Research Article

Circulating endothelial microparticles and miR-92a in acute myocardial infarction

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Microparticles (MPs) and miRNAs have been shown to play important roles in coronary artery disease (CAD) by monitoring endothelial dysfunction. The present study aims to investigate the diagnostic value of endothelial MPs (EMPs) and miRNAs (miR-92a or miR-23a) as biomarkers in distinguishing patients with acute myocardial infarction (AMI) from those with CAD. Plasma samples from 37 patients with AMI, 42 patients with stable CAD (SCAD), and 35 healthy adults were collected for investigation in the present study. The numbers of CD31+/CD42b− MPs, CD31+/CD42b+ MPs, and CD31−/CD42b− MPs were measured by flow cytometry and the levels of miR-92a and miR-23a were analyzed using reverse transcription-quantitative PCR. Moreover, cardiac troponin I (cTnI) expression was detected by ELISA to serve as a routine diagnostic parameter. The number of CD31+/CD42b− was higher in AMI group than those in SCAD and healthy groups. Besides, the expression of miR-92a was higher in AMI group compared with two other groups. Furthermore, evidence showed that there was a positive correlation between the levels of CD31+/CD42b− MPs and miR-92a. Finally, the receiver operating characteristic (ROC) curve revealed that the area value under the curve of CD31+/CD42b− MPs, miR-92a and cTnI was 0.893, 0.888, and 0.912 respectively. CD31+/CD42b− MPs and miR-92a might have great potential to provide diagnostic value for AMI and could probably regulate the endothelial dysfunction in AMI patients.

Introduction

It is noteworthy that coronary heart disease, particularly acute myocardial infarction (AMI), is the leading cause of death and disability worldwide [1,2]. AMI is characterized by cardiac cell death resulted from exposure to prolonged ischemia after occlusion of a coronary artery. Rapid and correct diagnosis of AMI is believed to be a key role in therapy and prognosis for this disease [3]. At present, cardiac troponin I (cTnI) is the golden standard used by clinicians to diagnose patients. However, cTnI cannot distinguish between patients with stable coronary artery disease (SCAD) and patients at risk for AMI [4]. Therefore, it is essential to identify novel biomarkers with high sensitivity and specificity for early diagnosis of AMI.

Microparticles (MPs) are small membrane vesicles released from many different cell types in response to cellular activation or apoptosis. MPs shedding from endothelial cells are called endothelial MPs (EMPs) [5]. Some evidence suggest a higher expression of EMPs in patients with SCAD and AMI than that in healthy people [6]. However, little is known about the difference of EMPs expression between SCAD and AMI.

Moreover, in recent years, a large number of data suggest that microRNAs (miRNAs, miR) are important for the development of various disorders, including cardiovascular diseases [7,8]. The role of plasma
miRNAs in AMI has been recently investigated [9]. Some evidence demonstrated that \textit{miR-92a} and \textit{miR-23a} could regulate endothelial dysfunction and atherosclerosis [10,11]. However, the expression difference of both \textit{miR-23a} and \textit{miR-92a} between SCAD and AMI is largely undetermined.

Therefore, the present study was designed to determine the diagnostic value of EMPs and \textit{miR-92a} or \textit{miR-23a} as biomarkers in distinguishing patients with AMI from those patients with coronary artery disease (CAD).

**Materials and methods**

**Study subjects**

We recruited 37 patients with AMI, 42 patients with SCAD, and 35 healthy adults in the present study from September 2015 to December 2015. Patients with SCAD were diagnosed based on presentation with exertional substernal chest pressure without recent acceleration in symptoms and evidence of a significant angiographic stenosis \( \geq 70\% \), in the absence of myocardial necrosis. Patients with AMI were diagnosed according to the universal definition of myocardial infarction [12]. All subjects including patients and controls with a history and clinical features of infectious disease, auto-immune, malignant tumor, peripheral vascular disease, lung diseases, hepatic or hematologic disorders, or chronic renal failure requiring dialysis were excluded from the present study. The present study’s protocol was approved by the Ethics Committee (Beijing Anzhen Hospital of the Capital University of Medical Sciences) of and informed written consent was obtained from all these subjects. Clinical and laboratory data were collected from all subjects.

