MiR-223 or miR-126 predicts resistance to dual antiplatelet therapy in patients with ST-elevation myocardial infarction

Xiaojing Li1,*, Qi Yao2,*, Hanbin Cui1, Jun Yang2, Nan Wu1, Yahui Liu3, Ying Zhou3, Yinwei Zhang2, Jia Su1, Yezi Xia2 and Xiaomin Chen1

Abstract
Objective: To explore the role of miR-223 and miR-126 in predicting treatment responses to dual antiplatelet therapy (DAPT) in patients with ST-elevation myocardial infarction (STEMI).
Methods: Plasma miR-223 and miR-126 levels were measured before treatment. Treatment responses and 2-year survival were determined. In vitro experiments were performed to explore the mechanism of action.
Results: Patients with resistance to DAPT had a lower level of miR-223 and miR-126. Cardiac-event-free survival was shorter in patients with lower miR-223 or miR-126 levels. MiR-223 and miR-126 independently predicted DAPT resistance. Modulating miR-223 or miR-126 in platelets in vitro significantly changed the response to clopidogrel by regulating platelet aggregation.
Conclusion: MiR-223 and miR-126 play a role in DAPT resistance and may provide potential biomarkers in patients with STEMI.

Keywords
MicroRNA, ST-elevation myocardial infarction, dual antiplatelet therapy, resistance, biomarker, platelet aggregation

Date received: 20 August 2020; accepted: 19 April 2021

*These authors contributed equally to this paper.

Corresponding author:
Xiaoming Chen, Cardiovascular Center of Ningbo First Hospital, No. 59 Liuting Street, Ningbo 315000, China.
Email: chxmin@hotmail.com

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
Introduction

Clot formation induced by platelet activation, adhesion, and aggregation is an important event in the pathogenesis of acute coronary syndromes (ACSs) and their complications. Dual antiplatelet therapy (DAPT) with aspirin and clopidogrel has been demonstrated to be effective for the treatment of ACS, prevention of myocardial infarction, and reduction in ACS-related deaths. However, patients with ACS who receive DAPT show different response rates, and the underlying mechanisms remain unclear. Therefore, it is important to identify predictors for the efficacy of DAPT and understand the underlying mechanisms.

MicroRNA (miR) is a small non-coding RNA that modulates the expression of several mRNAs by regulating their translation and degradation. Platelets are an important source of circulating miRs, among which miR-223 has gained particular attention as it is found predominantly in platelets. An important achievement in platelet research is the discovery that activated platelets participate in cell communication by releasing microparticles containing complexes of miR-223 and Argonaute 2, which are internalized by other platelets.

Previous results regarding the effects of miR-223 on responses to DAPT (aspirin combined with clopidogrel or another P2Y12 antagonist) are controversial. It has been reported that miR-223 interacts with the 3'-untranslated region of the mRNA encoding the P2Y12 adenosine 5'-diphosphate (ADP) receptor to inhibit P2Y12 expression. Decreased levels of miR-223 in platelets and plasma have been observed in patients with troponin-negative non-ST-elevation ACS treated with DAPT who have concurrent high on-treatment platelet reactivity, indicating a poorer outcome. In contrast, it has been shown that reduced levels of miR-223 are associated with enhanced platelet inhibition during DAPT. Therefore, further study is required to explore the role of miR-223 in predicting DAPT outcomes.

The levels of other miRs, such as miR-126, have been reported to be altered in patients with ACS during DAPT. For instance, circulating levels of miR-126 were decreased after DAPT therapy. However, increased levels of miR-126 before therapy served as an indicator of adverse events after percutaneous coronary intervention in patients receiving DAPT. Whether the plasma levels of miR-126 before treatment can be used to predict responses to DAPT in patients with ACS has not been explored.

In the present study, we measured the plasma levels of miR-223 and miR-126 in patients with ST-elevation myocardial infarction (STEMI) and with or without DAPT resistance to confirm whether circulating miR-223 and miR-126 can predict treatment resistance and patient survival. More importantly, we performed in vitro studies by modulating the level of miR-223 or miR-126 in platelets collected from patients to observe the response to DAPT. Our results provide novel insight into predicting the prognosis of patients with STEMI after DAPT.

