Clinical Significance of MiR-27a Expression in Serum Exosomes in Patients with Heart Failure

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ABSTRACT

To study the clinical significance of miR-27a expression in serum exosomes in patients with heart failure (HF), this study was carried out. Totally 101 chronic heart failure (CHF) patients (research group) admitted to our hospital from April 2016 to January 2019 were enrolled, and another 30 healthy subjects (control group) who underwent physical examination during the same period were selected. The difference of miR-27a expression level in serum exosomes between the two groups of subjects was compared, so as to analyze the diagnostic value of miR-27a in HF and the relationship between miR-27a expression level and patient prognosis. The expression level of miR-27a in peripheral blood exosomes of subjects in the control group (CG) was significantly higher than that in the research group (RG) (P<0.05), while the miR-27a expression of the latter increased significantly 3 months after treatment (P<0.05). ROC analysis showed that the AUC, sensitivity, specificity, and critical level of miR-27a in the diagnosis of CHF were 0.835, 77.23%, 80.00%, and 1.060 respectively. While after 3 months of treatment, the indicators of the RG effectively improved (P<0.05). Pearson and Sparman correlation analysis revealed that there was a significant linear correlation between miR-27a and each indicator (P<0.05). K-M survival curve analysis demonstrated that the survival rate of patients with high expression of miR-27a was significantly higher than that of patients with low expression (P<0.05). The expression of miR-27a in serum exosomes is decreased in patients with HF and has important potential value in the diagnosis and prognosis of CHF.

Introduction

Heart failure (HF) is a kind of clinical syndrome caused by abnormal cardiac structure and function, which is characterized by dyspnea, ankle swelling and fatigue. The incidence of HF in developed countries is 1%-2%, but with the increase of age, the incidence of HF can increase to 10% (1,2). HF is prone to cause shock due to insufficient blood perfusion in important organs of the body, with poor prognosis and high mortality. Some studies have reported that the 5-year survival rate of patients with CHF is similar to that of patients with a malignant tumor, which is one of the important causes of human death (3,4). There is no obvious clinical symptom in the early stage of HF, nor there is any effective treatment in the late stage of it. Therefore, finding biomarkers related to HF is of great significance for early diagnosis and discovery of potential therapeutic targets.

MicroRNAs (miRNAs), a class of short non-coding RNA with a nucleotide length of about 20bp, are widely found in animals and plants and play an important part in cardiovascular diseases (including HF) by binding to the 3'-terminal non-translation region of targeted mRNA to regulate mRNA translation (5,6). However, the current research on miRNAs in HF is still very weak. Recent studies have shown that miR-27a gene polymorphism is associated with susceptibility to heart disease (7) and is involved in cardiac function remodeling (8). In a study on the expression profile of plasma miRNAs in HF, miR-27a expression was found to be significantly decreased in patients with HF and was associated with 180-day mortality, with a risk ratio of 1.379. However, to date, no study has been conducted to further analyze the clinical diagnostic value of miR-27a in HF. While exosomes are membrane-bound vesicles formed by inward germination of multi-vesicle inclusions, which

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Cellular and Molecular Biology, 2021, 67(5): 324-331
are secreted or fused to the cell membrane and are an important means of signal communication between cells (9). Some studies have displayed that the related pathological changes in HF can induce the expression of miRNAs in cardiac exosomes and affect angiogenesis and myocardial cell survival (10). Although MiR-27a is also expressed in cardiac exosomes (11,12), whether it is expressed in serum exosomes of patients with HF or whether its expression changes have some clinical significance remains unknown.

Therefore, this study detected the expression of miR-27a in serum exosomes of patients with HF, aiming to explore its clinical application value in this disease.

Materials and methods

Study subjects

This study adopted prospective analysis to select 101 patients with CHF (research group, or RG in short) admitted to our hospital from April 2016 to January 2019, and another 30 subjects who underwent physical examinations in our hospital during the same period (control group, or CG in short). Inclusion criteria: All subjects were aged 50-70 years. Patients in the RG met the 2016 European Society of Cardiology guidelines for the Diagnosis and Treatment of Acute and Chronic Heart Failure (1), who had not received any treatment, with no abnormal bleeding or abnormal coagulation function, nor functional impairment of other organs, and those with complete medical records. Exclusion criteria: Patients with acute myocardial infarction, cardiogenic shock or other causes of HF; Patients with valvular stenosis arrhythmia, atrial fibrillation, bundle branch block, unstable angina, or another cardiomyopathy; Patients with thyroid disease, severe infection, or hypokalemia. This study was approved by the Medical Ethics Committee of our hospital, and written informed consent was obtained from all study subjects or their families.

