Diagnostic value of miR-30d-5p and miR-125b-5p in acute myocardial infarction

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Abstract. Rapid and accurate differential diagnosis of acute myocardial infarction (AMI) is crucial for timely interventions and the improvement of prognosis. However, this is difficult to achieve using current methods. Therefore, the present study aimed to evaluate the suitability of circulating microRNAs (miRNAs) as AMI biomarkers in patients with acute coronary syndrome (ACS). miRNA profiling in plasma samples from patients with AMI (n=3) and healthy controls (n=3) was performed using microarrays. Results were then validated in five patients and five healthy controls. miRNA-125b-5p and miR-30d-5p expression levels were quantified in plasma samples from 230 patients with ACS and 79 healthy controls using reverse transcription-quantitative polymerase chain reaction. Routine diagnostic parameters were assessed, including creatinine kinase MB, cardiac troponin I (cTnI) and myoglobin. A total of 33 miRNAs were differentially expressed in patients with AMI and healthy controls. Following validation based on the previously established roles for these miRNAs, six miRNAs were validated. miR-125b-5p and miR-30d-5p were selected for further investigation. Expression levels of miR-125b-5p and miR-30d-5p in plasma were higher in patients with ACS compared with the healthy controls (P<0.001). Receiver operating characteristic curve analysis revealed that the area under the curve of miR-30d-5p was higher than that of cTnI (0.915 and 0.899). miR-125b-5p (sensitivity, 0.808; specificity, 0.845) and miR-30d-5p (sensitivity, 0.855; specificity, 0.810) were suitable diagnostic predictors of AMI. Kaplan-Meier survival analysis indicated that miR-125b-5p levels were associated with 6 month cardiovascular events in patients with ACS (11,12). In addition, the timing of troponin measurement with respect to symptom onset has an impact on the result (13,14). Therefore, novel biomarkers with high sensitivity and specificity for early diagnosis of AMI are urgently required to improve the prognosis of patients with acute chest pain.

Introduction

Acute coronary syndrome (ACS) covers the spectrum of acute myocardial ischemia and/or necrosis that is commonly secondary to reduced coronary blood flow. ACS also includes unstable angina pectoris (UAP) and acute myocardial infarction (AMI) (1-5). ACS is common in emergency rooms and each year it is responsible for 1 million hospital admissions in the USA and 2 million in Europe (5). Early diagnosis and intervention are crucial in order to minimize the damage to the cardiac muscle (1-5), as treatment may significantly reduce mortality and improve long-term prognosis (6). For accurate ACS diagnosis, physical examination, electrocardiography, radiologic studies and cardiac biomarker tests are essential, and these also aid in guiding treatment (1). Timely and appropriate treatment for ACS includes cardiac catheterization and primary percutaneous coronary intervention (7).

Since clinical presentation and echocardiography findings are often nonspecific in patients with chest pain, cardiac biomarkers, including cardiac troponin I (cTnI), creatine kinase MB (CK-MB) mass and myoglobin (Myo) are often used for diagnosis (8,9). Indeed, cTnI is the gold standard for diagnosis of AMI (10), however, serial testing is required as it is also frequently present in patients with chronic but stable coronary artery disease and may be detected in apparently healthy controls (11,12). In addition, the timing of troponin measurement with respect to symptom onset has an impact on the result (13,14). Therefore, novel biomarkers with high sensitivity and specificity for early diagnosis of AMI are urgently required to improve the prognosis of patients with acute chest pain.

Notably, the role of microRNAs (miRNAs) in acute myocardial infarction (AMI) has been previously investigated (15,16). Levels of muscle-specific miR-1, miR-133a and miR-499 in addition to cardiac-specific miR-208a were significantly higher in plasma samples from patients with AMI compared with controls (16,17). Circulating miRNAs are readily detectable, relatively stable and tissue-specific (14), making them attractive biomarker candidates.