Methods

Patients

In this prospective study, consecutive adult patients with STEMI were recruited between January and September 2017 after excluding those who did not meet the inclusion criteria. The exclusion criteria were as follows: (1) those who were allergic or intolerable to clopidogrel or aspirin; (2) those who experienced cerebral vascular events in the past 6 months; (3) those who had bleeding disorders; (4) those who had severe liver diseases, including liver fibrosis.
or cirrhosis; (5) those who suffered terminal
diseases, such as advanced tumors or
uremia; (6) those who were on platelet gly-
coprotein IIb/IIIa inhibitors; (7) those
who had hematological disorders; (7) those
whose platelet count was lower than
100 \times 10^9/L or greater than 450 \times 10^9/L;
and (8) those who refused to attend this
study. Patients were diagnosed with
STEMI according to the 2016 American
College of Cardiology/American Heart
Association guideline.\textsuperscript{12}

In addition to a dose of oral aspirin (100
mg daily), all patients received a loading
dose of oral clopidogrel (300 mg) and a
maintenance dose of oral clopidogrel (75
mg) daily after admission. All recruited
patients were followed up for at least 2
years after the initial administration. A
composite endpoint of heart disease-
related death, recurrent ACSs, and heart
failure was documented. Severe side
effects were also recorded. The major side
effect refers to major bleeding events, which
are defined as intracranial bleeding, a decrease
in hemoglobin concentration by \(>5\) g/dL,
or a decrease in hematocrit by at least
10\%. No healthy volunteers from the gen-
eral public were recruited.

**Determination of responses to clopidogrel**

Responses to clopidogrel were determined
by the VerifyNow method (Accumetrics,
Inc., San Diego, CA, USA) in accordance
with the manufacturer’s instruction and
previous publications.\textsuperscript{13} Briefly,
VerifyNow is designed to determine the
blockage of P2Y\textsubscript{12} in platelets after treat-
ment with ADP. Clopidogrel resistance
was defined as P2Y\textsubscript{12} reaction units
(PRU)\(>208\) based on our preliminary
results and publications.\textsuperscript{14–16}

Whole blood samples were collected
from each individual at 6 hours after the
loading dose and 7 days after clopidogrel
treatment. In accordance with the
manufacturer’s instructions, 2 mL of
whole blood was loaded into the system,
and PRU rates were recorded.

**miR quantification**

miRs were extracted from blood samples
before and after treatment. Plasma samples
were obtained by centrifugation at 4000\(\times\)g
for 30 minutes at 4\(^\circ\)C. A total of 400 \(\mu\)L of
plasma was used for miRNA extraction
with a commercial miRNA isolation kit
(Life Technologies, Carlsbad, CA, USA).
Reverse transcription targeting miRs was
performed using a TaqMan MicroRNA
Reverse Transcription Kit (Life
Technologies). Quantitative polymerase
chain reaction (qPCR) was conducted
using TaqMan primers and probes (Life
Technologies) following the manufacturer’s
guides. All samples were run in triplicate
and quantified using the comparative
threshold cycle method (\(2^{-\Delta\Delta CT}\)) method
with miR-16 as an endogenous control.

**Evaluation of the role of miR in vitro**

To perform gain-of-function or loss-of-
function assays, we transfected the collected
platelets with a miRNA overexpression
plasmid or siRNA targeting miRNA \textit{in}
\textit{vitro} in accordance with a published proto-

col.\textsuperscript{17} Platelets were purified as previously
published.\textsuperscript{18} Vectors for miR-223 or miR-
126 and non-targeting control siRNA were
purchased from Sigma-Aldrich (St. Louis,
MO, USA) and transfected into platelets
using a commercial transfection kit
(Lipofectamine 2000, Invitrogen,
Carlsbad, CA, USA) in accordance with
the manufacturer’s instruction. The expres-
sion levels of miR-223 and miR-126 were
validated by qPCR as described above.
Following transfection, platelets were dilut-
ed to the circulating concentration in
humans and collected for the VerifyNow
assay.
Light transmission aggregometry

Platelets were collected and transfected with miR mimics or inhibitors as described above. After transfection, platelets (200 × 10^9/L) were used for aggregation assays. Platelet aggregation was induced by adding ADP (purchased from Sigma-Aldrich) at a concentration of 2 μM in the presence or absence of clopidogrel (30 μM) in the medium, as previously reported.19,20 The process was performed on a Chronolog Optical Lumi-Aggregometer (Havertown, PA, USA) at 37°C and 240×g. Data were collected at 15 minutes after ADP stimulation.