**cTnl determination**

The preferred biomarker for each specific category of MI is cTnl, which has high myocardial tissue specificity as well as high clinical sensitivity. Detection of rise and fall of the measurements is essential to the diagnosis of AMI. An increased cTnl concentration is defined as a value exceeding the 99% of a normal reference population [13]. Blood samples were obtained after 1 h in the emergency department [14]. Plasma cTnl concentrations were determined by chemiluminescence immunoassays according to manufacturer’s protocol (Beckman Coulter, Fullerton, CA, U.S.A.).

**Sample preparation for MP analysis and miRNA measurement**

Whole blood samples were collected into 15-ml sterile centrifuge tube containing citrate anticoagulant and centrifuged at 1500 g for 20 min. One milliliter of plasma was drawn carefully by a micropipette into a 1.5-ml sterile Eppendorf tube and centrifuged at 13000 g for 2 min to obtain the platelet-poor plasma (PFP). Then, samples were stored respectively at \(-20\)^\circ C for a week and then at \(-80\)^\circ C until MP analysis.

PFP samples were transferred into new RNase/DNase-free tubes and stored at \(-80\)^\circ C until RNA extraction. It has been shown that miRNAs in frozen plasma remain stable for years and are reliable biomarkers of cardiac heart disease.

**MP detection through flow cytometry**

The top 50 \( \mu \)l of PFP was then incubated with fluorochrome-labeled antibodies specific for CD31 (BD Biosciences) and CD42b (BD Biosciences) for 20 min in the dark at 4\(^\circ\)C. Thereafter, the samples were diluted with 1 ml of PBS and then measured by flow cytometry. EMPs were identified as CD31+/CD42b— events within the MP size gate, platelet MPs (PMPs) as CD31+/CD42b+, and MPs derived from other cells as CD31—/CD42b—.

**miRNA analysis through qRT-PCR**

Quantitative real-time PCR (qRT-PCR)s were performed using the high-throughput BioMark Real-Time PCR system (Fluidigm, South San Francisco, CA, U.S.A.) according to the manufacturer’s protocol to determine the quality and abundance of miRNA. miRNA was quantified using NanoDrop spectrophotometer, and 10 ng of the total RNA was reversely transcribed using a TaqMan miRNA Reverse transcription kit (Applied Biosystems), according to the manufacturer’s protocol. \textit{miR-92a} and \textit{miR-23a} in plasma were detected using Taqman miRNA assays (Applied Biosystems) on a 7500 HT real-time polymerase chain reaction machine (Applied Biosystems). Cel-\textit{miR-39} was used as an endogenous control and the subsequent RNA isolation was then performed according to the manufacturers’ recommendation (final elution volume 50 \( \mu \)l). The addition of cel-\textit{miR-39} allows controlling sample-to-sample variations of the RNA isolation efficiency; it was demonstrated previously that estimation of cel-\textit{miR-39} is a suitable approach for normalization of plasma miRNAs in coronary heart disease [15].
Table 1 Clinical data of healthy subjects, SCAD, and AMI patients