It was hypothesized that specific miRNAs may be associated with AMI. The aim of the present study was to comprehensively assess the miRNAs released into circulation during AMI, and determine which may be used as biomarkers to detect and monitor myocardial injury. In addition, miRNA
expression levels were compared with established biomarkers, including CK-MB, Myo and cTnI.

**Patients and methods**

**Study design and patients.** Circulating miRNAs were profiled in 3 patients with AMI and 3 healthy controls. These results were then validated by profiling the same miRNAs in 5 additional patients with AMI and healthy controls. The selected miRNAs were further assessed with a larger sample size, including 230 consecutive patients with ACS and 79 healthy controls (normal electrocardiograms and no history of cardiovascular diseases). Exclusion criteria were as follows: i) Presence of chest pain for >3 h at admission; or ii) angiography was not performed.

Plasma samples from patients were collected at the TEDA International Cardiovascular Hospital Emergency Department (Tianjin, China) between September 2011 and September 2013. Serial blood samples were collected from individuals with AMI at 0-3, 3-6, 6-9, 9-12 and 12-24 h following admission. Diagnoses of ACS, UA, AMI, ST-elevated myocardial infarction (STEMI) and non-ST-elevated myocardial infarction (NSTEMI) were made according to the manufacturers' protocol. RNA integrity was assessed by electrophoresis on a 1.2% denaturing agarose gel at 120 V for 15 min. Intact total RNA was characterized by sharp 28S and 18S rRNA bands (eukaryotic samples). All plasma RNA preparations were quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Samples with absorbance (260/280) ratios >1.8 were considered to be optimal for microarray assays. RNA samples were labeled with the miRCURY Hy3 Power labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on the miRCURY LNA microRNA Array system. The slides were washed three times using a wash buffer kit (Exiqon), then dried by centrifugation at 1.5 x g, for 5 min at room temperature. Next, the slides were scanned on an Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). Scanned images were imported into the GenePix Pro software (version 6.0; Molecular Devices, LLC) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥30 in all samples were selected for calculating the normalization factor. Data were normalized using the Median normalization (18). Significantly differentially expressed miRNAs were identified through Volcano Plot filtering. Hierarchical clustering was also performed to indicate the various miRNA expression profiling among samples. The threshold value for significance of miRNA upregulation or downregulation was set at fold-change ≥2.0, with P<0.05 calculated by Student’s t-test. The miRNAs selected for investigation in the current study were further filtered based on their expression levels, described in previously published data, and their heart-specificity defined according to umm.uni-heidelberg.de/apps/zmf/mirwalk/disease.php (19).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA (5 µl) was reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Prior to RT, RNA was eluted in nuclease-free water in order to avoid DNA contamination. Temperatures used for RT were 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The primer sequences used were obtained from Invitrogen (Thermo Fisher Scientific, Inc.) and profiling was performed with plasma samples from patients with AMI (n=3) and healthy controls (n=3) using the mirCURY LNA microRNA Array system (version 18.0; Exiqon Inc., Woburn, MA, USA). Total RNA was prepared using TRIzol (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) and an miRNeasy Mini kit (Qiagen GmbH, Venlo, Netherlands) according to the manufacturers’ protocol. RNA integrity was assessed by electrophoresis on a 1.2% denaturing agarose gel at 120 V for 15 min. Intact total RNA was characterized by sharp 28S and 18S rRNA bands (eukaryotic samples). All plasma RNA preparations were quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Samples with absorbance (260/280) ratios >1.8 were considered to be optimal for microarray assays. RNA samples were labeled with the miRCURY Hy3 Power labeling kit (Exiqon, Vedbaek, Denmark) and hybridized on the miRCURY LNA microRNA Array system. The slides were washed three times using a wash buffer kit (Exiqon), then dried by centrifugation at 1.5 x g, for 5 min at room temperature. Next, the slides were scanned on an Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). Scanned images were imported into the GenePix Pro software (version 6.0; Molecular Devices, LLC) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥30 in all samples were selected for calculating the normalization factor. Data were normalized using the Median normalization (18). Significantly differentially expressed miRNAs were identified through Volcano Plot filtering. Hierarchical clustering was also performed to indicate the various miRNA expression profiling among samples. The threshold value for significance of miRNA upregulation or downregulation was set at fold-change ≥2.0, with P<0.05 calculated by Student’s t-test. The miRNAs selected for investigation in the current study were further filtered based on their expression levels, described in previously published data, and their heart-specificity defined according to umm.uni-heidelberg.de/apps/zmf/mirwalk/disease.php (19).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA (5 µl) was reverse-transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Prior to RT, RNA was eluted in nuclease-free water in order to avoid DNA contamination. Temperatures used for RT were 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The primer sequences used were obtained from Invitrogen (Thermo Fisher Scientific, Inc.) and