Statistical analysis

Data were presented as the mean±standard deviation, median (range), or n (%), according to the type of variables. Student t-tests were used to compare continuous data when two groups were compared. One-way ANOVA with the Bonferroni post-hoc test was used to compare data among more than two groups. Chi-squared tests were used to analyze categorical variables. Event-free survivals were assessed with the Kaplan–Meier method. Area under the curve (AUC) analysis was also performed to determine the ability of miR-223 and miR-126 to predict the resistance to DAPT in patients with STEMI. The correlation between variables and treatment responses to DAPT was analyzed by logistic regression. The effects of variables on event-free survival were analyzed by Cox regression. Normal distributions were confirmed by Shapiro–Wilk tests. Homogeneity of variance was analyzed by Hartley tests. All data were analyzed using GraphPad Prism 5 (Windows Edition, GraphPad Software, San Diego, CA, USA) and SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.

Ethical consideration

The Ethics Committee of Ningbo First Hospital at Ningbo University reviewed, approved, and supervised this study (approval number: 20150014). All patients involved in this study agreed to their participation and signed written consent forms.

Results

Patient characteristics

The selection of patients involved in this study is presented in Figure 1. A total of 120 consecutive patients with STEMI were included in the present study. Thirty-six patients had a PRU larger than 208 U and were considered resistant to DAPT (referred to as non-responders), and the remaining patients were responders. All recruited patients received lose-dose daily aspirin (75–100 mg oral daily) prior to hospital admission. The characteristics of responders and non-responders are compared in Table 1. Non-responders were older and more frequently used proton-pump inhibitors than responders. No severe side effects were observed in either group.

Association of plasma miR-223/miR-126 levels with resistance to DAPT

As shown in Figure 2a, non-responders had a significantly lower plasma level of miR-223 compared with that of responders before the initiation of DAPT (responders vs. non-responders, 1.117±0.02 vs. 0.342±0.045, p < 0.0001). Similarly, a lower level of miR-126 before treatment was observed in non-responders (responders vs. non-responders, 1.34±0.025 vs. 0.8±0.1265, p < 0.0001) (Figure 2b). These data indicate the non-responders to DAPT have lower
levels of miR-223 and miR-126 in the plasma before treatment.

Because the PRU value is an important indicator of DAPT resistance, we then determined whether there was a correlation between the PRU and plasma levels of miR-223 or miR-126. We performed linear regression analyses between these variables. As indicated in Figure 2c and d, there were significant correlations between the PRU and levels of miR-223 or miR-126 (p < 0.0001), further confirming that miR-223 and miR-126 are associated with DAPT responses.

**Predictive values of plasma miR-223 or miR-126 for DAPT resistance**

AUC analysis was then performed using miR-223 and miR-126 to predict the resistance to DAPT in patients with STEMI. As shown in Table 2, both plasma miR-223 and miR-126 had significant value in predicting DAPT resistance. The AUC of miR-223 for predicting resistance to DAPT was 0.996, which was superior to miR-126 (0.905). The cut-off value of plasma miR-223 levels was 0.70 with a sensitivity of 88.9% and a specificity of 97.6%
Table 1. Patient characteristics.

