Circulating MicroRNAs as Novel Biomarkers for Platelet Activation

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Rationale: **MicroRNA (miRNA) biomarkers are attracting considerable interest. Effects of medication, however, have not been investigated thus far.**

Objective: **To analyze changes in plasma miRNAs in response to antiplatelet therapy.**

Methods and Results: **Profiling for 377 miRNAs was performed in platelets, platelet microparticles, platelet-rich plasma, platelet-poor plasma, and serum. Platelet-rich plasma showed markedly higher levels of miRNAs than serum and platelet-poor plasma. Few abundant platelet miRNAs, such as miR-24, miR-197, miR-191, and miR-223, were also increased in serum compared with platelet-poor plasma. In contrast, antiplatelet therapy significantly reduced miRNA levels. Using custom-made quantitative real-time polymerase chain reaction plates, 92 miRNAs were assessed in a dose-escalation study in healthy volunteers at 4 different time points: at baseline without therapy, at 1 week with 10 mg prasugrel, at 2 weeks with 10 mg prasugrel plus 75 mg aspirin, and at 3 weeks with 10 mg prasugrel plus 300 mg aspirin. Findings in healthy volunteers were confirmed by individual TaqMan quantitative real-time polymerase chain reaction assays (n=9). Validation was performed in an independent cohort of patients with symptomatic atherosclerosis (n=33), who received low-dose aspirin at baseline. Plasma levels of platelet miRNAs, such as miR-223, miR-191, and others, that is, miR-126 and miR-150, decreased on further platelet inhibition.**

Conclusions: **Our study demonstrated a substantial platelet contribution to the circulating miRNA pool and identified miRNAs responsive to antiplatelet therapy. It also highlights that antiplatelet therapy and preparation of blood samples could be confounding factors in case-control studies relating plasma miRNAs to cardiovascular disease. (*Circ Res*. 2013;112:595-600.)**

Key Words: antiplatelet therapy ■ biomarkers ■ microRNAs ■ platelets ■ platelet inhibitors

Pioneering studies have revealed the presence of endogenous microRNAs (miRNAs) in the circulation that are not cell-associated.[1] The cellular origin and the biological function of circulating miRNAs, however, are less clear.[2]

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We have previously quantified circulating miRNAs in a large population-based cohort, the Bruneck study.[3,4] Using concepts of network topology,[5] we identified altered miRNA signatures in patients with type 2 diabetes mellitus[3] and with future myocardial infarction.[4] In addition, we subjected healthy volunteers to limb ischemia-reperfusion generated by thigh cuff inflation.[4] Computational analysis identified 6 distinct miRNA clusters.[4] One cluster included all miRNAs associated with risk of myocardial infarction and consisted of miRNAs predominantly expressed in platelets. Microarray screening revealed that miR-126, miR-197, miR-223, miR-24, and miR-21 are among the most highly expressed miRNAs in platelets and platelet microparticles (PMPs), and their circulating levels correlated with PMPs as quantified by flow cytometry.

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Nonstandard Abbreviations and Acronyms	
miRNA	microRNA
PMP	platelet microparticles
PPP	platelet-poor plasma
qPCR	quantitative real-time polymerase chain reaction

On the basis of our recent finding that circulating miRNAs may reflect platelet activation, we further investigate the platelet contribution to circulating miRNAs. We provide proof-of-concept for the potential of platelet miRNAs as a surrogate marker of efficacy of antiplatelet therapy.

Methods

An expanded Methods section is available in the Online Data Supplement.

Study Subjects

The following samples were obtained: (1) platelets, PMPs, serum, platelet-rich plasma, and platelet-poor (PPP) plasma were isolated from healthy volunteers (n=3)[6]; (2) serum and PPP were collected from patients with type II diabetes mellitus (n=19, Online Table I); (3) PPP was collected from healthy young men (<40 years, n=9) participating in a dose-escalation study of dual antiplatelet therapy at 4 timepoints[7]; (4) validation was performed in PPP of patients with symptomatic carotid atherosclerosis[8] (n=33, Online Table II).

