ORIGINAL ARTICLE

The expression profile of platelet-derived miRNA in coronary artery disease patients with clopidogrel resistance

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Abstract

Clopidogrel is widely used for antiplatelet therapy in patients with coronary artery disease (CAD), but clopidogrel resistance (CR) is relatively common in these patients. The goal of our study was to explore the platelet-derived miRNA expression profile of CR in CAD patients. In this study, 66 CAD patients treated with dual antiplatelet therapy (clopidogrel 75 mg once daily plus aspirin 100 mg once daily) were included. According to inhibition of platelet aggregation (IPA), we divided these patients into CR group (IPA <30%) and control group (IPA ≥30%). The concentrations of clopidogrel and clopidogrel active metabolites in plasma were obtained using UHPLC-Q-Orbitrap HRMS method. The platelet-derived miRNA expression profiles of these subjects were detected by high-throughput sequencing and qRT-PCR. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used for function prediction of differentially expressed miRNAs. Our results suggested no significant difference of clopidogrel and active metabolic derivative concentrations between CR group and control group. Correlation analysis showed no significant association between clopidogrel concentration and IPA; active metabolic derivative and IPA. In addition, 67 platelet-derived miRNAs were differentially expressed between three CR and three control patients. After adjusting, eight miRNAs might be related to CR in CAD. In our validation cohort (30 CR patients and 30 control group), miRNA-142-3p and miRNA-24-3p expression levels were significantly upregulated, and miRNA-411-3p expression was significantly downregulated in the CR group. In conclusion, the miRNA-142-3p, miRNA-24-3p, and miRNA-411-3p might be potential markers for CR in CAD patients.

KEYWORDS
antiplatelet therapy, clopidogrel resistance, coronary artery disease, platelet-derived miRNA

1 | INTRODUCTION

Coronary artery disease (CAD) is the common cause of death in the world, which is featured on myocardial ischemia and hypoxia caused by atherosclerotic plaque and stenosis.[1] Once the coronary atherosclerotic plaque ruptures, a mass of subendothelial matrix exposes and causes platelet activation and aggregation, eventually giving rise to arterial thrombosis and stenosis.[2] It follows that platelet plays a central role in this complicated pathological process of CAD. And antiplatelet therapy is an effective treatment, which could improve the prognosis of CAD patients. In the current clinical practice, antiplatelet drugs mainly include thromboxane A2 inhibitor, platelet glycoprotein IIb/IIIa receptor antagonist, P2Y12 receptor inhibitor, etc.[3]

Clopidogrel is a frequently used P2Y12 receptor inhibitor, which needs to be metabolized into active products in vivo to exert the antiplatelet role. The active product could bind to the P2Y12 receptor irreversibly, and inhibit ADP-induced platelet aggregation selectively.[4–7] Previous studies have shown that clopidogrel could effectively reduce the incidence of myocardial infarction and hospitalization rate.[8] Although the antiplatelet effect of clopidogrel has been recognized, different response to clopidogrel exists among individuals, even taking the same dose of clopidogrel.[9] There is an apparent racial discrepancy in the responsiveness of different populations to clopidogrel.[10] Low response or no response to clopidogrel might exist in 4%–30% of patients, which is called clopidogrel resistance (CR).[11] And CR is associated with a higher incidence of clinical adverse events (cardiovascular death, reinfarction, or severe bleeding). However, the exact mechanisms of the diversity of platelet response to clopidogrel are still unclear.

The miRNAs are small endogenous non-coding RNAs that are highly conserved genetically.[12] It can bind with the 3' untranslated region (UTR) of mRNA to promote mRNA degradation or inhibit transcription.[13] Platelets are small pieces of cytoplasm derived from mature megakaryocytes. Though there is no nucleus in the platelet, a large number of transcripts and miRNAs exist to participate in the regulation of transcription and translation in the platelet.[14,15] Mounting evidence indicates that platelet-derived miRNA plays a regulatory role in various biological functions and diseases including cardiovascular diseases.[16,17] For instance, platelet-derived miR-22/-320b can regulate the expression of proinflammatory mediator ICAM1 in ST segment elevation myocardial infarction patients.[18] In diabetic mice, decreased expression of platelet-derived miR-223 could enhance dedifferentiation of vascular smooth muscle cells and abnormal hyperplasia of vascular intima.[19] Unfortunately, the evidence of association between platelet-derived miRNAs and CR is scarce.