Treatment

All patients were treated with β-blockers, diuretics, angiotensin receptor blockers, aspirin, statins, angiotensin-converting enzyme inhibitors or angiotensin receptor antagonists for three months, depending on individual condition.

Follow-up

Patients in the RG were followed for 2 years. The follow-up was conducted either through home visits or telephone calls at the frequency of every 3 months, and patient survival information was recorded.

Extraction and identification of serum exosomes

Peripheral blood of 5ml was centrifuged at 4 °C and 2000g for 10min to collect the serum, which was then transferred to a new test tube, centrifuged for 2000g for 30min to obtain the clear serum (excluding cells and fragments). Then the clear serum was transferred to another test tube, followed by the addition of the Total Exosome Isolation Reagent according to the volume ratio of reagent to the serum of 1:5, mixed evenly, incubated at 4 °C for 30 minutes, and then centrifuged at 10000 g at room temperature for 10min. After that, the supernatant was discarded, and the precipitate was resuspended in 1x PBS buffer. Finally, a Western blot was applied to detect Tsg101 and CD63, and the size and shape of exosomes were observed by electron microscope. The Total Exosome Isolation Reagent was purchased from Thermo Fisher, with the article number of 4478360, and the FEI Tecnai G2 Spirit Bio TWIN electron microscope was supported by Nanjing Medical University.

QRT-PCR

The total RNA of the tissues was extracted using the TRIzol kit, while that of the peripheral blood was collected by the miRNeasy Micro Kit, whose purity, concentration and integrity were then tested by UV spectrophotometer and agarose gel electrophoresis at the requirement of 28s:18s≥2 and A260/A280 within 1.8-2.1. The RNA was amplified by the One-Step method in this study, and the reaction system was as follows: RNA Template: 1μg, Forward GSP: 0.4μL, Reverse GSP: 0.4μL, 2*One-Step Reaction Mix: 10μL, EasyScript One-Step Enzyme Mix: 0.4μL, and RNase-free Water was added to complete the reaction volume of 20μL. The amplification conditions were 40℃ for 30min, 94℃ for 5min, 94℃ for the 30s, 60℃ for 30s, 72℃ for 2kb/min, and 72℃ for 10min, totaling 42 cycles. GAPDH was used as the internal reference for lncRNA, and U6 as the internal reference for miRNA. The TRIzol kit was purchased from Invitrogen Company, USA, with the article number of 15596018.
EasyScript One-Step RT-PCR SuperMix kit was acquired from Beijing TransGen Biotechnology Co., Ltd., with the article number AE411-02, and the microplate reader was obtained from Shanghai Flash Spectrum Biotechnology Co., Ltd. Primer sequences are shown in Table 1.

**Western blot**

The protein in exosomes was extracted by repeated freeze-thaw method and protein concentration was detected by BCA assay. Next, the protein concentration was adjusted to 4ug/μL, electrophoretically separated by 12% SDS-PAGE with the initial voltage of 90V, and an increased one of 120V to move the sample to the suitable position of the separation gel. Upon the completion of electrophoresis, the membrane was transferred under a 100V constant pressure for 60min and then sealed at 37℃ for 600min. Then the transferred membrane was placed in 5% skim milk for sealing before the conduction of the immune reaction. The membrane was incubated overnight with primary antibody (1:1000) at 4°C, followed by the triple warm washing with PBS the next day, each time for 5min, and then incubated with secondary antibody (1:1000) at room temperature for 1h. After that, ECL luminescent reagent was employed to develop and fix. Quantity One software was adopted for statistical analysis of the bands after film scanning, and the relative expression level of protein was equal to the gray value of the bands/the grays value of the internal parameters. BCA protein kit, ECL luminescence kit and trypsin were purchased from Thermo Scientific™, with the corresponding article number 23250, 35055, 90058. Rabbit anti-Tsg101, CD63 polyclonal antibody and goat anti-rabbit IgG secondary antibody were all obtained from Abcam, and their article numbers were the USA. MA5-32463, PA5-92370, and ab6721 respectively.

**Observation Indicators**

The difference of miR-27a expression levels in serum exosomes was compared between the two groups. In addition, the diagnostic value of miR-27a in CHF was analyzed, so was the relationship between miR-27a expression level and patient prognosis.