Real-Time Polymerase Chain Reaction

MiRNAs were measured as described previously.[3,4] The layout of custom-made quantitative polymerase chain reaction (qPCR) plates is shown in Online Table III. For details and statistics or bioinformatics analysis, see the Online Data Supplement.

Results

Platelet Contribution to Circulating miRNAs

TaqMan miRNA fluidic cards (Human Pool Cards A v2.1) were used to assess a total of 377 miRNAs in platelets, PMPs, serum, platelet-rich plasma, and PPP of healthy individuals. Consistent with the expression data from platelets and PMPs (Online Figure I), miR-223 was the most differentially expressed miRNA in platelet-rich plasma (Figure 1). Similarly, when a panel of 28 miRNAs was compared in PPP and serum of diabetic patients (n=19), miR-223 showed the highest degree of classification potential (Figure 2A). On examination of the minimum miRNA signature that could discriminate serum from PPP, circulating levels of miR-223 provided the best efficiency of prediction (Figure 2B). Other miRNAs present in platelets, such as miR-24, miR-191, and miR-197, had less efficient prediction accuracy.

Pharmacological Intervention in Healthy Volunteers

Given the lack of a gold standard for assessing platelet function, we explored the potential of platelet miRNAs as a

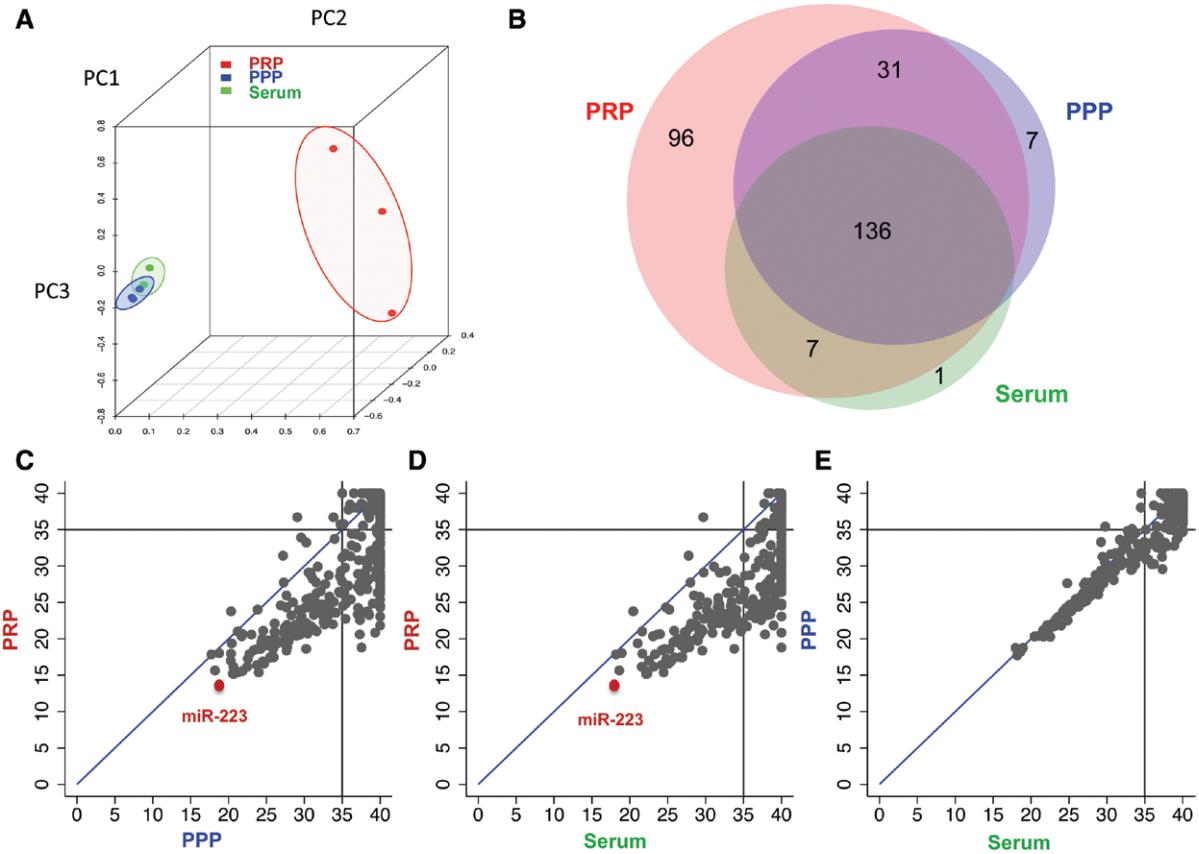

Figure 1. Platelet contribution to circulating miRNAs. Three hundred seventy-seven miRNAs were assessed in platelet-rich plasma (PRP), platelet-poor plasma (PPP), and serum of the same healthy individuals (n=3). A principal component analysis revealed clear discrimination of the samples (**A**) with most miRNAs being detected in PRP (cycle threshold [Ct] <35) (**B**). Levels of miRNAs were consistently higher in PRP compared with PPP (**C**) and serum (**D**). Differences between PPP and serum were much less pronounced (**E**). Blue lines are lines of equal levels. Added black lines on *x* and *y* axis indicate the detection threshold, assumed to be at a Ct value of 35.