In the present study, we are dedicated to exploring the platelet-derived miRNA expression profile of CR in CAD patients and predicting their functions by GO enrichment and KEGG pathway analysis. In addition, the expression of four platelet-derived miRNAs was validated in a large cohort. However, further studies should be conducted to clarify the relationship and mechanism between platelet-derived miRNAs and CR in CAD patients.

2 | MATERIALS AND METHODS

2.1 | Study population

In this study, 66 CAD patients were recruited from the Ningbo First Hospital through August 2018 to January 2019. All the patients were diagnosed as CAD according to the latest clinical guideline and have undergone percutaneous coronary interventions. Patients were subsequently treated with dual antiplatelet therapy (clopidogrel 75 mg once daily plus aspirin 100 mg once daily) for 7 days to stabilize the effect of antiplatelet aggregation. This study was approved by the Ethics Committee of Ningbo First Hospital. All the subjects have signed the informed consent form.

2.2 | Sample collection and storage

We collected 10 ml blood sample from all the patients after taking clopidogrel for 3 hours. Subsequently, 5 ml blood sample was used to sort platelets by magnetic activated cell sorting (MACS) and then stored in a refrigerator at −80℃. The tests of platelet function, clopidogrel concentration, and clopidogrel active metabolite concentration in plasma were performed within 4 h.

2.3 | Platelet function

The platelet function test was performed using VerifyNow analyzer (Accumetrics). At present, there is no uniform standard for the definition of CR. Common concepts include clinical CR and laboratory CR.[20,21] Clinical CR is generally considered to be that thromboembolic events still occur under standard clopidogrel treatment. The laboratory CR is identified by the results of platelet function testing. Unfortunately, it still lacks a uniform and reasonable threshold for IPA to determine CR. A review of previous studies suggested that we could distinguish CR by the cut-off value of inhibition of platelet aggregation (IPA) between 10% and 40%.[22] Thus, we used an empirically defined cut-off value of 30% based on our patients' conditions. The subjects with IPA <30% were included in the CR group and the subjects with IPA ≥30% were included in the control group.

2.4 | The concentration of clopidogrel and clopidogrel active metabolites

We added 40 µl 2,2'-Methylenebis (6-tert-butyl-4-methylphenol) into the blood sample in heparin anticoagulant tubes and separated plasma by centrifugation at 4000 r/min for 5 min. The concentrations of clopidogrel and clopidogrel active metabolites in plasma were determined using high-performance liquid chromatography quadrupole-Orbitrap mass spectrometry (UHPLC-Q-Orbitrap HRMS).

2.5 | Separation and purification of platelets and RNA isolation

MidiMACS™ magnetic cell sorting (Miltenyi) was used to obtain the purified platelets. The platelet-rich plasma samples were added in TRIZOL (Thermo Fisher) for 7 min at room temperature, and then used RNA-Solv reagent (Omega bio-tek) to isolate the total miRNA.

2.6 | miRNA high-throughput sequencing and function prediction

The platelet-derived miRNA expression profiles of three CR patients and three control patients were detected by using BGISEQ-500 sequencing platform (Huada Gene). After cDNA library construction, the high-throughput sequencing of the cDNA was accomplished by Combinatorial Probe Anchor Synthesis (cPAS). The differentially expressed miRNAs between the CR and control groups were identified by DEGseq package. The biological functions of differentially expressed miRNAs were analyzed by Gene Ontology (GO). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was used to determine the signal pathways involved in the corresponding target genes of miRNAs.

2.7 | Quantitative reverse transcription-polymerase chain reaction

Reverse transcription reaction was performed in the GoScript RT System (Promega). The real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using SYBR Green qPCR SuperMix Kit (Invitrogen) on the Mx3005P QPCR System (Stratagene) following the manufacturer's instructions. The primers are listed in Table 1. The $2^{-\triangle\triangle Ct}$ method was used to analyze the data.

2.8 | Statistical analysis

The primary endpoint was defined as the incidence of CR.[23] The power for the primary endpoint weight change is calculated based on a two-sided t-test with a significance level of 5%. The power with regard to the co-primary dichotomous endpoints was calculated based on a two-sided Chi-square test. With a sample size of 30 CR patients and 30 controls, the trail might have more than 90% power

to detect the difference between CR and controls. Our conclusions need further verification in a wide range of population.