| Variable                        | Responders (n = 84) | Non-responders (n = 36) | p value |
|---------------------------------|---------------------|-------------------------|---------|
| Age (years)                     | 60.05 ± 1.03        | 67.44 ± 1.54            | 0.0002***|
| Men                             | 50 (65.79%)         | 24 (66.67%)             | >0.999  |
| BMI (kg/m²)                     | 24.09 ± 0.27        | 24.26 ± 0.37            | 0.7257  |
| Smokers                         | 52 (61.90%)         | 22 (61.11%)             | >0.999  |
| Previous MI                     | 22 (26.19%)         | 14 (38.89%)             | 0.366   |
| Previous PCI                    | 14 (16.67%)         | 10 (27.78%)             | 0.482   |
| Previous coronary artery bypass | 8 (9.52%)           | 4 (11.11%)              | >0.999  |
| Previous heart failure          | 8 (9.52%)           | 2 (5.56%)               | >0.999  |
| Previous cancer                 | 4 (4.76%)           | 2 (5.56%)               | >0.999  |
| Anemia                          | 4 (4.76%)           | 2 (5.56%)               | >0.999  |
| LVEF < 35%                      | 4 (4.76%)           | 2 (5.56%)               | >0.999  |
| Diabetes mellitus               | 14 (16.67%)         | 14 (38.89%)             | 0.094   |
| High cholesterol                | 52 (61.90%)         | 24 (66.67%)             | 0.7781  |
| Hypertension                    | 60 (71.43%)         | 26 (72.22%)             | >0.999  |
| COPD                            | 8 (9.52%)           | 4 (11.11%)              | >0.999  |
| Use of PPI                      | 18 (21.43%)         | 22 (61.11%)             | 0.006** |

BMI, body mass index; COPD, chronic obstructive pulmonary disease; MI, myocardial infarction; PCI, percutaneous coronary intervention; LVEF, left ventricular ejection fraction; PPI, proton-pump inhibitor.

Figure 2. Association of plasma miR-223/miR-126 levels with resistance to DAPT. a. Serum levels of miR-223 in responders and non-responders before DAPT. b. Serum levels of miR-126 in responders and non-responders before DAPT. c. Correlation between PRU and relative miR-223 levels in the serum. d. Correlation between PRU and relative miR-126 levels in the serum. ** ** ** p < 0.0001, by two-tailed Student t-tests. STEMI, ST-elevation myocardial infarction; PRU, P2Y₁₂ reaction units; DAPT, dual antiplatelet therapy.
and the cut-off value of plasma miR-126 levels was 1.02 with a sensitivity of 88.9% and a specificity of 95.2% ($p < 0.0001$).

### Table 2. Predictive values of serum miR-223 and miR-126 in DAPT resistance.

| Variable                  | AUC  | Cut-off value | Sensitivity | Specificity | $p$ value |
|---------------------------|------|---------------|-------------|-------------|-----------|
| miR-223                   | 0.996| 0.70          | 88.9%       | 97.6%       | $<0.0001$ |
| miR-126                   | 0.905| 1.02          | 88.9%       | 95.2%       | $<0.0001$ |

DAPT, dual antiplatelet therapy; AUC, area under the curve.

### Table 3. Logistic regression analyses of risk factors for DAPT resistance.

| Variable                              | Univariable logistic regression | Multivariable logistic regression |
|---------------------------------------|----------------------------------|-----------------------------------|
|                                       | OR     | 95% CI       | $p$ value | OR     | 95% CI       | $p$ value |
| Age (≤64 vs. >64 years)               | 5.687  | 1.591–20.330 | 0.007*** | 3.177  | 0.144–70.271 | 0.464    |
| Sex (men vs. women)                   | 1.115  | 0.343–3.624  | 0.856     |        |              |          |
| BMI (kg/m2) (≤24.7 vs. >24.7)         | 0.968  | 0.319–2.940  | 0.955     |        |              |          |
| Smoker (no vs. yes)                   | 0.967  | 0.311–3.005  | 0.954     |        |              |          |
| Previous MI (no vs. yes)              | 1.793  | 0.556–5.784  | 0.328     |        |              |          |
| Previous PCI (no vs. yes)             | 1.923  | 0.518–7.144  | 0.329     |        |              |          |
| Previous coronary artery bypass (no vs. yes) | 1.187  | 0.197–7.149  | 0.851     |        |              |          |
| Previous heart failure (no vs. yes)   | 0.559  | 0.058–5.381  | 0.615     |        |              |          |
| Previous cancer (no vs. yes)          | 1.176  | 0.100–13.862 | 0.897     |        |              |          |
| Anemia (no vs. yes)                   | 2.500  | 0.324–19.302 | 0.380     |        |              |          |
| LVEF <35% (no vs. yes)                | 1.176  | 0.100–13.862 | 0.897     |        |              |          |
| Diabetes mellitus (no vs. yes)        | 3.182  | 0.914–11.079 | 0.069     |        |              |          |
| High cholesterol (no vs. yes)         | 1.231  | 0.385–3.930  | 0.726     |        |              |          |
| Hypertension (no vs. yes)             | 1.040  | 0.304–3.557  | 0.950     |        |              |          |
| COPD (no vs. yes)                     | 1.187  | 0.197–7.149  | 0.851     |        |              |          |
| Use of PPI (no vs. yes)               | 5.762  | 1.735–19.140 | 0.004**** | 7.782  | 0.352–172.086 | 0.194   |
| Serum miR-223 (>0.70 vs. ≤0.70)       | 328.00 | 27.772–3873.897 | <0.0001**** | 81.480 | 3.744–1773.249 | 0.005** |
| Serum miR-126 (>1.02 vs. ≤1.02)       | 160.000 | 20.723–1235.324 | <0.0001**** | 36.260 | 1.652–795.936 | 0.023*   |