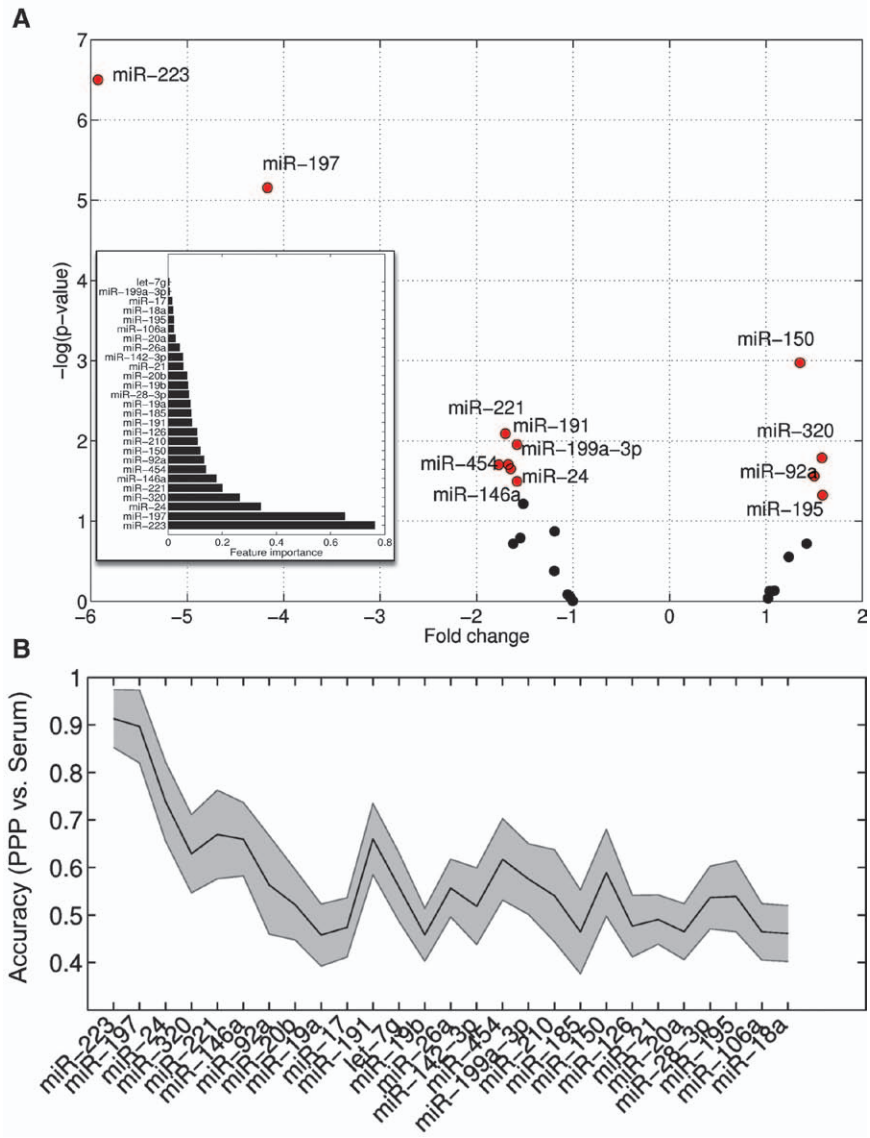

Figure 2. MiRNAs in patients with type 2 diabetes mellitus. A panel of 28 miRNAs was assessed in platelet-poor plasma (PPP) and serum of the same diabetic patients (n=19) by individual TaqMan quantitative real-time polymerase chain reaction assays. Levels of miR-24, miR-191, miR-197, and miR-223 were significantly higher in serum compared with PPP (**A**). miR-223, miR-197, and miR-24 showed the highest degree of classification potential (**inset**), with miR-223 levels giving the best efficiency of prediction (**B**).

surrogate marker of efficacy of antiplatelet therapy. Healthy young men (<40 years, n=9) were given 10 mg prasugrel (week 1), followed by a combination therapy with low-dose aspirin (75 mg, week 2) and higher-dose aspirin (300 mg, week 3). This dose escalation of aspirin in combination with prasugrel resulted in increasing platelet inhibition. As reported previously,[7] platelet function was assessed by 96-well plate aggregometry and the formation of thromboxane A_2 (measured as thromboxane B_2) by clotting blood, with response to treatment being additionally assessed by VerifyNow, and urine samples being retained for quantification of prostanoid metabolites on days 0, 7, 14, and 21 (Online Figure II). Using custom-designed qPCR plates preplated by the TaqMan custom plating service, 92 miRNAs were tested in PPP of a subset of 6 individuals. An exogenous spike-in control (*Caenorhabditis elegans* miRNA *cel-miR-39*) was used for normalization. Significant differences were identified for 15 miRNAs over the 4 time points (Online Figure III). To confirm the effect of dual antiplatelet therapy, selected miRNAs were quantified in all participants by individual TaqMan qPCR assays (Figure 3). Correlation coefficients with custom-made qPCR plates were >0.9. Notably, U6, a noncoding RNA frequently used for normalization, was affected by antiplatelet medication at week 1 and week 2 ($P<0.05$ in paired t test).

Pharmacological Intervention in Patients

Findings in healthy volunteers were corroborated by miRNA measurements in patients with recently symptomatic carotid atherosclerosis (n=33, Online Table II) participating in a randomized trial to determine whether treatment with dipyridamole or clopidogrel, in addition to aspirin, was more effective at reducing embolization.[8] Both treatment regimens had similar efficacy in reducing embolization, as evaluated using transcranial Doppler detection of cerebral embolic signals.[8] Twelve patients were randomly selected for miRNA analysis. All patients were on 75 mg of aspirin at baseline. Eight were randomized and received dipyridamole and 4 clopidogrel in

