Cardiovascular Drugs and Therapy (2019) 33:129–137
https://doi.org/10.1007/s10557-019-06855-3

ORIGINAL ARTICLE

Platelet MicroRNA 365-3p Expression Correlates with High On-treatment Platelet Reactivity in Coronary Artery Disease Patients

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Published online: 19 February 2019

Abstract

Purpose The expression level of platelet microRNAs (miRNAs) correlates with heart disease and may be altered by antiplatelet therapy. This study aims to assess whether certain miRNAs are associated with treatment response by platelets in patients who received percutaneous coronary intervention and antiplatelet therapy. The dynamic expression of certain miRNAs in patients receiving different antiplatelet regimens was also investigated.

Methods Healthy subjects ($N = 20$) received no-stent or antiplatelet therapy (as control), and patients ($N = 155$) who underwent stent implant and received treatment regimens that included aspirin plus clopidogrel, ticagrelor, or cilostazol were included. The association of miR-96-5p, miR-495-3p, miR-107, miR-223-3p, miR-15a-5, miR-365-3p, and miR-339-3p levels with treatment response, SYNTAX score, and HTPR was determined.

Results Of the different treatment regimens, ticagrelor was the most efficacious. At 24 h following drug administration, ROC analysis revealed that miR-339-3p and miR-365-3p had the highest sensitivity (74.3% and 90.0%, respectively) and specificity (71.4% and 93.3%) for detecting HTPR compared with the five other miRNAs. The SYNTAX score positively correlated with miR-223-3p and miR-365-3p levels at 24 h ($P \leq 0.006$) and with miR-365-3p levels 7 days following drug administration ($P = 0.014$). The expression of all three miRNAs reached the highest levels in hyperresponsive (P2Y12 reaction unit < 85) followed by hyporesponsive (P2Y12 reaction unit ≥ 208) and then normoreactive. The normoreactive value was very close to that of controls.

Conclusions Our data suggest that miR-365-3p expression level correlates with the antiplatelet treatment response.

Clinical Trial Registration NCT02101437

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10557-019-06855-3) contains supplementary material, which is available to authorized users.

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Keywords miRNA · Platelet reactivity unit · Antiplatelet therapy · SYNTAX score · P2Y12

Introduction

Blood platelets play a central role in normal hemostasis and in thrombotic and occlusive vascular disorders [1]. Following injury, platelets at the wound site are activated through contact with adhesion molecules. Activated platelets release bioactive components such as ADP, serotonin, and thromboxane A2, leading to further platelet recruitment, aggregation, and plug formation. While this process is essential for normal healing, platelet aggregation also is the key pathophysiological factor in arterial thrombus formation. Thus, antiplatelet therapy is commonly used in patients with cardiovascular disease. Dual therapy with aspirin and clopidogrel is the standard treatment for attenuating platelet function. However, a significant percentage of patients display no demonstrable antiplatelet effect after treatment with aspirin [2] or clopidogrel [3], known as high on-treatment platelet reactivity (HTPR), leaving these patients at increased risk for recurrent cardiovascular events. A greater understanding of the mechanisms underlying the action of current antiplatelet drugs on platelets is needed to guide the development of further therapeutic agents.

Lacking nuclei and DNA, platelets carry genetic information in an mRNA transcriptome and a diverse repertoire of regulatory microRNAs (miRNAs) [4] inherited from parent megakaryocytes [5, 6]. MicroRNAs are small, non-coding molecules that act to posttranscriptionally regulate gene expression either by promoting mRNA degradation or inhibiting translation [7]. Hundreds of miRNAs have been identified in platelets [8]. While the specific functions of most of these miRNAs are unknown, growing evidence indicates their great importance in health and disease. Recent studies show that in addition to regulating the expression of miRNAs within platelets [9], miRNAs released upon platelet activation can be taken up by other cells, altering their gene expression [10]. The levels of several miRNAs present in plasma or platelets correlate with vascular pathologies, including coronary artery disease [11–13], acute coronary syndrome [14], and myocardial infarction [10]. In addition, the levels of specific circulating miRNAs are positively associated with the degree of platelet activation [15]. Activated platelets exhibit altered expression of numerous miRNAs, including miR96-5p, miR495-3p [8, 16, 17], miR107 [8, 16], miR223-3p [16], miR15a-5 [17], miR365-3p [17], and miR339-3p [17].