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=35)</th>
<th>SCAD (n=37)</th>
<th>AMI (n=42)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% males)</td>
<td>57.1</td>
<td>59.5</td>
<td>66.7</td>
<td>0.083</td>
</tr>
<tr>
<td>Age (mean ± S.D.)</td>
<td>52.6 ± 5.1</td>
<td>53.7 ± 5.6</td>
<td>52.8 ± 5.6</td>
<td>0.125</td>
</tr>
<tr>
<td>BMI</td>
<td>23.1 ± 2.2</td>
<td>23.8 ± 2.9</td>
<td>24.2 ± 2.9</td>
<td>0.255</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>20.0</td>
<td>21.6</td>
<td>23.8</td>
<td>0.130</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>5.7</td>
<td>18.9</td>
<td>19.0</td>
<td>0.314</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>4.4 ± 0.4</td>
<td>4.3 ± 0.6</td>
<td>4.4 ± 0.8</td>
<td>0.268</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>2.4 ± 0.4</td>
<td>3.1 ± 0.8</td>
<td>3.5 ± 0.9</td>
<td>0.091</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>0.132</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.1 ± 0.2</td>
<td>1.5 ± 0.4</td>
<td>1.5 ± 0.5</td>
<td>0.178</td>
</tr>
<tr>
<td>ACE-I/ARB (%)</td>
<td>-</td>
<td>21.6</td>
<td>14.3</td>
<td>-</td>
</tr>
<tr>
<td>Calcium-channel blockers (%)</td>
<td>-</td>
<td>32.4</td>
<td>28.6</td>
<td>-</td>
</tr>
<tr>
<td>ß-blockers (%)</td>
<td>-</td>
<td>64.8</td>
<td>69.0</td>
<td>-</td>
</tr>
<tr>
<td>Statin (%)</td>
<td>-</td>
<td>59.5</td>
<td>64.3</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are described as mean ± S.D. or as the number of subjects. ACE-I/ARB: angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker. Comparison was made between SCAD and AMI group and P-value was shown, a value of P<0.05 was considered with significant difference.

Figure 1. Representative flow cytometry analysis of MPs in plasma

Statistical analysis
Data analysis was performed using the software of IBM SPSS Statistics version 18.0. Variables were evaluated using the Kolmogorov–Smirnov test or Shapiro–Wilks test to assess normality. Normally distributed data were expressed as the mean ± S.D. or as numbers. Comparison among groups was assessed by the chi-square test or ANOVA. Spearman’s correlation test was used to analyze correlations between EMPs and miR-92a. Each cardiac biomarker was examined; receiver operating characteristic (ROC) curves and optimal cut-off values were obtained. ROC curves were used for evaluation of diagnostic accuracy of EMPs, miR-92a, and cTnI. A value of P<0.05 was considered significant. Besides, the sensitivity and specificity value of the candidate biomarkers were determined.

Results
Characteristics of study subjects
In the present study, a total of 37 patients with AMI, 42 patients with SCAD, and 35 healthy people were recruited. As shown in Table 1, there were no significant differences in the following clinical characteristics between patients with AMI and patients with SCAD: age, gender, body mass index (BMI), hypertension, diabetes, history of smoking, total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglyceride (TG). Patient characteristics are detailed in Table 1.

Expression of MPs in plasma
As shown in Figure 1 and Table 2, we found the CD31+/CD42b− MPs were markedly higher in patients with AMI than patients with SCAD (P=0.0003) and controls. Besides, there was no significant difference between patients with
Table 2 Characteristics of MPs in healthy subjects and patients with AMI or CAD

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>AMI</th>
<th>CAD</th>
<th>Healthy controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31+/CD42b− MPs</td>
<td>12.5 ± 5.7</td>
<td>0.08 ± 0.07</td>
<td>0.04 ± 0.03</td>
<td>0.0003*</td>
</tr>
<tr>
<td>CD31+/CD42b+ MPs</td>
<td>3.4 ± 2.4</td>
<td>3.9 ± 1.8</td>
<td>3.8 ± 2.1</td>
<td>0.18</td>
</tr>
<tr>
<td>CD31−/CD42b− MPs</td>
<td>79 ± 8.9</td>
<td>82 ± 11.9</td>
<td>84 ± 10.6</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Comparison was conducted between SCAD and AMI group and P-value was shown, * a value of P<0.05 was considered with significant difference.

Figure 2. The relative expression of miR-92a and miR-23a in healthy subjects and patients with CAD or AMI
Comparisons were made between CAD and healthy group, or CAD and AMI group. A value of P<0.05 was considered with significant difference.

SCAD and controls. However, CD31+/CD42b+ MPs and CD31−/CD42b− MPs showed no significant difference among the three groups.