**miRNA array analysis.** In order to assess the differential miRNA expression in patients with AMI, miRNA expression

**Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.**

| microRNAs       | Forward (5'-3')       | Reverse (5'-3')       |
|-----------------|-----------------------|-----------------------|
| hsa-miR-125b-5p | GCTCCCTGAGACCCCTAAC   | GTGCCTGTCGTGGAGTCTG   |
| hsa-miR-136-5p  | GGAACCTCCATTGTTTTGA   | CAGTGCGTTCGTGGAGTCTG  |
| hsa-miR-129-1-3p| GAAGCCCTACCACCA      | CAGTGCGTTCGTGGAG      |
| hsa-miR-30d-5p  | GGTGTAACACCTCCCCGAC   | CAGTGCGTTCGTGGAGAG    |
| hsa-miR-27a-5p  | GAGGGGCTTACGTGCTTGT   | GTGCGTTCGTGGAGTCTG    |
| hsa-miR-1291    | TCGCCCTGACTGAGACC     | CAGTGCGTTCGTGGAGTCTG  |
Table II. MicroRNAs were differentially expressed in patients with AMI compared with healthy controls by microarrays.

| microRNAs          | Fold-change | P-value |
|--------------------|-------------|---------|
| hsa-miR-24-3p      | 7.563       | 0.034   |
| hsa-miR-29a-5p     | 7.615       | 0.005   |
| hsa-miR-125b-5p    | 40.002      | 0.004   |
| hsa-let-7b-5p      | 16.004      | 0.008   |
| hsa-let-7g-5p      | 5.058       | 0.038   |
| hsa-miR-125a-5p    | 12.485      | 0.002   |
| hsa-miR-149-5p     | 6.772       | 0.035   |
| hsa-miR-330-5p     | 9.886       | 0.004   |
| hsa-miR-101-3p     | 9.706       | 0.024   |
| hsa-miR-34c-5p     | 2.325       | 0.048   |
| hsa-miR-139-5p     | 39.982      | 0.028   |
| hsa-miR-29b-1-5p   | 2.179       | 0.034   |
| hsa-miR-132-5p     | 10.184      | 0.023   |
| hsa-miR-212-3p     | 4.204       | 0.021   |
| hsa-miR-129-1-3p   | 16.264      | 0.003   |
| hsa-miR-138-5p     | 249.663     | 0.010   |
| hsa-miR-9-5p       | 31.712      | 0.016   |
| hsa-miR-30a-5p     | 6.233       | 0.026   |
| hsa-miR-29c-5p     | 6.756       | 0.038   |
| hsa-miR-138-2-3p   | 3.074       | 0.010   |
| hsa-miR-129-5p     | 21.802      | 0.024   |
| hsa-miR-124-3p     | 301.144     | 0.027   |
| hsa-let-7i-5p      | 10.096      | 0.002   |
| hsa-miR-22-3p      | 9.958       | 0.043   |
| hsa-miR-9-3p       | 16.504      | 0.020   |
| hsa-miR-136-5p     | 448.976     | 0.011   |
| hsa-miR-15a-3p     | 3.325       | 0.015   |

B, Downregulated

| microRNAs          | Fold-change | P-value |
|--------------------|-------------|---------|
| hsa-miR-500a-5p    | 0.385       | 0.047   |
| hsa-miR-27a-5p     | 0.046       | 0.012   |
| hsa-miR-142-5p     | 0.200       | 0.027   |
| hsa-miR-664a-5p    | 0.212       | 0.020   |
| hsa-miR-1246       | 0.173       | 0.046   |
| hsa-miR-1291       | 0.033       | 0.116   |