Mounting evidence indicates that platelet miRNA expression both affects the response to and is affected by antiplatelet agents. For example, miR223, the most abundant miRNA in platelets [8], regulates the expression of many genes, including P2Y12, a key target of clopidogrel antiplatelet drug therapy. Decreased platelet miR-223 expression was observed in patients with an abnormally low platelet response to clopidogrel [18].

To further elucidate the relationship between platelet miRNA expression and antiplatelet drugs, this study investigates the effect of several antiplatelet therapies on the expression of key platelet miRNAs. In platelets from percutaneous coronary intervention patients, we compare the abundance of specific miRNAs following clopidogrel, ticagrelor, and clopidogrel plus cilostazol treatment in the presence of aspirin before and after platelet activation. These results are stratified with respect to platelet responsiveness to the various treatments to determine whether the expression of specific miRNAs is associated with HTPR.

Methods

This is a prospective, single-blind, randomized comparative study (Clinical trial registration: NCT02101437). Patients from Taipei City Hospital were enrolled in the study. The protocol was approved by the Institutional Review Board of Taipei City Hospital, Taipei, Taiwan, ROC. The study was performed in accordance with the Declaration of Helsinki and all patients gave their written informed consent.

Study Population

Patients with stable angina who had received an elective stent implant and were subsequently treated with dual antiplatelet therapy consisting of aspirin plus either clopidogrel, clopidogrel and cilostazol, or ticagrelor were included in the study. Included patients had high on-treatment platelet reactivity (HTPR) indicating they had low drug reactivity, defined as having a P2Y12 reaction unit (PRU) ≥ 208 [19–28]. Although, some prior studies have used ≥ 235 or ≥ 240 as the HTPR [19–22, 28–31]. Only patients with de novo lesions and who had not received coronary angioplasty were included to remove the risk of bias of the fact that the SYNTAX score does not always reflect the status of a previously treated lesion.

Patients who were not suitable for antiplatelet therapy (e.g., active gastrointestinal ulcer or bleeding), or who had contraindications to or intolerance of aspirin, clopidogrel, ticagrelor, cilostazol, who were planning to receive major surgery in the next year, or who could not continue to receive medications (included non-compliance with regimens) were excluded from the study. Patients were also excluded if they had previously received other antiplatelet drugs other than aspirin, clopidogrel, ticagrelor, or cilostazol. We included healthy subjects who had no stent treatment and no antiplatelet drugs treatments, as the control group.

Study Design

Patients were randomized to one of three treatment groups: (1) aspirin plus the standard dose of clopidogrel (Group standard; Gr.s); (2) aspirin plus ticagrelor (Group ticagrelor; Gr.t); (3) aspirin plus the standard dose of clopidogrel and cilostazol (Group cilostazol, Gr.c). The following doses for each drug were used: aspirin (loading 300 mg, then 100 mg once daily); clopidogrel (standard dose: loading 300 mg, then 75 mg once daily); ticagrelor (loading 180 mg, then 90 mg twice daily); and cilostazol (100 mg twice daily). All patients except for the control received a stent and aspirin. Dual antiplatelet therapy was continued for 6 months, followed by aspirin alone. Randomized methods are described in Supplemental Material. Patients were treated for at least 1 month and followed up for clinical events for 12 months.

For the three treatment groups, the clinical outcomes, PRU, and miRNA measurements were made at 24 h and 7 days following the start of drug therapy. The control group assessment occurred within 24 h after recruitment.

PRU Measurements

Blood (2 mL) was collected and PRU were determined using VerifyNow (Accumetrics, San Diego, CA, USA) as described by the manufacturer's instructions. Briefly, the VerifyNow assay measures the ability of activated platelets (platelets were activated by addition of adenosine diphosphate (ADP) and prostaglandin E1 (PGE1) to bind to fibrinogen-coated beads, which results in aggregation of the beads). In the VerifyNow assay, the increase in light transmittance is proportional to platelet aggregation and inversely proportional to inhibition of platelets. Samples untreated with ADP/PGE1 were used as negative controls. The PGE1 was added to the ADP solution to inhibit non-specific aggregation and increase sensitivity [32].