**Statistical analysis**

Data analysis was performed using SPSS19.0 (Asia Analytics Formerly SPSS, China). The counting data were expressed as a percentage, and compared by the χ2 test. The measurement data were expressed as mean±sd, and the student's t-test was used for inter-group comparison. ROC analysis was employed to analyze the diagnostic value, Pearson's and Spearman's analyses were adopted for correlation analysis, and the K-M curve was applied to evaluate the relationship between miR-27a and patient survival. P<0.05 indicated that the difference was statistically significant.

**Results and discussion**

**General information**

There were 30 subjects in the CG, including 18 males and 12 females, aged (64.38±10.47) years. While in the study group, there were 101 patients, including 59 males and 42 females, aged (62.59±8.58) years. The gender ratio and age did not identify any significant difference between the two groups (P>0.05). Other basic information is shown in Table 2.

**Exosome identification results**

Western blot analysis revealed the presence of marker proteins Tsg101 and CD63 in exosomes. (Figure 1)

**Analysis of miR-27a expression level**

QRT-PCR showed the expression level of miR-27a in peripheral blood exosomes of subjects in the CG was significantly higher than that in the RG (P<0.05), while that of the latter increased markedly after 3 months of treatment (P<0.05). (Figure 2)

**Diagnostic value of miR-27a in CHF**

The ROC model was established by comparing the expression level of miR-27a in the CG with the level of miR-27a in the RG before treatment. The analysis results showed that the corresponding AUC, sensitivity, specificity and critical level of miR-27a in diagnosis of CHF were 0.835, 77.23%, 80.00% and 1.060. (Figure 3)
Correlation analysis between miR-27a and related indicators of CHF

After 3 months of treatment, the indicator in the RG effectively improved (P<0.05), as shown in Table 3. Pearson and Sparman correlation analysis exhibited that miR-27a was significantly linearly correlated with each indicator. (P<0.05). (Figure 4)

Relationship between miR-27a and prognosis of CHF

During the two-year follow-up, 15 patients died in the RG. With the median expression level of post-treatment miR-27a in the RG as the critical value, patients in the RG were divided into a high expression group (>median) and a low expression group (≤median). The K-M survival curve model was then established, whose results demonstrated that the survival rate of patients in the high expression group was significantly higher than that of patients in the low expression group (P<0.05). (Figure 5)

CHF is one of the important causes of death in humans, whose incidence increases in parallel with age. For your consideration, the incidence of CHF approximately doubles for every 10 years of increase in age (13). Therefore, early screening and accurate prognosis assessment of CHF are of great significance to guide clinical treatment and improve the prognosis of patients.

The results of this study showed that miR-27a was lowly expressed in peripheral blood exosomes of patients with CHF. Further analyzing the diagnostic value of miR-27a in exosomes for CHF, we found that when miR-27a<1.060, the AUC, sensitivity and specificity of miR-27a in the diagnosis of CHF were 0.835, 77.23% and 80.00% respectively. While after treatment, many indicators represented by LAD, LVEDD, LVEF, 6MWT of patients with CHF had effectively improved, and meanwhile we found that the miR-27a expression also increased significantly in exosomes. The correlation analysis revealed that there was a significant linear correlation between miR-27a and multiple indicators, which further suggested that miR-27a was associated with the development of CHF. What’s more, our results exhibited that miR-27a was associated with prognosis. We followed the patient for 2 years and 15 of 101 patients died. Then the expression level of miR-27a in the RG after treatment was utilized to analyze the relationship between miR-27a and the prognosis and death of the patients. The results displayed that the survival rate of the patients with high expression of miR-27a was significantly higher than that of the patients with low expression. Therefore, it can be preliminarily concluded that miR-27a is of great significance in the diagnosis and prognosis evaluation of CHF.

Exosomes are extracellular vesicles with a diameter of 30-100nm, containing proteins, nucleotides and other substances, which are important pathways for cell-to-cell communication, and are stably expressed in serum (14,15). Some studies reported that cardiac fibroblasts could complete signal communication with cardiac cells by releasing exosomes, thus causing cardiac hypertrophy and leading to heart failure (16). Tian (17) further found in their study that cardiac fibroblasts could release exosomes enriched in miRNAs and communicate with cardiomyocytes, which could dysregulate Nrf2/ARE signaling pathway, promote oxidative stress, resulting in myocardial dysfunction. MiR-27a is widely expressed in exosomes of various origins, such as myoblasts (18), skin keratinocytes (19), gastric cancer cells (20), and stem cells (21), which are implicated in cell osteogenic differentiation, migration, fibrosis, etc. It is also expressed in circulating exosomes and can be used as a biomarker for the diagnosis and prognosis of colorectal cancer (22). In heart disease, miR-27a is down-regulated in coronary sinus blood samples of patients with congestive heart failure (23) and is able to regulate extracellular matrix transformation, prevent myocardial fibrosis, and participate in the differentiation of stem cells into cardiomyocytes (24, 25). Moreover, miR-27a is capable of protecting cardiomyocytes from sepsis and oxidative stress (26,27).