The threshold value for significance used for miRNA upregulation or downregulation was set at fold change ≥2.0 with a P<0.05 calculated by t-test. All differentially expressed microRNAs are available upon request.

are described in Table I. Subsequently, 2.33 µl cDNA was used to assess miRNA expression by qPCR using the TaqMan microRNA Assay kits (hsa-miR-125b-5p, assay id. 477885_mir; hsa-miR-136-5p, assay id. 478307_mir; hsa-miR-129-1-3p, assay id. 478690_mir; cel-miR-39-3p, assay id. 478293_mir; Applied Biosystems; Thermo Fisher Scientific, Inc.) on a Light Cycler 480 Real Time PCR System (Roche Diagnostics, Basel, Switzerland). All reactions involved an initial denaturation step at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. The Cq value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (20). Values were normalized by spiking in 5 fmol/µl cel-miR-39-3p (Invitrogen; Thermo Fisher Scientific, Inc.) in the patient samples (21) from the cel-miR-39-3p TaqMan MicroRNA Assay kit and ratios were derived as previously proposed (22).

Biochemical analyses. Peripheral blood was collected in tubes containing EDTA and centrifuged at 820 x g for 5 min at room temperature. cTnI and Myo levels were determined in plasma samples by chemiluminescence immunoassays, using the accuTnI and Access Myoglobin kits respectively from Beckman Coulter, Inc. (Brea, CA, USA). CK-MB was assessed by a quantitative mass assay using the Access CK-MB kit (Beckman Coulter, Inc.).

Study endpoint and follow-up. The study endpoint was defined as the occurrence of cardiovascular death, myocardial infarction, hospitalization for unstable angina, stroke, coronary revascularization procedures, peripheral revascularization procedures or heart failure requiring hospitalization (23). To avoid multiple counting of patients with more than one event, each patient contributed only once to the composite endpoint. Endpoints or events were determined by reviewing the medical records or by follow-up telephone interviews for up to 12 months following the initial chest pain episode.

Statistical analysis. Statistical analysis was performed using SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, CA, USA). The normality of the data was assessed using Shapiro-Wilk test or Kolmogorov-Smirnov test. Normally distributed data is expressed as the mean ± standard error, and the differences between groups were compared by one-way analysis of variance with Tukey's honest significant difference test used for post-hoc analysis or independent samples t-test. The normally distributed data has been indicated in Table III. Data that was not normally distributed was expressed as the median (min, max) or median and interquartile range, and the differences between groups were compared using the non-parametric Kruskal-Wallis one-way analysis of variance. The differences between qualitative categorical data groups were compared using the Pearson's χ² test. Receiver operating characteristic (ROC) curves were established for discriminating AMI. Each cardiac biomarker was examined, and ROC curves and optimal cut-off values were obtained. In addition, the sensitivity, specificity, positive predictive value and negative predictive value of the candidate biomarkers were determined. The correlations of cardiac biomarkers with end point events at 1-, 6- and 12-month follow-ups were evaluated by the Kaplan-Meier, log-rank and Cox regression tests. Kaplan-Meier was used to draw the Kaplan-Meier curves, and log-rank tests were used to detect whether there were
significant differences between the Kaplan-Meier curves. Cox regression analysis was performed to assess the risk factors involved in the prognosis of patients with ACS Two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients and controls. Profiling of the circulating miRNAs was performed in 3 patients with AMI and 3 healthy controls. The results were then assessed in a validation cohort of additional patients with AMI (n=5) and healthy controls (n=5). Next, the study was extended to a larger sample size (patients, n=230; controls, n=79). Fig. 1 presents the patients' flowchart. Among 588 patients who were admitted for chest pain, 277 were excluded, as the time between pain onset and admission was >3 h; and 81 were excluded due to the cause of chest pain not being ACS. Therefore, 230 patients were included (98 with STEMI, 74 with NSTEMI and 58 with UAP). A total of 79 healthy controls were recruited.

