55829688	Sense	5'-ACATATGGTGCATGCGTGAC-3'
	Antisense	5'-TCACGGCTTGTAATCCCAGT-3'
Vector construction primers		
pmirGLO-GAS5-wt-primer	Sense	5'-tgtttaaacgagctcgctagcTTACCTCCTAGTGCTGAATGCATT-3'
	Antisense	5'-tgcctgcaggtcgactctagaGTCAGACATTTGATCAACATCATTACA-3'
pmirGLO-P2Y12-wt-primer	Sense	5'-tgtttaaacgagctcgctagcGTGTTCAGAACTCGTTAAAGCAAAG-3'
	Antisense	5'-tgcctgcaggtcgactctagaTAGGTCAGGATTTGGTTAGGGG-3'
pmirGLO-GAS5-mut-primer	Sense	5'-TCTTAATTAGCTCTAcagtcCTAAAGGCATTTGTT-3'
	Antisense	5'-AACAAATGCCTTTAGgactgTAGAGCTAATTAAGA-3'
pmirGLO-P2Y12-mut-primer	Sense	5'-TAAGTAAAAATATTAcagtcCGAAGAAGCAACTAA-3'
	Antisense	5'-TTAGTTGCTTCTTCGgactgTAATATTTTTACTTA-3'
Oligonucleotides sequences		
GAS5-si-1	Sense	5'-GACCUGUUAUCCUAAACUATT-3'
	Antisense	5'-TAGTTTAGGATAACAGGTCTT-3'
GAS5-si-2	Sense	5'-GCAGACCUGUUAUCCUAAATT-3'
	Antisense	5'-UUUAGGAUAACAGGUCUGCTT-3'
GAS5-si-3	Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
	Antisense	5'-ACGUGACACGUUCGGAGAATT-3'
miR-223-3p-mimic	Sense	5'-UGUCAGUUUGUCAAAUACCCCA-3'
	Antisense	5'-UGGGGUAUUUGACAAACUGACA-3'
miR-223-3p-inhibitor	Sense	5'-UGGGGUAUUUGACAAACUGACA-3'
qPCR primers		
GAPDH	Sense	5'-CTGCACCACCAACTGCTTAG-3'
	Antisense	5'-AGGTCCACCACTGACACGTT-3'
GAS5	Sense	5'-CCTGTGAGGTATGGTGCTGG-3'
	Antisense	5'-CTGTGTGCCAATGGCTTGAG-3'
P2Y12	Sense	5'-CACTGCTCTACACTGTCCTGT-3'
	Antisense	5'-AGTGGTCCTGTTCCCAGTTTG-3'

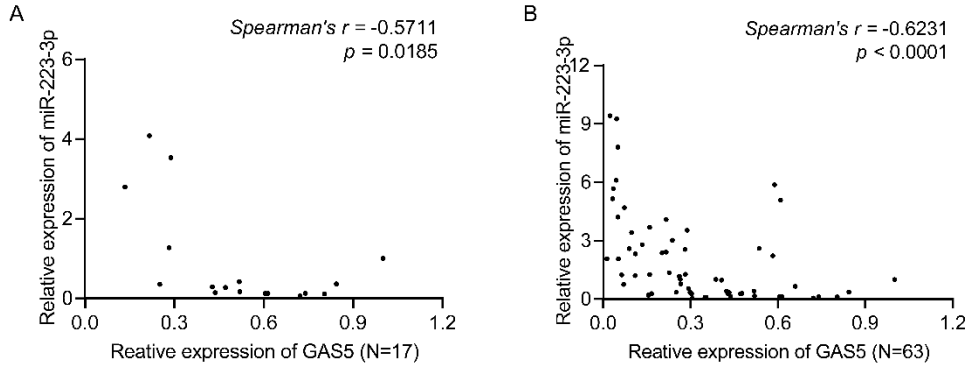
PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; CYP2C19, cytochrome P450 family 2 subfamily C member 19; P2Y12, purinergic receptor P2Y12; GAS5, growth arrest specific 5; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction

Figure S1. The correlation of GAS5 expression and mRNA expression of VASP and ITGB3.



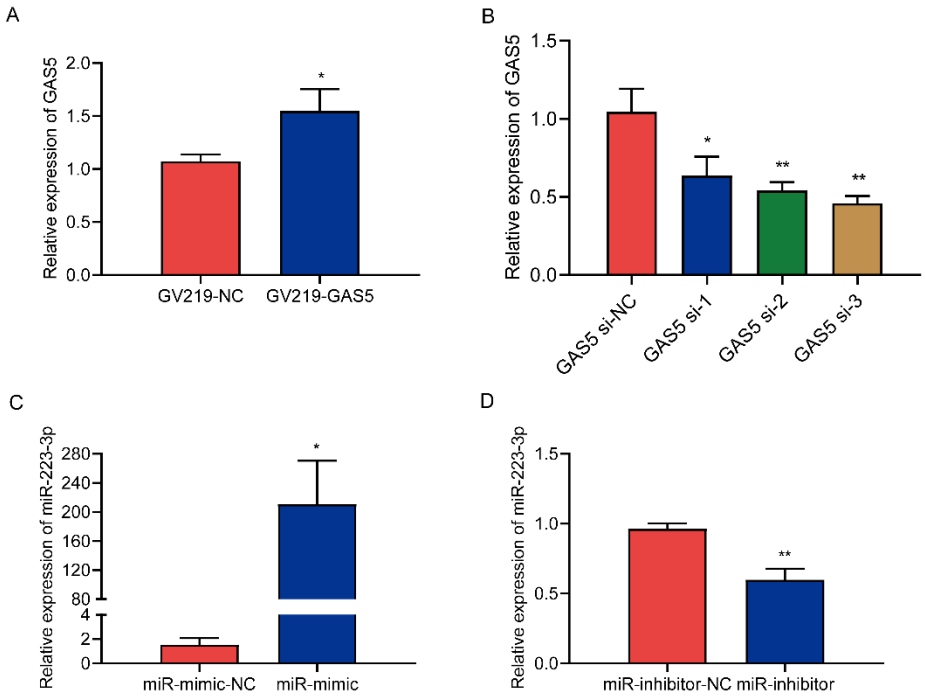
The correlation of GAS5 expression and mRNA expression of VASP (A) and ITGB3 (B). *P* values based on *Spearman's* rank correlation test.

Figure S2. The correlation of miR-223-3p expression and GAS5 expression.



The correlation of miR-223-3p expression and GAS5 expression in PRP of 17 healthy volunteers (A) and the combined subjects (17 healthy volunteers and 46 CAD patients) (B). PRP, platelet-rich plasma. *P* values based on *Spearman's* rank correlation test.

Figure S3. The efficiency of overexpression and interference.



(A) The efficiency of GAS5 overexpression. (B) The efficiency of GAS5 interference. (C) The efficiency of miR-223-3p overexpression. (D) The efficacy of miR-223-3p interference. Data were presented as mean ± SD. miR-mimic-NC, miR-223-3p-mimic-NC; miR-mimic, miR-223-3p-mimic; miR-inhibitor-NC, miR-223-3p-inhibitor-NC; miR-inhibitor, miR-223-3p-inhibitor. *$p < 0.05$, **$p < 0.01$. *P* values based on Student's *t*-test (A, B and D) and Welch's *t*-test (C).