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Original Article

Expression Levels of MicroRNAs-146b and Anti-Cardiac Troponin I in Serum of Children with Viral Myocarditis and Their Clinical Significance

Min YAN [1], ***Jing WANG*** [2], ***Suyun WANG*** [3], ***Yang ZHANG*** [4], ***Ling LIU*** [5], ****Hua ZHAO*** [6]

1. *Department of Pediatrics, Jinan City People's Hospital Affiliated to Shandong First Medical University, Jinan 271199, China*
2. *Department of Physical Examination, the People's Hospital of Zhangqiu Area, Jinan 250200, China*
3. *Ward of Emergency, the People's Hospital of Zhangqiu Area, Jinan 250200, China*
4. *Department of Respiratory Medicine, the People's Hospital of Zhangqiu Area, Jinan 250200, China*
5. *EEG Room, the People's Hospital of Zhangqiu Area, Jinan 250200, China*
6. *Department of Ophthalmology, People's Hospital of Weifang, Weifang 261041, China*

***Corresponding Author:** Email: hpyv5377@163.com

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Abstract

Background: To investigate the expression levels of microRNAs-146b (miR-146b) and cardiac troponin I (anti-cTnI) in serum of children with viral myocarditis and their clinical significance.

Methods: Forty-eight children with viral myocarditis (patient group) and 40 healthy physical examinees (healthy group), who were diagnosed in Jinan City People's Hospital Affiliated to Shandong First Medical University, China from Feb 2018 to May 2019, were enrolled as study subjects. Reverse transcription polymerase chain reaction (RT-PCR) was used to detect the level of miR-146b in serum of children. ELISA was used to detect the expression of anti-cTnI in serum of children. Pearson was used to analyze the correlation between the level of miR-146b and the level of anti-cTnI, and the factors affecting the prognosis.

Results: The levels of miR-146b and anti-cTnI in serum of children in patient group were statistically significantly higher than those of healthy group ($P<0.01$). The AUC of miR-146b was 0.741, (95% CI: 0.638-0.843), the specificity was 62.50%, the sensitivity was 82.50%, and the AUC of anti-cTnI was 0.720 (95% CI: 0.608-0.832), the specificity was 64.58% and the sensitivity was 92.50%. The level of miR-146b was positively correlated with the level of anti-cTnI ($r=0.601$, $P<0.05$). CK-MB, LVEF, miR-146b and anti-cTnI expression were independent risk factors affecting the prognosis.

Conclusion: The levels of miR-146b and anti-cTnI increased in serum of patients with viral myocarditis. They were related to the degree of myocardial injury, which indicated that miR-146b and anti-cTnI might be involved in the pathological process of viral myocarditis.

Keywords: MicroRNAs-146b; Cardiac troponin I; Viral myocarditis; Clinical significance

Introduction

Viral myocarditis is a common disease in pediatric clinic. In recent years, its morbidity has increased obviously, and it plays an important role in pediatric myocarditis (1). According to statis-

tics, about 20% of infants suddenly die because of viral myocarditis or the fatal ventricular arrhythmia caused by viral myocarditis. Patients with persistent viral myocarditis have a poor prognosis, and the 5-yr survival rate of them is approximately 50% (2). In addition, some viral myocarditis could develop into dilated cardiomyopathy due to viral damage, immunity, genetic susceptibility and other factors (3). However, at present, there is no specific treatment of viral myocarditis at home and abroad. The current treatment still focuses on controlling malignant arrhythmia and heart failure (4).