Platelet miRNA Measurements

For evaluating the levels of the specific mature miRNAs, platelet-rich plasma (white blood cell count to platelet ratio of < 0.4%) was used to minimize the influence of miRNAs from white blood cells [23, 24]. If the white blood cell count to platelet ratio was > 0.4%, the sample was re-centrifuged at 1000 rpm for 5 min then rechecked. This was repeated until the white blood cell count to platelet ratio was < 0.4% to minimize the influence of miRNAs from white blood cells [25, 30].

The assessment of platelet miRNAs expression can be affected by even a small amount of contamination by white blood cells present during the purification of platelet-rich plasma. While MiR-223, miR-197, miR574-3p, and Let-7a are expressed in both white blood cells and platelets [33], their expression is much higher in platelets than in white blood cells [21, 34]. To reduce the influence from white blood cells, we checked the WBC count after PRP isolation. The PRP was subjected to miRNA analysis if the percentage of WBC was less than 0.4%. We conducted a preliminary study to confirm miRNA expression in PRP and buffy coat plasma. The expression level of miRNA was much higher in PRP than in buffy coat plasma (unpublished data). Therefore, we think that any noise from the white blood cells can be ignored.

Total miRNA was isolated from the platelet-rich plasma using the High Pure miRNA Isolation Kit (Roche, Basel, Switzerland). The total miRNA concentration was evaluated using the WPA BIOWAVEII spectrophotometer (Biochrom, Cambridge, UK).

The following miRNAs were amplified by RT-PCR: miR-96-5p, miR-495-3p, miR-107, miR-223-3p, miR-15a-5p, miR-365-3p, and miR-339-3p. For each reaction, three oligomer primers were used and one oligo probe (see Supplemental Table S1and S2 for primer sequences and greater description of methods). The PRP volume employed for RNA extraction, as well as RNA input for preparing the RT, were recorded. The first primer contained a stem-loop structure required for synthesis of cDNA using the Taq DNA polymerase. The other two primers (a forward and reverse primer) were used for amplification using real-time PCR using the Roche Universal Probe Library system. The reverse primer for all the miRNAs was GTGCAGGGTCCGAGGT.

SYNTAX Score

SYNTAX score is an angiographic tool for grading the complexity of coronary artery disease, and in this study, it was calculated as described in Sianos et al. (2005) (see http://www.syntaxscore.com/index.php) [31]. The greater the SYNTAX score, the more severe the disease. SYNTAX score calculation was done by two of the current authors and the average of the two authors' results was presented. If there was a discrepancy of greater than > 3, a third author also evaluated the score and the final score represented an agreement among authors.

Statistical Analysis

Age, mean platelet volume (MVP), cholesterol, and low-density lipoprotein cholesterol (LDL-C) were expressed as mean ± standard deviation (SD) and other continuous variables were presented as median (interquartile range, P_{25}–P_{75}). Count and percentage were reported for categorical variables. Differences between groups were examined by analysis of variance (ANOVA) for age, MVP, cholesterol, and LDL-C. The Kruskal-Wallis test was used for other interval variables, and the Fisher's exact test for categorical variables. If a statistically significant difference was found after

ANOVA, post-hoc tests by Tukey's test and Dunnett's T3 test were carried out for variables with equal SDs and those with unequal SDs, respectively. Mann-Whitney *U* test was implemented if a significant difference was obtained by the Kruskal-Wallis test. Correlations between SYNTAX score and miRNA expression were estimated by Spearman's rank correlation.

Receiver operating characteristic (ROC) curves of the seven miRNAs of interest were generated to determine optimal cut-off points and to distinguish between the control and all other treatment groups. The point with analogous sensitivity and specificity was chosen as an optimal cut-off point. The likelihood ratio (LR) was calculated in order to evaluate the value of diagnostic tests regarding the different miRNAs. A LR > 1 indicated a higher probability of a target PRU being present; while, LR < 1 indicated a lower probability of target disorder being present. Five levels were defined by the LR: no change (LR = 1), minimal increase/decrease in the probability of a target disorder being present (LR < 2/LR between 0.5 and 1), small increase/decrease in the probability of the presence of a target disorder (LR between 2 and 5/LR between 0.2 and 0.5), moderate increase/ decrease in the probability of a target disorder being present (LR between 5 and 10/LR between 0.1 and 0.2), and large and often conclusive increase/decrease in the probability of the presence of a target disorder (LR > 10/ LR < 0.1). All tests were two-sided and $P < 0.05$ was considered statistically significant. When post-hoc tests were performed, significant levels were adjusted to values of 0.05 divided by the number of test (e.g., 0.05/3 = 0.017, 0.05/10 = 0.005, and 0.05/15 = 0.003). Data analyses were performed using IBM SPSS Statistics for Windows (Version 22.0, IBM Corp., Armonk, NY, USA).