*a p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

DAPT, dual antiplatelet therapy; OR, odds ratio; CI, confidence interval; BMI, body mass index; COPD, chronic obstructive pulmonary disease; MI, myocardial infarction; PCI, percutaneous coronary intervention; LVEF, left ventricular ejection fraction; PPI, proton-pump inhibitor.

### Risk factors for DAPT resistance

We subsequently aimed to identify risk factors for DAPT resistance using the logistic regression method. As revealed in Table 3, univariable logistic regression analyses showed that older patients (>64 years old), the use of proton-pump inhibitors, low plasma miR-223 levels (<0.70), and low plasma miR-126 levels (≤1.02) were associated with DAPT resistance. Moreover, we input these parameters into a multivariable logistic regression analysis, which demonstrated that the use of proton-pump inhibitors, low plasma miR-223, and
low plasma miR-126 were independent risk factors for DAPT resistance.

Patients with low levels of miR-223 or miR-126 had shorter cardiac-event-free survival

In addition to DAPT responses, we followed up all recruited patients to analyze heart disease-related events within 2 years. We divided patients into two groups according to the cut-off values of plasma miR-223 or miR-126 (Table 2). As shown in Figure 3a and b, patients with lower levels of miR-223 (≤0.70) or miR-126 (≤1.02) had significantly shorter cardiac-event-free survival than those with higher levels of miR-223 or miR-126 (p < 0.0001).

miR-223 and miR-126 mediate DAPT responses in vitro

To explore the role of miR-223 and miR-126 in mediating the response to DAPT, we manipulated the levels of these two miRs in vitro and evaluated the PRU. According to the cut-off values obtained in Table 2, we classified these patients into two groups based on miR-223 values (≥0.70 or ≤0.70) and miR-126 values (≥1.02 or ≤1.02). Accordingly, miR overexpression vectors were added to the platelets obtained from patients with low levels of the respective miR, whereas miR siRNAs were used to transfect platelets obtained from those with high levels of the corresponding miR. As shown in Figure 4a, miR-223 plasmid transfection into platelets from the
decreased miR-223 group potently reduced the average PRU from over 208 U to lower than 208 U (Figure 4a). Meanwhile, miR-223 siRNA transfection into platelets from the elevated miR-223 group increased the PRU (Figure 4b). Similar results were also observed in platelets transfected with miR-126 plasmids or siRNAs (Figure 4c and d). These data suggest that the platelet levels of miR-223 or miR-126 potently regulate the response of patients with STEMI to DAPT.

**miR-223 and miR-126 regulate platelet aggregation in response to clopidogrel**

Finally, we explored whether the contribution of miR-223 or miR-126 to DAPT responses was mediated by platelet aggregation in vitro. ADP (2 μM) was added to the medium 15 minutes prior to miR plasmid or siRNA transfections to stimulate platelet aggregation in the presence or absence of clopidogrel (30 μM). Using light transmission aggregometry, we determined that transfection with overexpression plasmids or siRNAs for either miR-223 or miR-126 had no effect on platelet aggregation in the absence of clopidogrel (Figure 5a–d). However, when we increased miR-223 levels in platelets collected from patients in the decreased miR-223 group, platelets treated with miR-223 mimics showed a substantial decrease in aggregation following clopidogrel treatment (Figure 5a), indicating the potential role of miR-223 in modulating platelet responsiveness to DAPT in patients with STEMI.
an improved response to clopidogrel. In contrast, when miR-223 levels were decreased in the elevated miR-223 group, platelets showed enhanced aggregation after clopidogrel treatment (Figure 5b). Similar results were observed when the platelet levels of miR-126 were manipulated in vitro (Figure 5c and d). Taken together, these data indicate that miR-223 and miR-126 play important roles in clopidogrel responses by regulating platelet aggregation.