Detection of circulating miRNAs by microarrays. A total of 33 miRNAs were differentially expressed in patients with AMI (n=3) and healthy controls (n=3) (Table II). Hierarchical clustering for the 33 differentially expressed miRNAs is indicated by a volcano plot (Fig. 2). Fig. 3 represents a heat map of these miRNAs. As indicated by Table II, a total of 27 miRNAs were upregulated and 6 were downregulated in patients with AMI compared with healthy controls.

Circulating miRNAs in the validation cohort. Among the 33 significantly differentially expressed miRNAs in patients with AMI (n=3) and healthy controls (n=3), 4 upregulated (miR-125b-5p, miR-30d-5p, miR-136-5p, miR-129-1-3p) and
Table III. Clinical characteristics of the study groups.

| Characteristic         | STEMI (n=98) | NSTEMI (n=74) | All AMI (n=172) | UAP (n=58) | HC (n=79) | P1       | P2       |
|------------------------|--------------|---------------|-----------------|------------|-----------|----------|----------|
| Gender, male (n, %)    | 72 (73.5)    | 50 (67.6)     | 122 (70.9)      | 34 (58.6)  | 50 (63.29) | 0.174    | 0.342    |
| Age (years)            | 61 (20-81)   | 63 (29-91)    | 62 (20-91)      | 65.5 (35-85) | 41 (21-68) | <0.001   | <0.001   |
| Smoking (n, %)         | 71 (72.4)    | 33 (44.6)     | 104 (60.5)      | 22 (37.9)  | 0 (0.0)   | <0.001   | <0.001   |
| Hypertension (n, %)    | 55 (56.1)    | 54 (73.0)     | 109 (63.4)      | 45 (77.6)  | 0 (0.0)   | <0.001   | <0.001   |
| Diabetes (n, %)        | 25 (25.5)    | 26 (35.1)     | 51 (29.7)       | 20 (34.5)  | 0 (0.0)   | <0.001   | <0.001   |
| Arrhythmia (n, %)      | 50 (51.0)    | 56 (75.7)     | 106 (61.6)      | 34 (58.6)  | 0 (0.0)   | <0.001   | <0.001   |
| SBP (mmHg)             | 130 (70-220) | 135 (92-210)  | 133 (70-220)    | 131.5 (95-195) | 120 (90-180) | <0.001   | <0.001   |
| DBP (mmHg)             | 80 (40-140)  | 81 (55-107)   | 80 (40-140)     | 76.5 (51-110) | 80 (59-120) | 0.246    | 0.567    |
| Glu (mmol/l)           | 8.3 (4.6-22.3) | 7.2 (4.2-20.9) | 7.8 (4.2-22.3)  | 6.0 (4.2-22.3) | 4.9 (4.0-11.4) | <0.001   | <0.001   |
| TC (mmol/l)            | 4.7 (2.9-7.5) | 4.4 (1.3-7.4) | 4.6 (1.3-7.5)   | 4.05 (2.7-6.3) | 4.8 (0.6-6.5) | <0.001   | 0.031    |
| TG (mmol/l)            | 1.55 (0.35-4.56) | 1.28 (0.53-6.15) | 1.41 (0.35-6.15) | 1.28 (0.58-4.2) | 1.13 (0.37-3.86) | 0.008    | 0.004    |
| HDL-C (mmol/l)         | 0.98 (0.48-1.78) | 0.97 (0.55-2.86) | 0.98 (0.48-2.86) | 1.08 (0.43-1.81) | 1.32 (0.76-2.12) | <0.001   | <0.001   |
| LDL-C (mmol/l)         | 3.04 (1.09-5.54) | 2.95 (1.04-5.65) | 3.01 (1.04-5.65) | 2.49 (1.33-4.33) | 3.11 (1.47-3.82) | 0.001    | 0.201    |
| UA (µmol/l)            | 352 (194-574) | 324 (144-546) | 336 (1447-574)  | 349 (192.625) | 309 (160-450) | 0.025    | 0.004    |
| CREA (µmol/l)          | 70.5 (39.0-138.0) | 67.5 (40.0-215.0) | 70 (39-215)     | 59.0 (40.0-68.0) | 63 (34-94) | 0.001    | 0.003    |
| eGFRd                  | 98.714 (43.453-191.629) | 104.969 (26.412-190.978) | 101.268 (26.412-191.629) | 121.152 (35.745-180.456) | 122.74 (69.22-238.25) | <0.001   | <0.001   |
| LVEF                    | 55 (28-64)   | 58 (24-68)    | 56 (24-68)      | 59 (43-69)  | 64 (60-68) | <0.001   | <0.001   |
| CK-MB mass (ng/ml)     | 3.6 (0.4-302.0) | 11.1 (1.3-302.0) | 6.1 (0.4-302.0) | 1.4 (0.4-8.4) | -         | -        | -        |
| Myo (ng/ml)            | 95.8 (4.5-1703.6) | 59.7 (12.6-2325.1) | 77.5 (4.5-2325.1) | 23.7 (8.5-240.2) | -         | -        | -        |
| cTnI (µg/ml)           | 0.131 (0.000-102.000) | 2.201 (0.000-50.837) | 0.45 (0.000-102.000) | 0.013 (0.000-1.020) | 0.000 (0.000-0.011) | <0.001   | 0.001    |
| miR-125b-5p            | 3.725 (0.949-1403.769) | 4.679 (0.949-1403.769) | 4.378 (0.949-1403.769) | 1.017 (0.040-8.939) | 1         | <0.001   | <0.001   |
| miR-30d-5p             | 9.818 (1.365-11492.847) | 8.286 (1.064-357.669) | 8.692 (1.064-11492.847) | 0.949 (0.053-11.518) | 1         | <0.001   | <0.001   |