Results

Baseline Characteristics, Hematological Index, SYNTAX Score, Comorbidities, and Medication

The overall design of the study and patient characteristics are shown in Fig. 1. No significant difference was observed between the three treatment groups with respect to age, sex, glucose, creatinine, triglyceride, cholesterol, low-density lipoprotein cholesterol (LDL-C), SYNTAX score, hypertension, diabetes, chronic renal failure, ACEI/ARB, or the use of beta blockers or statins (Table 1). The control group had greater levels of high-density lipoprotein cholesterol (HDL-C) than did the clopidogrel group ($P < 0.001$). The platelet-rich plasma (PRP) platelet count in the clopidogrel group was lower than that of the ticagrelor group ($P < 0.001$). Of the different treatments, ticagrelor was the most efficacious at 24 h and at 7 days (see Supplemental Material for description of treatment results).

Expression Levels of All Seven miRNAs According to PRU Levels

The expression levels of all seven miRNAs differed significantly between PRU levels, as defined by the 2013 ESC criteria, at 24 h and 7 days after the initiation of treatment (Table 2). The one exception was miRNA-107, which was expressed at similar levels in platelets of all PRU levels after 24 h ($P = 0.211$). Similar results were seen in in vivo experiments using cultured cells, showing that all the drugs tested in this study altered the expression of all seven miRNAs to some degree (unpublished data).

After 24 h of treatment, the expression levels of miR-365-3p and miRNA-393-3p were significantly higher in the hyperresponsive (PRU < 85) and hyporesponsive (PRU ≥ 208) platelets than in controls ($P < 0.001$) (Table 2). miR-365-3p expression was significantly higher in the PRU ≥ 208 group than in the other PRU groups ($P < 0.001$). miR-365-3p expression in the PRU < 85 group was higher than that of the PRU 85–207 group, while the expression of miRNA-393-3p was significantly higher in the PRU < 85 group than in the other two PRU groups ($P < 0.001$). miRNA-393-3p expression was lower in the PRU ≥ 208 group than in the PRU < 85 group ($P < 0.001$). After 7 days of treatment, the findings for miR-365-3p were similar (Table 2).

miRNA-223-3p expression at 24 h was similar to that of controls in all PRU groups except the PRU 85–207 group, in which the levels were significantly lower than that of controls ($P < 0.001$) (Table 2).The expression of miRNA-233-3p in the PRU-85-207 group was also significantly lower than that of the other two PRU groups ($P < 0.001$). The findings were similar at 7 days, except that the levels of miRN-223-3p were higher than that of controls.

miRNA-15a expression was significantly higher in the PRU < 85 group than in controls after 24 h of treatment, and miRNA-495-3p expression was significantly higher in the PRU ≥ 208 group than in the PRU 85–207 group. No difference in expression of these two miRNA was observed between groups on day 7.

Assessment of miRNAs as Biomarker for Determining High On-treatment Platelet Reactivity

ROC analysis found that miR-339-3p and miR-365-3p had the highest sensitivity (74.3% and 90.0%, respectively) and specificity (71.4% and 93.3%) for detecting HTPR compared with the five other miRNAs 24 h following drug administration. The findings were similar 7 days post drug dosing. For both these miRNAs, the LR (+) was > 2.0 and the LR (−) was < 0.5 indicating that if a patient had Log2 miR-339-3p or miR-365-3p greater than the cut-off value 24 h (− 6.69 and − 6.85, respectively) or 7 days (− 6.63 and − 6.41, respectively)