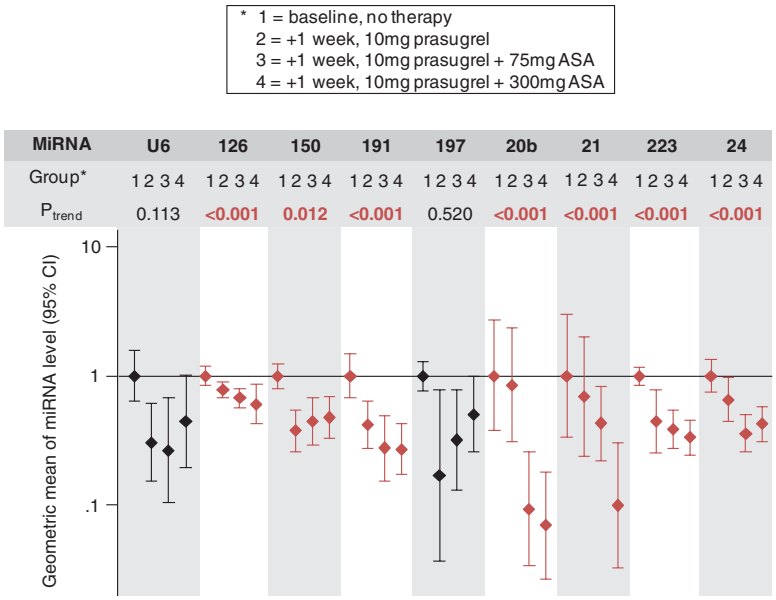

Figure 3. Response to antiplatelet therapy in healthy individuals. After screening of 92 miRNAs using custom-made quantitative real-time polymerase chain reaction (qPCR) plates (Online Figure III), a panel of 8 miRNAs plus U6 was assessed by individual Taqman qPCR assays in platelet-poor plasma from all healthy volunteers (n=9) participating in a dose-escalation study for antiplatelet therapy. Probability values are from linear mixed models with random intercepts comparing miRNA levels over the 4 time points. ASA indicates acetylsalicylic acid.

addition to aspirin. After 48 hours, effects on miRNAs were assessed in PPP using custom-designed miRNA qPCR plates based on Exiqon's locked nucleic acid technology to further ensure robustness of data independent of the technological platform (Online Figure IV). Selected miRNAs were quantified in all patients (n=33) by individual TaqMan qPCR assays (Figure 4). As in healthy volunteers, more potent platelet inhibition resulted in a reduction of miR-126 (P<0.001), miR-150 (P=0.003), miR-191 (P=0.004), and miR-223 (P=0.016), providing independent confirmation of our findings in a patient cohort, who were not naive for antiplatelet agents. In comparison, changes in ex vivo measurements of peak aggregation to ADP and collagen were less pronounced (Online Table II).

Discussion

The present study identified circulating platelet miRNAs that are responsive to antiplatelet therapy. Particular strengths of our study are as follows: (1) the repeated measurements in healthy individuals; (2) the additional investigation in patients on dual antiplatelet therapy; (3) the large number of miRNAs that were assessed by using custom-designed miRNA qPCR plates based on 2 different technologies (locked nucleic acid and TaqMan).[9]

Circulating Platelet miRNAs

Platelets represent the second most abundant cell type in blood. Although their miRNA content is low compared with other cells, platelets contribute substantially to the circulating miRNA pool. Any inconsistencies in plasma preparation will have profound effects on the miRNA content. Also, platelets shed microparticles on activation. Reduced microparticle shedding on platelet inhibition is likely to be responsible for the observed decrease in plasma miRNAs. Antiplatelet therapy was probably a confounding factor in previous case–control studies

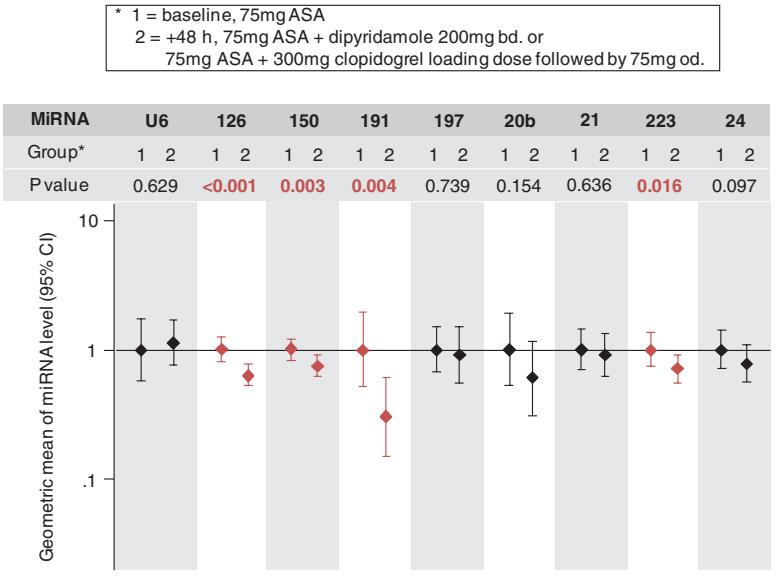

Figure 4. Response to antiplatelet therapy in patients. After screening of 92 miRNAs using custom-made quantitative real-time polymerase chain reaction plates (Online Figure IV), a panel of 8 miRNAs plus U6 was assessed in patients with symptomatic carotid atherosclerosis (n=33) who were on 75 mg aspirin (ASA) at baseline. Samples were taken 48 h after initiation of dual antiplatelet therapy with either dipyridamole or clopidogrel. *Groups depicted in red differ significantly in their average ΔCt value from the baseline group (tested by paired t tests, critical probability value of 0.05).