SPSS version 18.0 (SPSS, Inc.) was used for statistical analysis. Normally distributed quantitative variables were expressed by mean ± standard deviation, which were tested by t test when comparing differences between two groups. Non-normally distributed quantitative variables were expressed as medians and interquartile ranges, which were assessed using the Wilcoxon rank sum test when comparing differences between the two groups. Pearson correlation was used to evaluate the correlation between IPA and the concentrations of clopidogrel and clopidogrel active metabolites. $p < .05$ was considered a statistically significant difference.

TABLE 2 The clinical characteristics of control and clopidogrel-resistant patients

Characteristics	CR (n = 33)	Control (n = 33)	p Value
Age, years	60.91 ± 10.96	63.33 ± 11.16	.377
Male	25	23	.583
Smoking	19	17	.624
Hypertension	16	20	.326
Diabetes mellitus	7	10	.402
WBC counts, 10^9/L	8.16 ± 3.15	7.74 ± 3.56	.617
Hemoglobin, g/L	136.21 ± 19.30	139.15 ± 14.50	.487
Platelet counts, 10^9/L	242.39 ± 78.10	215.39 ± 50.70	.101
Serum creatinine, μmol/L	74 (65.5, 81)	71 (66, 82)	.542
LDL-C, mmol/L	2.73 ± 0.61	2.60 ± 0.67	.44
Total cholesterol, mmol/L	4.29 ± 0.89	4.13 ± 0.88	.482
HDL-C, mmol/L	1.03 ± 0.23	0.95 ± 0.24	.204
Triglyceride, mmol/L	1.35 (1.00, 1.97)	1.53 (1.20, 2.16)	.323
CRP, mg/L	3.45 (1.04, 10.56)	3.10 (1.03, 10.13)	.863
ALT, U/L	26.00 (17.50, 45.00)	28.00 (17.50, 40.50)	.954
AST, U/L	26.00 (19.50, 44.00)	26.00 (19.00, 72.00)	.817

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

TABLE 1 The sequences of primers used for qRT-PCR

	Forward	Reverse
miR-3184-3p	5′-AATAGAAAAGTCTCGCTC-3′	5′-AAGTTAGGCTGAGGGGCA-3′
miR-24-3p	5′-ACAGCAGGCACAGAGAGGGG-3′	5′-CTGGCTCAGTTCAGCAGGAACAG-3′
miR-142-3p	5′-GTCGTATCCAGTGCAGGG-3′	5′-CGACGTGTAGTGTTTCCTA-3′
miR-411-3p	5′-CCGAGTATGTAACACGGTC-3′	5′-TATGTAACACGGTCCACTAACC-3′
GAPDH	5′-GCACCGTCAAGGCTGGAAC-3′	5′-TGGTGAAGCGCCAGTGGA-3′

TABLE 3 The platelet-derived miRNAs with statistically significant difference in expressions between the clopidogrel resistance group and control group