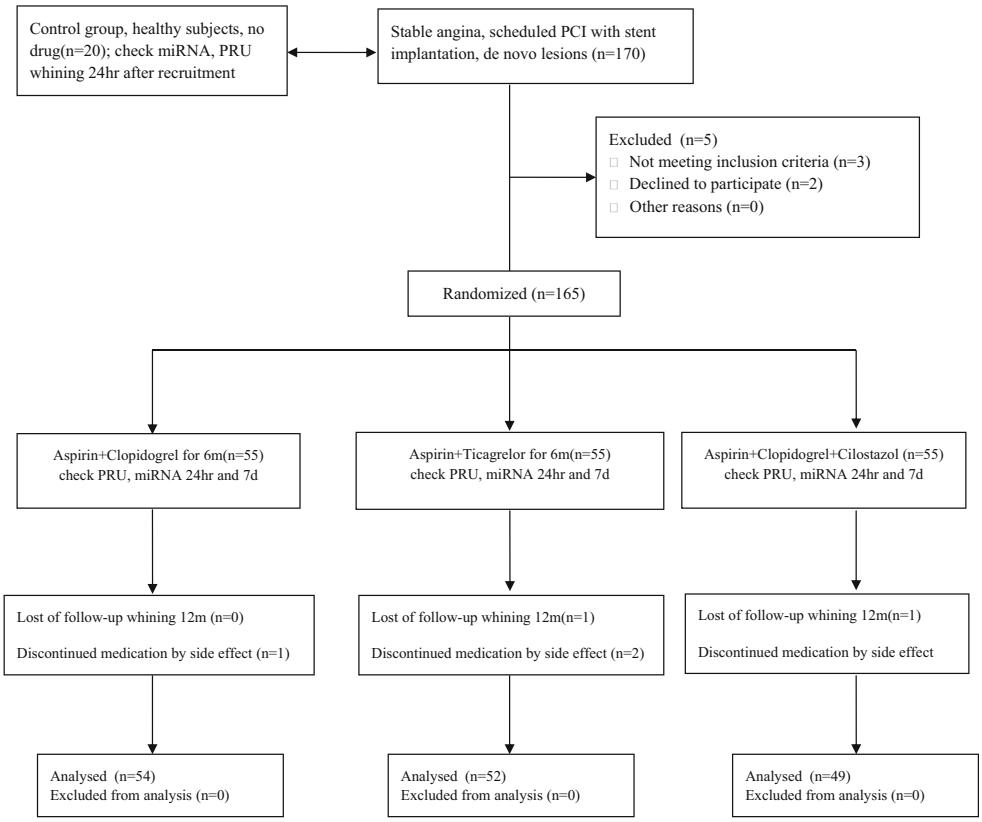

Fig 1 Diagram of study design

following dosing there was weak evidence that they had stable angina (Fig. 2 and data not shown).

Correlations Between SYNTAX Score and Expressions of 7 miRNA

We assessed if any of the seven miRNAs were associated with the SYNTAX score. The SYNTAX score correlated with levels of miR-223-3p and miR-365-3p (see Supplemental Material Table S3). The SYNTAX score positively correlated with miR-223-3p and miR-365-3p levels at 24 h (P values $\leq$ 0.006). After 7 days following drug administration, the SYNTAX score positively correlated with miR-365-3p levels ($P = 0.014$).

Discussion

This study investigates the efficacy of different antiplatelet drugs together with the expression of seven specific miRNAs involved in platelet activation and treatment response. Of the different treatment regimens, ticagrelor was the most efficacious. At 24 h following drug administration, ROC analysis revealed that miR-339-3p and miR-365-3p had the highest sensitivity (74.3% and 90.0%, respectively) and specificity (71.4% and 93.3%) for detecting HTPR compared with the five other miRNAs. The SYNTAX score correlated positively with miR-223-3p and miR-365-3p levels at 24 h ($P \leq 0.006$) and with miR-365-3p levels 7 days following drug administration ($P = 0.014$). The expression of these three miRNAs was highest in hyperresponsive platelets (PRU < 85), followed by hyporesponsive (PRU ≥ 208), then normoreactive platelets. These findings suggest that the miR-365-3p expression level correlates with the antiplatelet treatment response.