Expression of miR-92a and miR-23a in plasma
Two coronary heart disease-related miRNAs, miR-92a and miR-23a, expressed differently among the three groups. As shown in Figure 2, the expression of miR-92a rather than miR-23a between patients with SCAD and controls, and between patients with AMI and SCAD both showed a significant difference.

Correlations between circulating EMPs and miR-92a in AMI patients
To evaluate the possible link between EMPs and miR-92a in AMI patients, we analyzed whether there was a correlation between the high levels of expression of miR-92a and EMPs. It was revealed that the level of expression of miR-92a was significantly correlated with EMPs (see in Figure 3).

Circulating EMPs and miR-92a expression as potential predictor of AMI
To further investigate the efficiency of EMPs and miR-92a as potential biomarkers of AMI, we performed ROC curve analysis between patients with AMI and patients with SCAD. Surprisingly, according to the outcome of ROC curve analysis, we found that the areas under the curve (AUC) of EMPs, miR-92a, and cTnI were 0.893, 0.888, and 0.912 respectively (see in Figure 4).

Discussion
The high level of EMPs in plasma directly demonstrates a condition of endothelial dysfunction. Endothelial dysfunction has been associated with increased circulating EMPs [16], and high level of EMPs in plasma indicates endothelial activation [17]. In the present study, the level of EMPs was highly expressed in AMI rather than in SCAD, which indicated that the extent of endothelial cells activation was higher in AMI than in SCAD. In our study, for the first time we found that the expression of miR-92a in plasma was higher in AMI than SCAD, and in SCAD than controls. Since high level of miR-92a expression promotes endothelial activation and the development of atherosclerotic lesions, and miR-92a can serve as a valuable therapeutic target in angiogenesis and functional recovery of ischemic tissues in mice [18], we speculated that miR-92a might probably relate to myocardial damage. The mechanism under this
hypothesis is unknown yet, but we postulated that endothelial cells-derived miR-92a might be transferred by EMPs from endothelial cells to myocardial cells and thus cause damage to the myocardial cells.

Furthermore, we explored the relationship between EMPs and miR-92a in AMI patients. It is known that EMPs have intricate functions in intercellular communication and compound exchange and can regulate target cells by carrying certain non-coding RNA and protein into them [19]. Interestingly, we showed a significantly positive correlation between EMPs and miR-92a, which implied that EMPs might regulate endothelial dysfunction and other types of cells through miR-92a in AMI.

Last, we examined the diagnostic value of EMPs and miR-92a in discriminating patients with AMI from patients with SCAD. Our results showed that the AUC of circulating EMPs, miR-92a, and cTnI were 8.925, 8.883, and 9.123 respectively. This evidence demonstrated that the high levels of expression of miR-92a and EMPs could be potential biomarkers to distinguish patients with AMI from patients with SCAD. However, since the sample size in the present study was relatively small, larger clinical studies are required to thoroughly establish the case.

Conclusion
The present study indicates that the high level of plasma EMPs and miR-92a could reflect the extent of endothelial cells activation and more importantly, they could serve as novel biomarkers to diagnose patients with AMI from stable coronary heart disease.
Competing interests
The authors declare that there are no competing interests associated with the manuscript.

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Author contribution
Y.L. and Y.C.Z. conceived and designed the experiments. Y.C.Z., J.J.C., F.C., C.Y.W., J.M.Z., and X.J.R. performed the experiments. Y.P., B.N., and Q.L. analyzed the data. Y.L. and Y.C.Z. wrote the paper.

Abbreviations
AMI, acute myocardial infarction; CD31, Platelet endothelial cell adhesion molecule-1; cel-miR-39, Caenorhabditis elegans microRNA 39; CD42, cluster of differentiation 42; MI, myocardial infarction; AUC, areas under the curve; BMI, body mass index; CAD, coronary artery disease; cTnI, cardiac troponin I; EMP, endothelial microparticle; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; MP, microparticle; PFP, platelet-poor plasma; ROC, receiver operating characteristic; SCAD, stable coronary artery disease; TC, total cholesterol; TG, triglyceride.

References