miRNA	Log$_2$ FC	p value	miRNA	Log$_2$ FC	p value
Upregulated			Downregulated		
has-miR-103a-2-5p	2.13	.035	hsa-miR-150-5p	1.04	.046
hsa-miR-548av-3p	3.77	.034	hsa-miR-11401	2.60	.024
hsa-miR-3184-3p	6.54	<.001	hsa-miR-6749-3p	1.57	.028
hsa-miR-17-5p	0.91	.013	hsa-miR-100-5p	2.93	.001
hsa-miR-339-5p	1.06	.033	hsa-miR-483-3p	3.68	.005
hsa-miR-454-3p	1.48	.001	hsa-miR-4647	3.90	.022
hsa-miR-106b-5p	1.15	.026	hsa-miR-4676-5p	2.77	.044
hsa-miR-32-5p	1.08	.019	hsa-miR-3181	4.66	.022
hsa-miR-10401-3p	2.80	.001	hsa-miR-4446-3p	1.69	.003
hsa-miR-140-5p	1.08	.024	hsa-miR-6772-5p	4.05	.034
hsa-miR-30e-5p	1.61	.038	hsa-miR-6850-5p	3.67	.004
hsa-miR-330-5p	2.48	.041	hsa-miR-411-3p	5.53	<.001
hsa-miR-138-5p	3.15	.006	hsa-miR-6732-5p	4.87	.039
hsa-miR-181a-5p	1.19	.026	hsa-miR-6837-5p	1.85	.050
hsa-let-7i-3p	1.89	.014	hsa-miR-27b-5p	1.10	.042
hsa-miR-324-5p	1.97	.022	hsa-miR-23b-5p	1.17	.022
hsa-miR-660-5p	0.77	.045	hsa-miR-1236-3p	2.18	.044
hsa-miR-24-3p	1.86	.001	hsa-miR-2110	1.01	.008
hsa-miR-3074-5p	1.52	.002	hsa-miR-485-5p	1.79	.040
hsa-miR-30d-5p	1.03	.017	hsa-miR-485-3p	1.99	.024
hsa-miR-142-3p	1.51	.001	hsa-miR-654-5p	2.00	.027
hsa-miR-186-5p	1.67	.014	hsa-miR-1180-3p	1.46	.002
hsa-miR-25-3p	0.78	.03	hsa-miR-10a-3p	3.86	<.001
hsa-miR-107	1.69	.048	hsa-miR-378f	7.13	<.001
hsa-miR-378e	3.51	.013	hsa-let-7c-5p	0.86	.047
			hsa-let-7b-5p	0.96	.019
Downregulated			hsa-miR-574-5p	1.09	.022
hsa-let-7d-3p	0.93	.045	hsa-miR-342-5p	2.58	.008
hsa-miR-6819-3p	0.71	.048	hsa-miR-320a-3p	0.87	.039
hsa-let-7e-5p	1.51	.034	hsa-miR-3064-5p	1.61	.035
hsa-miR-490-5p	1.71	.025	hsa-miR-320c	1.07	.040
hsa-miR-6721-5p	2.64	.031	hsa-miR-190b-5p	1.38	.020
hsa-miR-6729-3p	2.98	.032	hsa-miR-1260b	1.34	.013
hsa-miR-505-5p	1.39	.013	hsa-miR-7-5p	1.72	.005

Abbreviation: FC, fold change.

3 | RESULTS

3.1 | Clinical characteristics of the patients and group design

In this study, 66 patients with CAD were enrolled, including 48 males and 18 females. The clinical characteristics of the subjects were shown in Table 2. The results of VerifyNow analysis were shown in Table 3. We used IPA to reflect the inhibition effect of clopidogrel on platelet aggregation function. The 66 patients were divided into CR group (n = 33) and control group (n = 33) with the IPA cutoff of 30%. The median IPA of CR group was 16%, and the median IPA of control group was 50%.

3.2 | Concentrations of clopidogrel and active metabolic derivative

The concentrations of clopidogrel and active metabolic derivative were detected using UHPLC. The results showed that there was no significant difference between the two groups in the concentrations

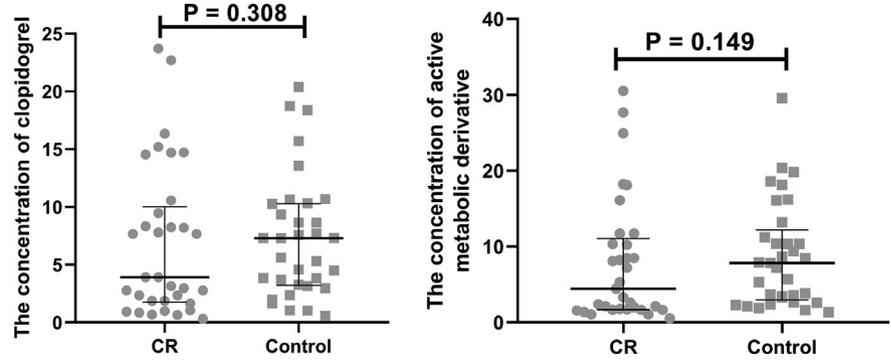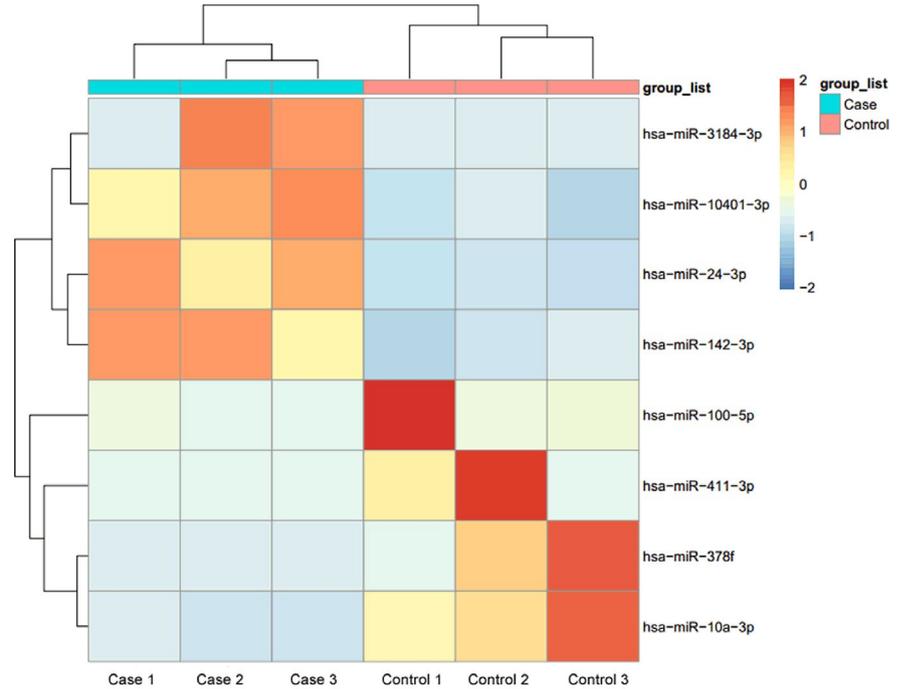FIGURE 1 The concentration of clopidogrel and its active metabolite in CR and control group