Of the seven miRNAs, miR-365-3p expression showed the greatest correlation with treatment type and platelet activity, with higher expression levels associated with higher platelet activity. In a similar study, Kondkar et al. (2010) found an inverse relationship between miR-96 expression and platelet reactivity, with higher pre-miR-96 (mir-96) expression levels in hyporesponsive than in hyperresponsive platelets [35]. Bioinformatics and molecular experiments suggest that miR-96 regulates platelet reactivity by reducing the levels of vesicle-associated membrane protein (VAMP8) mRNA and consequent protein levels of VAMP8, a key regulator of platelet granulation. In our study, miR-96-5p showed expression trends similar to that seen in the Kondkar et al. study [35], with levels highest in the PRU < 85 group. No difference in miR-96-5p expression was observed between treatment groups ($P > 0.05$) (see Supplemental Material Fig. S3).

Table 1 Baseline characteristics, hematological index, SYNTAX score, comorbidities, and medication measured 24 hours following treatment.

	Control (n = 20)	Clopidogrel[#] (n = 54)	Ticagrelor[#] (n = 52)	Clopidogrel + Cilostazol[#] (n = 49)	P value
Demoraphics					
Age	66.67±9.81	69.39±10.68	67.99±13.56	65.35±11.55	0.362
Male gender	12 (60.0)	38 (70.4)	38 (73.1)	28 (57.1)	0.306
Hematological index					
MPV, fL	9.82±0.68	10.56±0.96	10.4±0.81	10.35±0.91	*0.016*
Glucose, mg/dl	96(85.25,112.5)	101(87,114)	93.5(82.25,111.75)	102(85,118)	0.732
BUN, mg/dl	16.4(11.85,19.2)	20.5(16.18,28.68)	18.3(14.85,22.9)	19(14,25.5)	*0.032*
Creatinine, mg/dl	0.8(0.53,0.98)	0.9(0.68,1.2)	1(0.8,1.2)	0.8(0.6,1.1)	0.053
Triglyceride, mg/dl	134(88.5,187.5)	119(89.75,194)	163(105,203.25)	127(87,179)	0.409
Cholesterol, mg/dl	187.65±41.35	198.35±48.89	205.04±45.37	194.08±43.94	0.455
HDL-C, mg/dl	59(51.75,63.75)	47.5(36,55.75)[ǂ]	48(41,62.75)	48(38,58.5)	*0.018*
LDL-C, mg/dl	117.6±36.63	122.98±38.7	134.15±36.84	122.57±38.29	0.252
PRP platelet count	410.5(297.5,598.75)	425(331.25,550.5)[*]	533.5(403.75,748)	457(321,563.5)	*0.004*
SYNTAX (mean (sd))	NA	16(12,19.25)	13(11,17.75)	13(8,18.5)	0.054
Comorbidities					
Hypertension = yes	NA	53 (98.1)	52 (100.0)	46 (93.9)	0.119
Diabetes = yes	NA	23 (42.6)	22 (42.3)	24 (49.0)	0.742
Chronic renal failure = yes	NA	16 (29.6)	17 (32.7)	14 (28.6)	0.905
Medication					
ACEI/ARB = yes	NA	47 (87.0)	44 (84.6)	37 (75.5)	0.289
Beta blocker = yes	NA	32 (59.3)	32 (61.5)	38 (77.6)	0.109
Statin = yes	NA	53 (98.1)	52 (100.0)	49 (100.0)	NA

Data on age, MPV, cholesterol, and LDL-C are presented as mean ± standard deviation; other variables are shown as median (interquartile range). Gender, hypertension, diabetes, choronic renal failure, ACEI/ARB, beta blocker, and statin are expressed as count (%). NA indicaties no available data.

Italic *P* value indicated significantly among various PRU level (*P* value < 0.05).

[#] These treatment groups also received aspirin.

[ǂ] Indicates significantly different between control group and a given group, P < 0.001.

[*] Indicates significantly different between Ticagrelor group and a given group, P < 0.001.

ACEI/ARB = angiotensin converting enzyme inhibitor/ angiotensin II receptor blocker; BUN = blood urea nitrogen; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; MPV = mean platelet volume; PRP = platelet-rich plasma;

We observed that the expression levels of three target miRNAs (miR-339-3p, miR-365-3p, and miR-495-3p) differed between treatment groups, suggesting that changes in the expression of these miRNAs may result from the effects of therapy (see Supplemental Material. Fig. S3). These results are consistent with another study in which the circulating levels of several miRNAs changed significantly when acute coronary syndrome patients switched from a dual antiplatelet treatment with clopidogrel to ticagrelor, a stronger P2Y12 inhibitor [36]. The authors concluded that this observed modulation in the levels of specific microRNAs in response to increasing platelet inhibition is further the confirmation of the pathophysiological role played by platelet-derived microRNAs in cardiovascular disease.