reporting a loss of miRNAs in patients with coronary artery disease.[10] Similarly, the transcoronary concentration gradients of noncardiac miRNAs may be related to platelet adhesion in the coronary circulation.[11] Given our limited knowledge about circulating miRNAs,[12] well-controlled intervention studies are needed to fill the significant gaps in our current knowledge about the effects of medication on circulating miRNAs.

Effects of Antiplatelet Therapy

Antiplatelet therapy plays a prime role in treatment and prevention of myocardial infarction and strokes. Yet, there is still no widely agreed and ideal measure of platelet activation to assess antiplatelet efficacy.[13] Importantly, the combination therapy of aspirin plus $P2Y_{12}$ inhibitors is associated with a significant bleeding risk, which can be life-threatening in a small but significant number of patients.[14] Combination therapy is commonly used for the management of non–ST-elevation acute coronary syndromes and ST-elevation myocardial infarction. Aspirin inhibits the production of thromboxanes. $P2Y_{12}$ inhibitors, such as clopidogrel, prasugrel, and ticagrelor, act by inhibiting ADP receptors. Thus, their mechanisms are complementary but variability in response to clopidogrel has been associated with gastrointestinal absorption, drug interactions, and P450 isoenzyme activity.[15] There is currently no one measure or standard definition in tests for antiplatelet efficacy.[13] Our study provides evidence that platelet miRNAs should be explored as a point-of-care test for tailoring antiplatelet therapies. These findings could become even more important as new potent antiplatelet agents are currently developed and tested in clinical trials.

Clinical Implications

miRNAs are stable and readily determined by qPCR, a technique widely used for viral diagnostics in clinical laboratories. Furthermore, quantification of miRNAs may well provide information about platelet reactivity within the circulation, which is a result of a balance between pro- and antiaggregatory stimuli. Tests of platelet reactivity to date have concentrated on assessing platelet responses ex vivo. Such approaches have notably failed to provide any useful guidance to antiplatelet medication. Thus, a clinical introduction of platelet miRNA assays could address the need to provide tailored effective antiplatelet therapy. Future studies will need to explore whether platelet miRNA-guided dosing of therapy can improve therapeutic outcomes.

Conclusions

Repeated measurements in healthy volunteers and patients on antiplatelet therapy provided proof-of-concept that miRNAs could serve as novel biomarkers of platelet activation and codiagnostic for efficacy of antiplatelet therapy. This awaits confirmation in larger studies. Our findings also highlight that antiplatelet therapy is a potential confounding factor for miRNA measurements in case–control studies of cardiovascular disease.

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Disclosures

The authors filed patent applications related to circulating miRNAs as cardiovascular biomarkers.

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Novelty and Significance

What Is Known?

- Platelets and platelet microparticles contain microRNAs.
- Platelets shed microparticles on activation.
- Platelet microparticles correlate to plasma microRNAs.

What New Information Does This Article Contribute?

- Extent of platelet contribution to circulating microRNAs.
- Identification of plasma microRNAs responsive to platelet inhibition

Tests of platelet reactivity to date have concentrated on assessing platelet responses ex vivo. Such approaches have limited use in guiding antiplatelet medication. Platelets shed microparticles on activation. Microparticles contain platelet microRNAs, which can be readily determined by real-time polymerase chain reactions. Thus, a clinical introduction of platelet microRNA assays could be used to tailor antiplatelet therapy and reduce the risk of life-threatening bleeding complications. Future studies are required to determine whether tailoring antiplatelet therapies according to platelet microRNA levels might improve therapeutic outcomes in patients on dual antiplatelet therapy.