FIGURE 2 Heatmap of differentially expressed platelet-derived miRNAs from the control and clopidogrel-resistant patients

of clopidogrel and active metabolic derivative (clopidogrel: p = .308; active metabolic derivative: p = .149, Figure 1). Correlation analysis showed no significant association between clopidogrel concentration and IPA (p = .958); active metabolic derivative and IPA (p = .698).

3.3 | Differential expression of platelet-derived miRNA

There were six samples from the CR group (n = 3) and the control group (n = 3) selected for high-throughput sequencing of platelet-derived miRNAs. A total of 67 differentially expressed miRNAs were identified using DEGseq package in R software (Table 3). After FDR correction, we obtained eight miRNAs with significantly different expression (Figures 2 and 3). Compared with the control group, there were four upregulated miRNAs (has-miRNA-3184-3p, has-miRNA-10401-3p, has-miRNA-24-3p, and has-miRNA-142-3p), and four downregulated miRNAs (has-miRNA-100-5p, has-miRNA-411-3p, has-miRNA-378f, and has-miRNA-10a-3p) in the CR group.
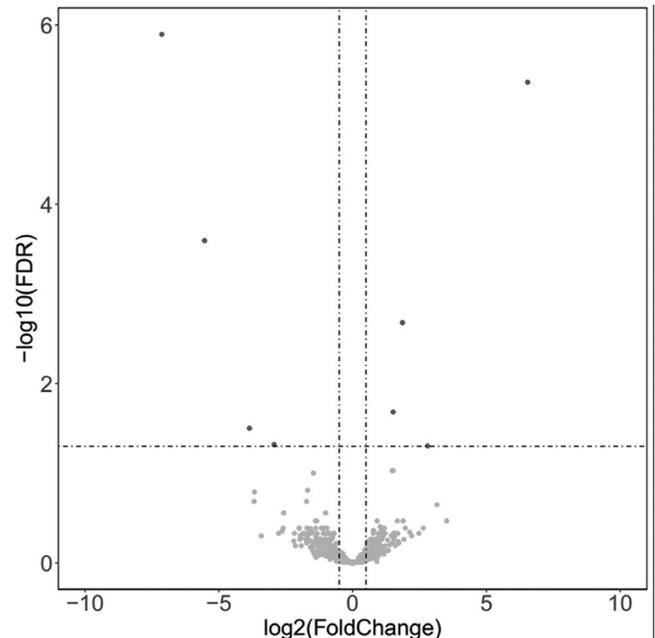