Discussion
In the present study, we presented the following results: (1) patients with STEMI exhibited a response rate of 70% to DAPT; (2) patients with lower levels of plasma miR-223 or miR-126 at the start of DAPT showed treatment resistance; (3) lower plasma concentrations of miR-223 or miR-126 and the use of proton-pump inhibitors are risk factors for DAPT resistance; (4) patients with STEMI and low levels of plasma miR-223 or miR-126 have shorter cardiac-event-free survival; and (5) more importantly, reducing the levels of miR-223 or miR-126 in platelets collected from patients with high concentrations in the circulation impaired the responsiveness of platelets to clopidogrel.

Only a certain number of patients show a good response to clopidogrel measured by
the inhibition of platelet aggregation.\textsuperscript{21–25} More importantly, those who have a poor response to clopidogrel are at high risk for ischemic events and other cardiac events.\textsuperscript{21–25} With respect to these previous reports, we found that approximately 70\% of patients in our cohort showed a good response to DAPT, which was determined using the VerifyNow system. Our data, in addition to previous publications, highlight the importance of predicting responses to DAPT before treatment.

Platelets and platelet microparticles (PMPs) are a source of miRs, including miR-126, miR-197, miR-223, miR-24, and miR-21, which can be detected in the circulation.\textsuperscript{10} More importantly, the concentrations of individual miRs in the circulation are correlated with their levels in platelets,\textsuperscript{10} as these miRs are exclusively expressed in platelets. Platelet-derived miRs may have several important physiological effects. For instance, after antiplatelet therapy, the concentrations of certain miRs are reduced in the circulation,\textsuperscript{10,26} which likely results from reduced PMP shedding during platelet inhibition. However, further investigations, including pre-clinical and clinical trials, are warranted to understand the roles of circulating miRs expressed in platelets.

Antiplatelet therapy, including DAPT, is critical for the treatment of ischemic cardiac diseases. The two medications used in this regimen have different but synergetic inhibitory effects on platelets. Aspirin inhibits thromboxanes produced by platelets, whereas P2Y\textsubscript{12} inhibitors (such as clopidogrel) suppress ADP-induced platelet aggregation. In the present study, we found that patients with high plasma miR-223 or miR-126 levels showed a good response to DAPT and longer cardiac-event-free survival, consistent with previous reports.\textsuperscript{27} It has been reported that miR-223 binds to the 3’ untranslated region of P2Y\textsubscript{12}, which results in the suppression of protein expression.\textsuperscript{27,28} However, the precise roles of these platelet-derived miRs in DAPT responses warrant further experiments.

In this report, we found that decreased levels of miR-223 or miR-126 predicted undesired outcomes in patients with STEMI, although the results from a previous study show that increased levels of miR-223 or miR-126 are correlated with ischemic events within 30 days or 1 year.\textsuperscript{29} It is worth noting that there may be differences in the design of these two studies. For instance, the ethnicity of the patients in the study mentioned above is unknown but is presumably Caucasian or Eastern European, whereas our cohort was a Han Chinese group. Differences in the genetics between these two cohorts might explain the varied results. Furthermore, a fraction of patients in the Prague-18 study received two medications (ticagrelor and clopidogrel or prasugrel and clopidogrel).\textsuperscript{29} Another major difference between their study and ours is the definition of endpoints. Hromadka et al.\textsuperscript{29} used a combined endpoint of ischemic events. However, using an individual endpoint rather than a combined one may change the results. Regardless of these differences, an international collaboration involving more ethnic groups at multiple institutes is warranted to explore the value of miR-223 or miR-126 in predicting adverse events in patients with STEMI.