Data are presented as the median (min, max) or proportion, as appropriate. STEMI, ST-elevated myocardial infarction; NSTEMI, non-STEMI; AMI, acute myocardial infarction; UAP, unstable angina pectoris; HC, healthy controls; P1, AMI vs. UAP vs. HC; P2, STEMI vs. NSTEMI vs. HC; SBP, systolic blood pressure; DBP, diastolic blood pressure; Glu, glutamine; TC, total cholesterol; TG, total glycoside; HDL, high-density lipoprotein; LDL, low-density lipoprotein; UA, uric acid; CREA, creatinine; eGFR, estimated glomerular filtration rate; LVEF, left ventricular ejection fraction; CK-MBmass, creatine kinase MB mass; Myo, myoglobin; cTnI, cardiac troponin I. *P<0.05 vs. UAP, †P<0.05 vs. HC, ‡P<0.05 vs. NSTEMI; ‡Data was normally distributed for these variables.
Figure 3. Profiling of circulating miRNAs in patients with AMI and healthy controls. RNA was isolated from the plasma of healthy controls (N1, N2, N3) and AMI patients (M1, M2, M3). The heat map diagram represents the clustering of the 33 differentially expressed miRNAs. Red indicates higher expression, and green low expression. Data is summarized in Table II. AMI, acute myocardial infarction; M1, AMI patient 1; M2, AMI patient 2; M3, AMI patient 3; N1, healthy control 1; N2, healthy control 2; N3, healthy control 3.