FIGURE 3 Volcano plot of platelet-derived miRNA expression profiles between the control and clopidogrel-resistant patients
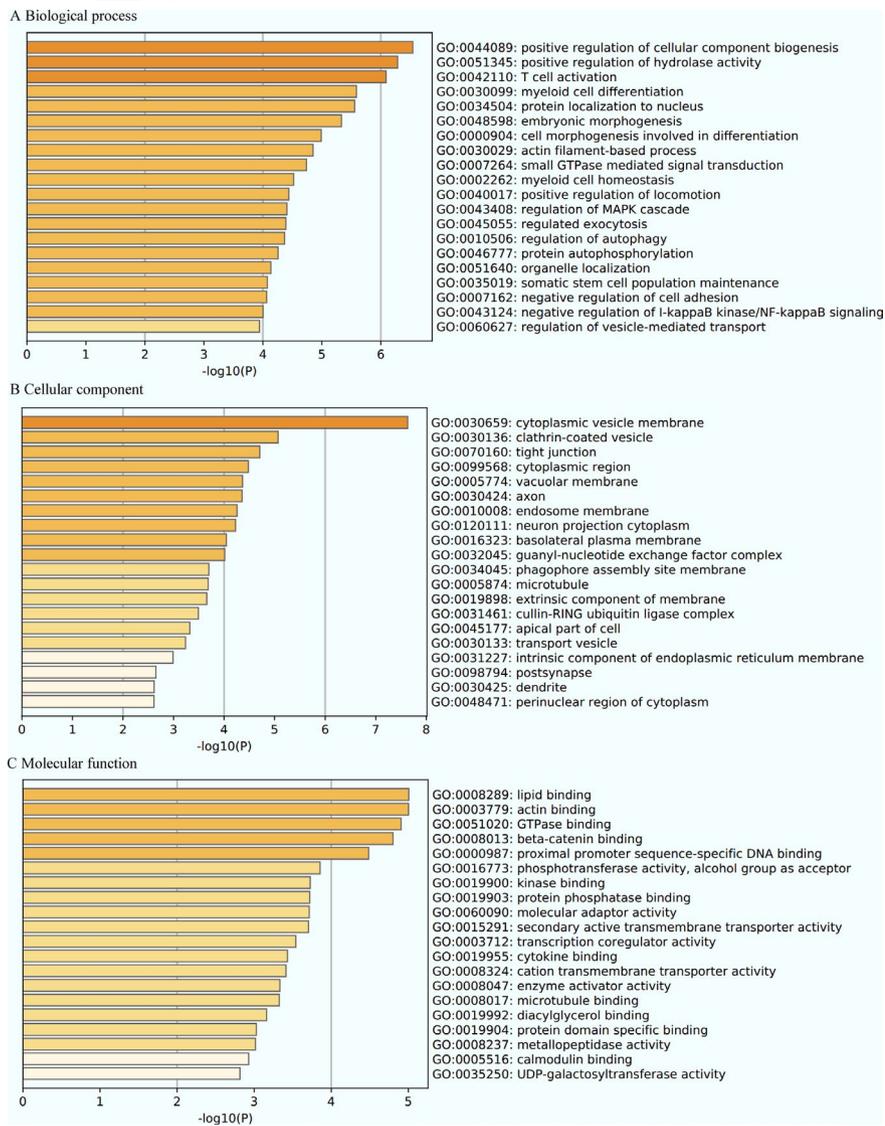
FIGURE 4 Top 20 significantly enriched GO categories for the differentially expressed platelet-derived miRNA target genes. Bar charts show the enrichment of differentially expressed miRNA target genes in biological process (A), cellular component (B), and molecular function (C). Y-axis represents GO category, and x-axis represents enrichment significance
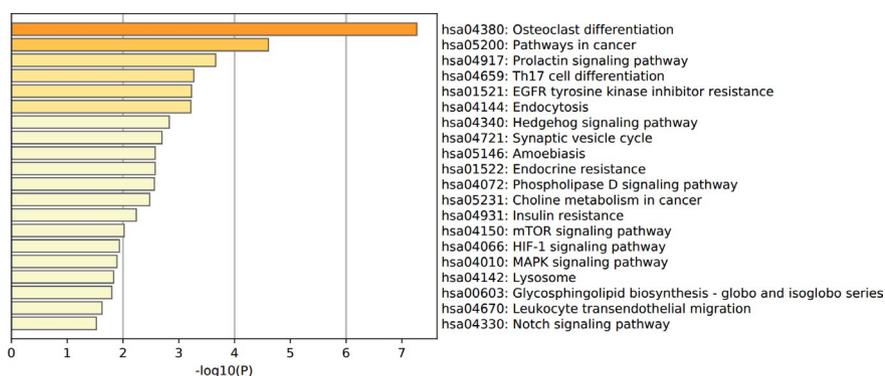