Kaudewitz et al. demonstrated that the expression level of miRNA was associated with platelet function but not modulated by the choice of the medication [37], which is similar to our results. Aspirin likely influences miRNA expression; however, no studies investigating the underlying mechanism or degree of the effect were found. Because nearly all patients with coronary syndrome take aspirin (with or without other P2Y12 inhibitors), we found no studies that examined miRNA expression levels in coronary syndrome patients who did not take aspirin.

The elucidation of the mechanism of action of several platelet miRNAs has begun. Landry et al. identified a putative binding site for miR-223 in the 3′UTR of P2Y12 and showed that miR-223 repressed the translation of a reporter gene containing the P2Y12 3′UTR, suggesting that this miRNA regulates P2Y12 expression in vivo [4]. Similar to the results of our study, Nagalla et al. found a correlation between platelet miRNA expression and platelet reactivity [8]. They showed that the miRNAs miR-200b, miR-495, and miR-107 influenced the expression of protein kinase cAMP-dependent regulatory type II beta (PRKAR2B), Kelch-like protein 5 (LKHL5), and circadian locomotor output cycles kaput (CLOCK) proteins. All three of

Table 2. Expression of seven miRNAs in patients with various PRU level defined by 2013 ESC criteria.

	Control	PRU < 85	PRU 85-207	PRU ≥ 208	P value
24 hr (n = 175)					
miRNA-223-3p	7.54(6.68,8.15)	9.32(7.13,13.32)	6.09(4.42,6.9) †,*	7.85(6.79,8.91)§	*< 0.001*
miRNA-339-3p	-8.05(-8.48,-7.61)	-3.87(-5.83,-1.97)†	-7.53(-8.41,-6.48)*	-5.28(-6.97,-4.24) †,§	*< 0.001*
miRNA-15a-5	1.92(1.34,2.37)	4.83(4.2,6)†	1.87(0.55,4.09)	1.35(0.42,3.42)	*0.001*
miRNA-365-3p	-8.14(-9.08,-7.81)	-6.9(-7.11,-6.23) †	-8.43(-9.46,-7.84) *	-4.89(-5.96,-4.14) †,*,§	*< 0.001*
miRNA-96-5p	-5.44(-6.54,-4.8)	-3.76(-5.37,-0.68)	-7.15(-8.15,-4.6)	-5.42(-7.59,-3.67)	*0.016*
miRNA-495-3p	-4.61(-5.51,-3.74)	-6.18(-10.85,1.05)	-5.81(-8.2,-3.37)	-3.96(-5.13,-1.51) §	*0.002*
miRNA-107	-2.07(-2.71,-1.59)	-1.86(-3.74,1.68)	-3.5(-4.93,0.72)	-3.53(-4.43,-0.73)	0.211
Day 7 (n = 175)					
miRNA-223-3p	7.54(6.68,8.15)	9.51(8.86,10.25) †	6.01(5.14,6.83) †,*	8.89(8.37,9.65) †,§	*< 0.001*
miRNA-339-3p	-8.05(-8.48,-7.61)	-3.46(-5.1,-0.63) †	-7.54(-8.27,-6.81) *	-3.97(-5.26,-3.62) †,§	*< 0.001*
miRNA-15a-5	1.92(1.34,2.37)	3.96(1.3,6.68)	1.99(0.04,4.36)	2.04(0.56,3.37)	*0.017*
miRNA-365-3p	-8.14(-9.08,-7.81)	-6.58(-7.2,-5.65) †	-8.75(-10.07,-7.73) *	-5.08(-6.03,-4.48) †,*,§	*< 0.001*
miRNA-96-5p	-5.44(-6.54,-4.8)	-3.59(-7.95,-1.16)	-6.95(-8.67,-4.72)	-5.13(-7.54,-3.97)	*0.005*
miRNA-495-3p	-4.61(-5.51,-3.74)	-4.61(-6.68,-2.36)	-4.85(-6.75,-3.32)	-3.72(-4.89,-1.26)	0.051
miRNA-107	-2.07(-2.71,-1.59)	-1.16(-4.09,0.84)	-3.21(-4.6,-0.29)	-3.67(-5.1,-1.51)	*0.044*

Log2 transformation was applied to miRNA data and presented as median (interquartile range).