Figure 4. Circulating (A) miR-125b-5p, (B) miR-30d-5p, (C) miR-136-5p, (D) miR-27a-5p, (E) miR-129-1-3p and (F) miR-1291 expression levels in the validation cohort. MicroRNAs levels were determined by reverse transcription-quantitative polymerase chain reaction. Values were normalized to cel-miR-39 and ratios obtained by the 2^(-ΔΔCq) method. Data are presented as the mean ± standard error (n=5 each group). AMI, acute myocardial infarction.
2 downregulated (miR-27a-5p and miR-1291) miRNAs were selected as targets for further investigation based on previously published data (14,23,24). Their expression levels in samples from patients with AMI (n=5) and healthy controls (n=5) were determined by RT-qPCR, with cel-miR-39 used as an internal control. As indicated in Fig. 4, fold-changes of miR-125b-5p, miR-30d-5p, miR-136-5p and miR-129-1-3p were 4.46 (P=0.008), 4.29 (P=0.012), 1.42 (P=0.036) and 1.75 (P=0.016) while miR-27a-5p and miR-1291 were 0.68 (P=0.151) and 0.93 (P=0.691), respectively. According to the threshold value defined as fold-change ≥2.0 and P<0.05, miR-125b-5p and miR-30d-5p were selected for further investigation.

Clinical characteristics of the study population. A total of 230 patients with ACS and 79 healthy controls were assessed. Baseline characteristics of the ACS patients at admission are provided in Table III. Gender distribution and diastolic blood pressure were similar between the patients and the healthy controls. However, compared with healthy controls, patients with AMI and UAP were older, were more likely to be smokers, also suffered from hypertension, diabetes and arrhythmia or had elevated blood glucose, lipid, and uric acid levels, impaired renal function, and decreased heart function (all P<0.05). In addition, cTnI levels were higher in patients with AMI and UAP compared with healthy controls (P<0.001).

Plasma miR-125b-5p and miR-30d-5p levels in AMI, UAP and healthy control groups. Plasma levels of miR-125b-5p and miR-30d-5p were higher in patients with AMI compared with healthy controls (all P<0.001; Fig. 5). Furthermore, levels of miR-125b-5p and miR-30d-5p in plasma varied among the following sub-groups: miR-125b-5p levels in the STEMI, NSTEMI and UAP groups were 3.73, 4.68 and 1.02, while miR-30d-5p levels were 9.82, 8.29 and 0.95-fold higher than in healthy controls, respectively (Fig. 5).

Changes of miR-125b-5p, miR-30d-5p and cTnI levels at different time points following chest-pain onset in AMI patients. As presented in Fig. 6, miR-125b-5p and miR-30d-5p were detected in AMI patients as early as 3 h following chest pain onset. Notably, the expression levels peaked at 3-6 h and then dropped following 9 h. Meanwhile, cTnI peaked from 6-9 h and a decrease in levels followed at 12 h.

Specificity and sensitivity of miR-125b-5p and miR-30d-5p as diagnostic biomarkers. ROC analysis was performed to assess whether circulating miR-125b-5p and miR-30d-5p may be used as diagnostic biomarkers for AMI. Fig. 7 and Table IV
JIA et al. DIAGNOSTIC ROLES OF miR-30d-5p AND miR-125b-5p IN AMI

Indicates the ROC analysis of CK-MB, Myo and cTnI. The higher area under the curve (AUC) of miR-30d-5p may provide diagnostic information for patients with AMI on admission, with the ability to distinguish AMI from other diseases associated with chest pain. Based on these data, miR-125b-5p is of a similar specificity and sensitivity to cTnI; however, miR-30d-5p exceeded the performance of cTnI. Therefore, miR-125b-5p, and miR-30d-5p may be used to diagnose AMI in patients admitted to the emergency room with symptoms of ACS.

Prognostic value of miR-125b-5p and miR-30d-5p. To further investigate the efficiency of miR-125b-5p and miR-30d-5p as potential biomarkers of AMI, Kaplan-Meier survival analysis was performed for patients with or without AMI. Optimal cut-off values for miR-125b-5p and miR-30d-5p were determined from the corresponding ROC curves to be 2.061 and 2.599, respectively. Patients were then divided into positive (>cut-off value) and negative (<cut-off value) groups. At 6 months, the Kaplan-Meier curve predicted the miR-125b-5p-positive group to have a lower cumulative survival rate than the negative group (P=0.045; Fig. 8B), but there was no significant difference for miR-30d-5p groups (Fig. 8).