FIGURE 5 Top 20 significantly enriched signaling pathways for the target genes of eight platelet-derived miRNAs

3.4 | GO and KEGG pathway analysis of eight miRNAs target genes

We adopted the GO to determine the main biological functions of eight differently expressed platelet-derived miRNAs corresponding to the target genes. The top 20 significantly enriched results of different categories are shown in Figure 4, including biological process, cellular competent, and molecular function. KEGG enrichment analysis indicated that the target genes were significantly enriched in top 20 signal pathways shown in Figure 5, mainly including osteoclast differentiation, pathways in cancer, prolactin signal pathway, Th17 cell differentiation, EGFR tyrosine kinase inhibitor resistance, Hedgehog signal pathway, phospholipase D signal pathway, insulin resistance, mTOR signal pathway, MAPK signal pathway, Notch signal pathway, etc.

FIGURE 6 Expression levels of four platelet-derived miRNAs in CR and control groups
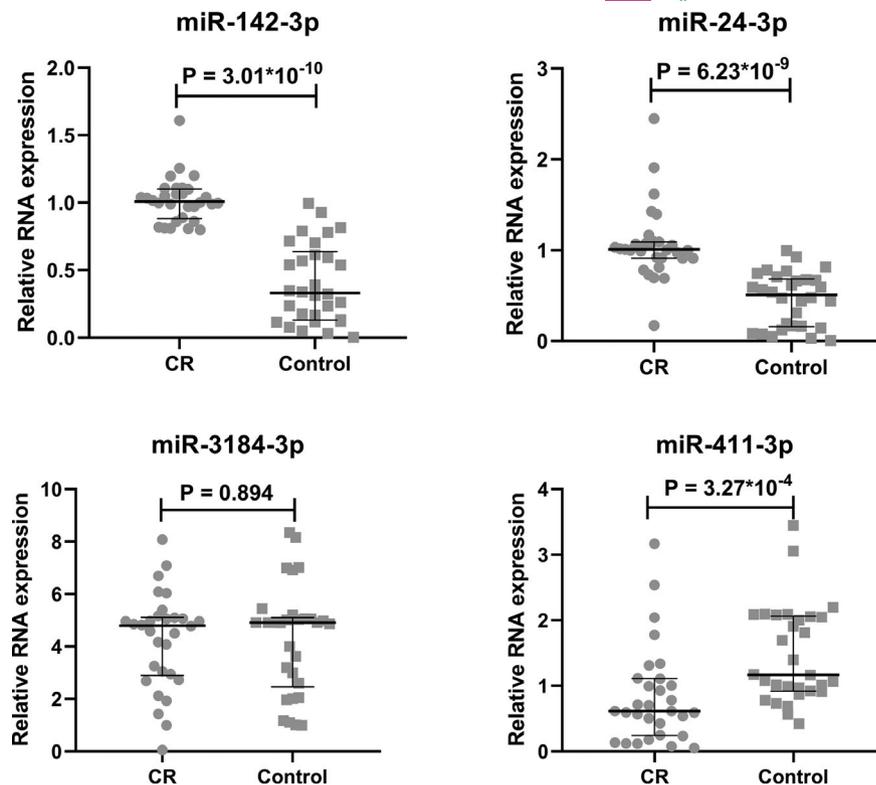
3.5 | Validation of differentially expressed miRNA

To explore the promising biomarkers for CR in CAD patients, we screened four specific miRNA markers (has-miRNA-3184-3p, has-miRNA-24-3p, has-miRNA-142-3p, and has-miRNA-411-3p) for evaluation in the CR group ($n = 30$) and control group ($n = 30$). Our results showed that the expression of has-miRNA-411-3p in CR group was downregulated ($p < .001$). The has-miRNA-142-3p and has-miRNA-24-3p showed significant higher expression levels in the CR group than controls (both $p < .001$, Figure 6). However, there was no significant difference in the has-miRNA-3184-3p expression level between the two groups ($p = .894$).

4 | DISCUSSION

As the widely used for antiplatelet therapy, clopidogrel could effectively reduce the risk of thrombosis. Nevertheless, some patients are resistant to clopidogrel.[24] Previous studies have shown that polymorphisms of *CYP2C19*, *ABCB1*, and *PON1* genes significantly might affect platelet reactivity by influencing the pharmacokinetics of clopidogrel.[25–27] A randomized, open-label, assessor-blinded trial found that patients under *CY2C19* genotype-guided strategy for selection of oral P2Y12 inhibitor therapy resulted in a lower incidence of bleeding but no increased thrombotic events compared with those with standard treatment with ticagrelor or prasugrel.[28] Therefore, gene-guided therapy might be a promising antiplatelet therapy in the future.