Table 1. Primer sequences

| Primer | Forward primer | Reverse primer |
|--------|---------------|---------------|
| miR-27a | 5'-TGGGATTTACAGCTGGGTAAAG-3' | 5'-CTACAAGGTTGTGCTGGA-3' |
| U6     | 5'-GCGCGTCGTAAGCGTGTCG-3' | 5'-GTGAGGGTCCCGAGG-3' |

All these studies mentioned above provide a possible action mechanism of miR-27a in the pathogenesis of heart failure, however up to now, there are few reports on the relationship between miR-27a and HF. Here in the current study, though certain
results have been achieved, there are still some deficiencies. For instance, whether miR-27a can be used as an early screening indicator for CHF still needs to be verified, which requires the design of an earlier blood collection time point.

To sum up, the expression of miR-27a in serum exosomes is reduced in patients with HF and has important potential value in the diagnosis and prognosis evaluation of CHF.

Figure 1. Analysis of miR-27a expression level. A: Expression of miR-27a in peripheral blood exosomes of patients with CHF. B: Changes in miR-27a expression in peripheral blood exosomes of patients with CHF after treatment. * indicated P<0.05.

Figure 2. Analysis of miR-27a expression level. A: Expression of miR-27a in peripheral blood exosomes of patients with CHF. B: Changes in miR-27a expression in peripheral blood exosomes of patients with CHF after treatment. * indicated P<0.05.

Figure 3. Diagnostic value of miR-27a in CHF. The AUC, sensitivity, specificity, and critical level of miR-27a in the diagnosis of CHF were 0.835, 77.23%, 80.00%, and 1.060.

Table 2. General information

|                      | The control group (n=30) | The research group (n=101) | χ²/t | P     |
|----------------------|--------------------------|---------------------------|------|-------|
| Gender (n,%):        |                          |                           |      |       |
| Male                 | 18 (60.00)               | 59 (58.42)                | 0.024| 0.877 |
| Female               | 12 (40.00)               | 42 (41.58)                |      |       |
| Age                  | 64.38±10.47              | 62.59±8.58                | 0.953| 0.343 |
| Body mass index (kg/m²) | 22.54±3.17             | 23.15±2.48                | 1.107| 0.271 |
| Smoking history      |                          |                           | 0.588| 0.443 |
| Yes                  | 11 (36.67)               | 45 (44.55)                |      |       |
| No                   | 19 (63.33)               | 56 (55.45)                |      |       |
| Drinking history     |                          |                           | 0.649| 0.421 |
| Yes                  | 8 (26.67)                | 20 (19.80)                |      |       |
| No                   | 22 (73.33)               | 81 (80.20)                |      |       |
| Hypertension         |                          |                           | 0.247| 0.619 |
| Yes                  | 25 (83.33)               | 80 (79.21)                |      |       |
| No                   | 5 (16.67)                | 21 (20.79)                |      |       |
| Diabetes mellitus    |                          |                           | 1.102| 0.294 |
| Yes                  | 11 (36.67)               | 48 (47.52)                |      |       |
| No                   | 19 (63.33)               | 53 (52.48)                |      |       |
| Hemoglobin (%)       | 144.37±14.18             | 132.38±18.14              | 3.328| 0.001 |
| Glutamicoxaloacetic transaminase (U/L) | 22.24±6.46 | 34.28±5.13 | 10.610| <0.001 |
| Creatinine (g/L)     | 67.53±13.28              | 84.55±16.39               | 5.199| <0.001 |
| Total cholesterol (mmol/L) | 4.41±0.89 | 3.62±0.68 | 5.187 | <0.001 |
| Triglyceride (mmol/L) | 1.53±0.57               | 1.44±0.64                 | 0.693| 0.490 |
| Fasting blood glucose (mmol/L) | 6.22±0.63 | 6.14±0.83 | 0.487| 0.617 |
| B-type natriuretic peptide (ng/mL) | 0.14±0.03 | 0.57±0.18 | 12.997| <0.001 |
| LAD (mm)             | 34.12±4.34               | 44.58±4.34                | 11.597| <0.001 |
| LVEDD (mm)           | 48.38±2.27               | 59.43±4.58                | 11.778| <0.001 |
| LVEF(%)              | 59.66±5.17               | 44.59±6.06                | 12.343| <0.001 |
| Systolic pressure (mmHg) | 124.33±11.46 | 131.95±12.31 | 3.023| 0.003 |
| Diastolic blood pressure (mmHg) | 68.38±8.31 | 72.78±10.34 | 2.133| 0.035 |
| 6MWTest(m)           | 475.91±17.53             | 325.84±15.06              | 46.120| <0.001 |