Finally, Cox regression analysis was performed to assess the risk factors involved in the prognosis of patients with ACS. The levels of circulating miR-125b-5p and miR-30d-5p were not significantly associated with the risk of endpoint events at 1, 6 and 12 months (Table V), indicating that they may not reflect the prognosis in patients with ACS.

Discussion

The aim of the present study was to evaluate circulating microRNAs and their suitability as AMI biomarkers in patients

Table IV. Cardiac biomarkers on admission and diagnostic values.

| Variable | CK-MB (ng/ml) | cTnl (ng/ml) | Myo (ng/ml) |
|----------|--------------|--------------|-------------|
| Cut-off value | 2.650 | 38.500 | 0.049 |
| Sensitivity (95% CI) | 0.721 (0.696-0.802) | 0.698 (0.623-0.764) | 0.837 (0.722-0.887) |
| Specificity (95% CI) | 0.897 (0.822-0.957) | 0.914 (0.803-0.968) | 0.945 (0.771-0.992) |
| PPV % (95% CI) | 889 (0.844-0.933) | 917 (0.856-0.969) | 939 (0.880-0.986) |
| NPV % (95% CI) | 721 (0.673-0.765) | 834 (0.706-0.908) | 796 (0.639-0.903) |
| PLR (95% CI) | 5.495 (3.258-9.288) | 5.395 (0.970-1.891) | 2.650 (0.484-1.497) |
| NLR (95% CI) | 0.227 (0.166-0.311) | 0.193 (0.136-0.272) | 0.727 (0.116-0.274) |
| AUC (95% CI) | 0.897 (0.822-0.957) | 0.914 (0.803-0.968) | 0.837 (0.722-0.887) |

CK-MB, creatine kinase MB mass; Myo, myoglobin; cTnI, cardiac troponin I; CI, confidence intervals; PPV, positive predictive value; NVP, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; AUC, area under curve.
with ACS. The results indicated that 33 miRNAs were differentially expressed in patients with AMI and healthy controls. Following validation based on previously published roles for these miRNAs, six miRNAs were validated in an additional five patients and healthy controls. Finally, miR-125b-5p and miR-30d-5p were selected for a more detailed investigation with a larger sample size. Plasma levels of miR-125b-5p and miR-30d-5p were higher in patients with ACS compared with healthy controls (all \( P < 0.001 \)). ROC curve analysis revealed miR-125b-5p and miR-30d-5p as diagnostic predictors of AMI. Additionally, miR-30d-5p may have a higher diagnostic value than cTnI. Patients with higher levels of miR-125b-5p had poor prognosis compared with those with lower levels.

In the present study, miR-125b-5p, miR-30d-5p, miR-136-5p and miR-129-1-3p were upregulated, while miR-27a-5p and miR-1291 were downregulated. These findings are supported
by previous studies demonstrating the involvement of circulating microRNAs in AMI, including miR-486-3p, miR-150-3p, miR-126-5p, miR-26a-5p, miR-191-5p, miR-125b-5p, miR-663b, miR-1, miR-133a, miR-499 and miR-208a (15-18). These miRNAs are associated with muscle tissues in general and more specifically, cardiac muscle. Therefore, they were selected for further validation. miR-125b-5p and miR-30d-5p were selected for further investigation, and their expression was assessed in 230 patients with ACS and 79 healthy controls. In addition, miR-125b-5p and miR-30d-5p were detectable as early as 3 h after the onset of chest pain, prior to necrosis, thus may also have precise functions. This may point to paracrine signaling molecules (29), it is possible that circulating microRNAs (28) and data describing miRNAs as transport of miRNAs (28) and data describing miRNAs as paracrine signaling molecules (29), it is possible that circulating microRNAs are not merely byproducts of myocardial necrosis, thus may also have precise functions. This may point toward a cardioprotective role for these miRNAs in the case of myocardial infarction. These questions should be addressed thoroughly in future studies.

In conclusion, the current study identified that patients with AMI have distinct miRNA profiles compared with healthy controls. In addition, miR-125b-5p and miR-30d-5p may be used as potential early diagnostic biomarkers for AMI; however, their prognostic value may be limited.

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