This study has a few limitations. First, the cohort is relatively small; thus, a larger research population is needed in the future. Second, we only used the VerifyNow system to measure platelet functions. Although other measurement methods exist in practice, the above system is the most widely used and well tested in clinical trials with stable results.\textsuperscript{13} Lastly, the assay we used to determine the levels of plasma miRs is time-consuming, which might delay the therapeutic decision. Rapid test kits for miR levels in plasma are needed in clinical practice.

This study has clinical significance. Because ischemic cardiac events, such as
STEMI, require urgent therapeutic intervention, identifying non-responders in advance is crucial to select more effective and individual therapeutic options. Another issue is to identify these non-responders to avoid unnecessary side effects. Here, we showed that high plasma levels of miR-223 or miR-126 were correlated with a good response to DAPT, indicating their potential as promising predictors of treatment outcomes. Mechanistically, we determined that decreasing the levels of miR-223 or miR-126 in platelets collected from patients with high circulating miR concentrations induced therapy resistance. In contrast, increasing the levels of these miRs in platelets collected from patients with low circulating concentrations improved the responses of platelets to clopidogrel. These data were consistent with the observations in patients with STEMI. More importantly, these data indicate miR-223 or miR-126 as a direct regulator of platelet responses to clopidogrel, highlighting the potential of modulating their levels in clinical practice.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This study was supported by the Natural Science Foundation of Ningbo (2017A610198).

Author Contributions

XL and QY were responsible for the conceptualization and performance of experiments. HC, JY, NW, YL, YZ, YZ, and JS were responsible for sample collection and patient information organization. XC and YX were responsible for the conceptualization and supervision of the study.

ORCID iDs

Qi Yao https://orcid.org/0000-0002-3786-2814
Yezi Xia https://orcid.org/0000-0002-7604-0610

References

1. De Winter RJ, Windhausen F, Cornel JH, et al. Early invasive versus selectively invasive management for acute coronary syndromes. *N Engl J Med* 2005; 353: 1095–1104.
2. Serebruany VL, Steinhubl SR, Berger PB, et al. Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol* 2005; 45: 246–251.
3. Jaremo P, Lindahl TL, Fransson SG, et al. Individual variations of platelet inhibition after loading doses of clopidogrel. *J Intern Med* 2002; 252: 233–238.
4. Zampetaki A, Willeit P, Tilling L, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol* 2012; 60: 290–299.
5. Laffont B, Corduan A, Plé H, et al. Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles. *Blood* 2013; 122: 253–261.
6. Landry P, Plante I, Ouellet DL, et al. Existence of a microRNA pathway in anucleate platelets. *Nat Struct Mol Biol* 2009; 16: 961–966.
7. Zhang YY, Zhou X, Ji WJ, et al. Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome. *J Thromb Thrombolysis* 2014; 38: 65–72.
8. Shi R, Ge L, Zhou X, et al. Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity. *Thromb Res* 2013; 131: 508–513.
9. Matetzky S, Shenkman B, Guetta V, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. *Circulation* 2004; 109: 3171–3175.
10. Willeit P, Zampetaki A, Dudek K, et al. Circulating microRNAs as novel biomarkers for platelet activation. *Circ Res* 2013; 112: 595–600.
11. Yu XY, Chen JY, Zheng ZW, et al. Plasma miR-126 as a potential marker predicting
major adverse cardiac events in dual antiplatelet-treated patients after percutaneous coronary intervention. EuroIntervention 2013; 9: 546–554.

12. Levine GN, Bates ER, Bittl JA, et al. 2016 ACC/AHA Guideline Focused Update on Duration of Dual Antiplatelet Therapy in Patients With Coronary Artery Disease: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines: An Update of the 2011 ACCF/AHA/SCAI Guideline for Percutaneous Coronary Intervention, 2011 ACCF/AHA Guideline for Coronary Artery Bypass Graft Surgery, 2012 ACC/AHA/ACP/ATS/PCNA/SCAI/STS Guideline for the Diagnosis and Management of Patients With Stable Ischemic Heart Disease, 2013 ACCF/AHA Guideline for the Management of ST-Elevation Myocardial Infarction, 2014 AHA/ACC Guideline for the Management of Patients With Non-ST-Elevation Acute Coronary Syndromes, and 2014 ACC/AHA Guideline on Perioperative Cardiovascular Evaluation and Management of Patients Undergoing Noncardiac Surgery. Circulation 2016; 134: e123–e155.