Italic *P* value indicated significantly among various PRU level ($P < 0.05$).

† Indicates significantly different between control group and a given group, $P < 0.001$.

* Indicates significantly different between patients with PRU < 85 group and a given group, $P < 0.001$.

§ Indicates significantly different between patients with PRU between 85 and 207 and a given group, $P < 0.001$.

No significant difference derived in pairwise comparisons for miRNA-15a-5 at day 7, miRNA-96-5p at 24 hr and day 7, and miRNA-107 at day 7.

these proteins play key roles in modulating platelet activation [16].

This study has several limitations. Whether the patients were on other drugs (e.g., diabetes medications) that may have affected miRNA profiles is unknown. We did not investigate whether these miRNAs act to change the processing of a pre-miRNA or the turnover of a given mRNA, or whether any mRNAs are regulated by miR339-3p or miR365-3p. In-depth bioinformatics and molecular studies are required to address these questions. The control group was monitored for only 1 day and not throughout the entire study, which might confound some of the findings. The lack of a baseline assessment of circulating miRNA levels represents a limitation in the study design. Only 20 healthy subjects were included as the control group, resulting in inadequate power. Additional experiments are required to further evaluate the relationship of these miRNAs with platelet activity.

In summary, our findings indicate that the levels of all seven investigated miRNAs are influenced by platelet activity level. Coronary artery disease severity correlated with miR-365-3p levels after 24 h and 7 days of drug administration, with moderate to good sensitivity and specificity for both miR-339-3p and miR-365-3p in detecting HTPR. Together with the findings of previous studies, our results suggest that miRNAs play an important role in platelet

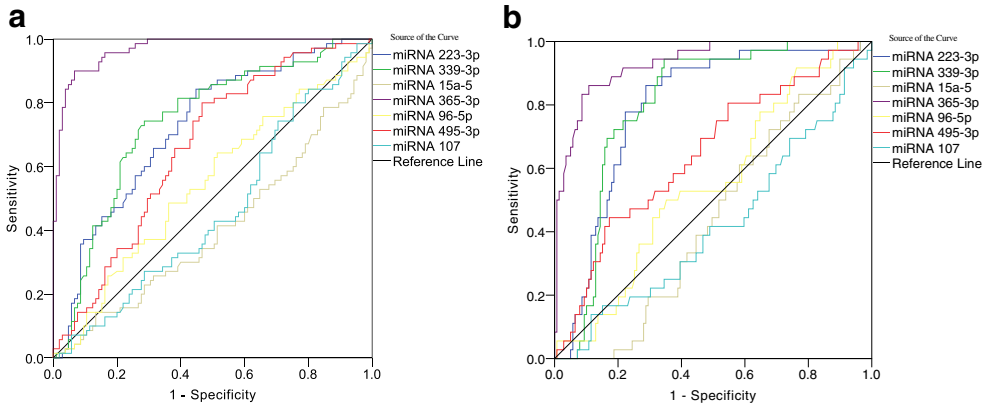

Fig. 2 Receiver operating characteristic (ROC) curves of seven miRNAs measured at **a** 24 h and **b** 7 days after treatment.

reactivity and that modification of their expression levels may alter the patient response to treatment.

Study Highlights

- What is the current knowledge on the topic?
  The expression level of platelet microRNAs (miRNAs) are correlated with various heart diseases and may be altered by antiplatelet therapy.
- What question did this study address?
  Are certain miRNAs associated with treatment response in platelets in patients who received percutaneous coronary intervention and antiplatelet therapy?
- What does this study add to our knowledge?
  miR-365-3p expression is correlated with antiplatelet treatment response.
- How might this change clinical pharmacology or translational science?
  The results suggest that modifying miRNA levels may be a strategy to alter the response of patients to antiplatelet treatment. Detection of miRNA levels may be a tool to predict antiplatelet treatment response.

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

Ethical Approval The protocol was approved by the Institutional Review Board of Taipei City Hospital, Taipei, Taiwan, ROC.

Informed Consent The study was performed in accordance with the Declaration of Helsinki and all patients gave their written informed consent.

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