Interestingly, there was no significant correlation between IPA and clopidogrel concentration or active metabolite derivative in the current study. Therefore, it is inappropriate to completely explain the difference of platelet reactivity to clopidogrel by affecting the pharmacokinetics of clopidogrel. The causes of CR need to be explored by more biological information. Previous studies have demonstrated that platelet-derived miRNA is related to CR.[29] For example, miR-223, the most abundant miRNA in platelets, was reduced in CR patients, ultimately affecting the expression of *P2Y12*.[30] In our study, we first profiled miRNA expression in the platelet of CR patients and controls. Also, we utilized GO and KEGG analysis to further explore the potential regulatory mechanism between platelet-derived miRNA and CR. By means of synthetic analysis, we selected four candidate miRNAs to validate the expression levels in large cohorts. It suggested that miRNA-142-3p and miRNA-24-3p expression levels were significantly upregulated, and miRNA-411-3p expression was significantly downregulated in the CR group.

In this study, we first reported that miRNA-411-3p was related to the CR in CAD patients. Inflammatory was essential for the hemostatic system in CAD patients, including increased platelet response and endothelial dysfunction, which may affect the antiplatelet efficacy.[31] It is worth noting that increased miRNA-411 suppresses the level of proinflammatory IL-18 by downregulating the JNK pathway, thereby reducing inflammation.[32] And an association between inflammation and reduced platelet response to clopidogrel has been demonstrated in the previous study.[33] In this context, we speculated that lower miRNA-411-3p was found in CR patients, which may be caused by changes in inflammation levels. And this inference needs further verification. Additionally, miR-24-3p has been reported to play a role in CR and platelet activity regulation, which could restrain

endogenous and exogenous coagulation pathways by inhibiting the synthesis of factor-X (FX).[34] In addition, inflammation and immune response can promote platelet activation and thrombosis.[35,36] The miR-142-3p could control the overexpression of TNF-α by downregulating protein kinase Cα (PKCα) or inhibiting the expression of IFN-γ.[37,38] miR-142-3p could reduce the induction of iTreg and significantly decrease the secretion of TGF-β and IL-10.[39] Moreover, miR-142-3p could downregulate the expression of Rac1 and Rac1-GTPase, thus activating antiplatelet and increasing the risk of hemorrhage.[40] Though the reasonable mechanisms of miRNAs in CR were briefly explored in the previous studies, further studies should be completed to explore the potential regulatory mechanism deeply to support our findings.

There are the following aspects of main strengths and limitations in our study. The concentrations of clopidogrel and active metabolic derivative in the peripheral blood were extremely low, so we used UHPLC-Q-Orbitrap HRMS to accurately determine their concentrations. Besides, this study mainly focused on the differences in pharmacodynamics of clopidogrel, so we try to eliminate the influencing factors of pharmacokinetics. In order to exclude interference from other sources of miRNA in peripheral blood, the object of this study was platelet-derived miRNA, which could better explain its effect on platelet activity. However, there were some limitations in the current study. First of all, due to the limitation of experimental material, our sample size was very small. And the applicability of relevant miRNA biomarkers needs further verification in a wide range of population. Besides, we only selected four platelet-derived miRNAs for subsequent verification, and then more differential miRNAs need to be clarified whether they are related to CR. Moreover, the effect of aspirin on CR and IPA was not evaluated, and needs further investigation. Finally, although we utilized GO and KEGG analysis to further explore the potential regulatory mechanism between platelet-derived miRNAs and CR, more studies should be carried out to confirm the regulatory mechanism.

In summary, our findings indicate that miRNA-142-3p, miRNA-24-3p, and miRNA-411-3p are relevant to CR. However, the applicability of relevant miRNA biomarkers needs further verification in a wide range of population.

DISCLOSURE

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this article.

AUTHOR CONTRIBUTIONS

XC, SL and HH contribute to the conception, design, and final approval of the submitted version. HH, JC, SL, XX, RC, YH and XC contribute to completing the figures, and writing the paper. All the authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

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

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