Table 3. Changes in indicators of patients in the research group before and after treatment

|                      | Before treatment | After treatment | χ²/t | P     |
|----------------------|------------------|----------------|------|-------|
| Hemoglobin (g/L)     | 132.38±18.14     | 140.25±22.38   | 2.745| 0.007 |
| Alanine aminotransferase (U/L) | 34.15±5.12 | 27.56±8.35 | 6.744 | <0.001 |
| Glutamicoxaloacetic transaminase (U/L) | 34.28±5.13 | 28.33±9.15 | 5.700 | <0.001 |
| Creatinine (g/L)     | 84.55±16.39      | 78.49±19.68    | 2.378| 0.018 |
| Total cholesterol (mmol/L) | 3.62±0.68 | 4.26±0.74 | 6.400 | <0.001 |
| B-type natriuretic peptide (ng/mL) | 0.57±0.18 | 0.28±0.13 | 13.126| <0.001 |
| LAD (mm)             | 44.58±4.34       | 38.43±5.62     | 8.704| <0.001 |
| LVEDD (mm)           | 59.43±4.58       | 48.38±5.14     | 16.131| <0.001 |
| LVEF(%)              | 44.59±6.06       | 53.11±6.39     | 9.723 | <0.001 |
| Systolic pressure (mmHg) | 131.95±12.31 | 125.76±10.83   | 3.794| <0.001 |
| Diastolic blood pressure (mmHg) | 72.78±10.34 | 69.67±9.45 | 2.232| 0.027 |
| 6MWTest(m)           | 325.84±15.06     | 442.18±20.71   | 45.660| <0.001 |
| NYHA classification  |                  |                | 46.076| <0.001 |

Note: LAD: left atrial diameter; LVEDD: left ventricular end-diastolic dimension; LVEF: left ventricular ejection fraction; 6MWTest: 6 minutes walking test.

Table 4. General information
Yuetao Xie et al. / MiR-27a Expression in Serum Exosomes in Patients with Heart Failure, 2021, 67(5): 324-331

Figure 4. Correlation analysis between miR-27a and related indicators of CHF. A: Correlation analysis between miR-27a and hemoglobin. B: Correlation analysis between miR-27a and alanine aminotransferase. C: Correlation analysis of miR-27a and glutamic-oxalacetic transaminase. D: Correlation analysis between miR-27a and creatinine. E: Correlation analysis between miR-27a and total cholesterol. F: Correlation analysis between miR-27a and B-type natriuretic peptide. G: Correlation analysis between miR-27a and LAD. H: Correlation analysis between miR-27a and LVEDD. I: Correlation analysis between miR-27a and LVEF. J: Correlation analysis between miR-27a and systolic blood pressure. K: Correlation analysis between miR-27a and systolic blood pressure. L: Correlation analysis between miR-27a and 6MWT. M: Correlation analysis between miR-27a and NYHA classification.

Figure 5. Relationship between miR-27a and prognosis of CHF. A: Difference in miR-27a expression in patients with CHF who survived and died. B: Relationship between miR-27a and two-year survival rate in patients with CHF.

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Interest conflict
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YX wrote the manuscript. YX and JZ conceived and designed the study. YX and NZ were responsible for the collection and analysis of the experimental
Yuetao Xie et al. / MiR-27a Expression in Serum Exosomes in Patients with Heart Failure, 2021, 67(5): 324-331

data. JZ and GL interpreted the data and drafted the manuscript. YX and GL revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Hebei General Hospital, China. Patients who participated in this research signed the informed consent and had complete clinical data. Signed written informed consents were obtained from the patients and/or guardians.

Consent for publication
Not applicable.

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