13. Saia F, Marino M, Campo G, et al. Incidence and outcome of high on-treatment platelet reactivity in patients with non-ST elevation acute coronary syndromes undergoing percutaneous coronary intervention (from the VIP [VerifyNow and Inhibition of Platelet Reactivity] study). Am J Cardiol 2013; 112: 792–798.

14. Storey RF, Angiolillo DJ, Bonaca MP, et al. Platelet Inhibition With Ticagrelor 60 mg Versus 90 mg Twice Daily in the PEGASUS-TIMI 54 Trial. J Am Coll Cardiol 2016; 67: 1145–1154.

15. Alexopoulos D, Stavrou K, Koniarli I, et al. Ticagrelor vs prasugrel one-month maintenance therapy: impact on platelet reactivity and bleeding events. Thromb Haemost 2014; 112: 551–557.

16. Trenk D, Stone GW, Gawaz M, et al. A randomized trial of prasugrel versus clopidogrel in patients with high platelet reactivity on clopidogrel after elective percutaneous coronary intervention with implantation of drug-eluting stents: results of the TRIGGER-PCI (Testing Platelet Reactivity In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel) study. J Am Coll Cardiol 2012; 59: 2159–2164.

17. Hong W, Kondkar AA, Nagalla S, et al. Transfusion of human platelets with short interfering RNA. Clin Transl Sci 2011; 4: 180–182.

18. Xiao H, Harvey K, Labarrere CA, et al. Platelet cryopreservation using a combination of epinephrine and dimethyl sulfoxide as cryoprotectants. Cryobiology 2000; 41: 97–105.

19. Leunissen TC, Wisman PP, Van Holten TC, et al. The effect of P2Y12 inhibition on platelet activation assessed with aggregation- and flow cytometry-based assays. Platelets 2017; 28: 567–575.

20. Weber AA, Reimann S and Schror K. Specific inhibition of ADP-induced platelet aggregation by clopidogrel in vitro. Br J Pharmacol 1999; 126: 415–420.

21. Parodi G, Marcucci R, Valenti R, et al. High residual platelet reactivity after clopidogrel loading and long-term cardiovascular events among patients with acute coronary syndromes undergoing PCI. JAMA 2011; 306: 1215–1223.

22. Price MJ, Endemann S, Gollapudi RR, et al. Prognostic significance of post-clopidogrel platelet reactivity assessed by a point-of-care assay on thrombotic events after drug-eluting stent implantation. Eur Heart J 2008; 29: 992–1000.

23. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, et al. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. J Am Coll Cardiol 2007; 49: 1505–1516.

24. Brar SS, Ten Berg J, Marcucci R, et al. Impact of platelet reactivity on clinical outcomes after percutaneous coronary intervention. A collaborative meta-analysis of individual participant data. J Am Coll Cardiol 2011; 58: 1945–1954.

25. Price MJ, Angiolillo DJ, Teirstein PS, et al. Platelet reactivity and cardiovascular outcomes after percutaneous coronary intervention: a time-dependent analysis of the
Gauging Responsiveness with a VerifyNow P2Y12 assay: Impact on Thrombosis and Safety (GRAVITAS) trial. Circulation 2011; 124: 1132–1137.

26. Fichtlscherer S, De Rosa S, Fox H, et al. Circulating microRNAs in patients with coronary artery disease. Circ Res 2010; 107: 677–684.

27. Chyrchel B, Totoń-Żurańska J, Kruszelnicka O, et al. Association of plasma miR-223 and platelet reactivity in patients with coronary artery disease on dual antiplatelet therapy: A preliminary report. Platelets 2015; 26: 593–597.

28. Warshaw AL, Laster L and Shulman NR. Protein synthesis by human platelets. J Biol Chem 1967; 242: 2094–2097.

29. Hromadka M, Motovska Z, Karpisek M, et al. 3300MiR-126-3P and MiR-223-3p in Prediction of Thrombotic Risk in Patients with Acute Myocardial Infarction and Primary Angioplasty, The Prague-18 Genetic Sub-study. Eur Heart J 2019; 40: ehz745.0064.