MicroRNAs (miRNAs) are non-coding, small-molecule, and single-stranded RNA and are encoded by the human genome, and their length is equal to the length of 19-24 nucleotides, besides they regulate the expression after genes are transcribed (5). The abnormal expression of miRNAs is closely related to cardiovascular diseases and the regulation of some biological processes (6-8). The diagnostic value and prognostic value of miRNAs for diseases are receiving more and more attention (9). MicroRNAs-146b (miR-146b), a member of the miR-146 family, primarily regulates inflammatory responses and plays an important role in the body's innate immune process (10, 11). miR-146b was highly expressed in animal models with viral myocarditis and myocardial tissues of patients (12, 13). Cardiac troponin I (anti-cTnI) only exists in thin filament of myocardial contractile proteins clinically used to diagnose myocardial necrosis (14). When myocardial cells are damaged, anti-cTnI is released into the blood. Anti-cTnI can activate the immune response as an antigen and produce anti-cTnI antibody as well as can exist in the blood for a long time (15). Anti-cTnI could be used to image other pathological conditions that are related to myocardial cell injury in patients with myocardial infarction (16). There were antibodies in serum of patients with myocardial infarction and the antibodies were associated with poor prognosis (17). Thus, the presence of anti-cTnI in serum of patients with viral myocarditis may indicate severe injury of cardiomyocytes and predict the poor prognosis of patients with viral myocarditis.

The gold standard of diagnosing viral myocarditis is myocardial biopsy, but specimens are difficult to obtain and it is difficult to diagnose viral myocarditis timely (18). MiRNAs and anti-cTnI are expected to be biomarkers of estimating the disease condition of children with viral myocarditis.

In this study, the expressions of miR-146b and anti-cTnI in children with viral myocarditis and healthy children were observed. The expressions of miR-146b and anti-cTnI and the diagnostic value of them for children with viral myocarditis as well as the correlation between the expression of miR-146b and the expression of anti-cTnI were analyzed, to provide references for clinical practice.

Materials and methods

General data

Overall, 48 children with viral myocarditis, who were diagnosed in Jinan City People's Hospital Affiliated to Shandong First Medical University, China from Feb 2018 to May 2019, were enrolled as patient group. The age of them was between 10 months old and 12 yr old. The average age of them was (6.46±2.17) yr old. Overall, 40 healthy children, who took physical examination in Jinan City People's Hospital Affiliated to Shandong First Medical University in the same period, were enrolled as healthy group. The age of them was between 1 yr old and 12 yr old. The average age of them was (6.37±2.26) yr old. Inclusion criteria: Children conformed to the diagnostic criteria of viral myocarditis (19); the age of children was or less than 12 yr old.

The program was inspected by the Ethics Committee of Jinan City People's Hospital Affiliated to Shandong First Medical University, and it was carried out after approved. The guardians of the children voluntarily signed an informed consent form. Exclusion criteria: children with primary diseases in lung, kidney, liver and blood system; children with congenital heart diseases, congenital neurological diseases, dilated cardiomyopathy, heart failure, cardiogenic shock, and malignant

tumors; children took some antibiotics such as azithromycin in a month.

Instruments and reagents

Anti-cTnI enzyme-linked immunosorbent kit (Huijia Bio, China, A-ALS12188); enzyme-linked immunometric meter (MolecularDevices, USA, SpectraMaxiD5); Light Cycler real-time fluorescent quantitative PCR instrument (Roche, Switzerland, 05815916001); total miRNAs extraction kit (Qiagen, Germany, HZ101-633); M-MLV reverse transcription kit (solarbio, USA, RP1100); UV spectrophotometer (Eppendorf, Germany, 6135000041); qReal-time PRC kit (Invitrogen, Grand Island, NY, USA, article number: C28025-032); SYBR Green qPCR Master Mix Kit (Medchemexpress, USA, HY-K0501 Ltd.); miR-146b internal reference primer and U6 internal reference primer were synthesized by Shanghai Bioengineering Co., Ltd..

Detection methods

Fasting venous blood (5 mL) of children in two groups was collected in the morning during admission (acute phase), and was placed in vacuum tubes. The serum was separated and was centrifuged at 3000 r/min for 5 min at 4 °C. The supernatant was collected. Anti-cTnI in the serum was detected by ELISA. The sample serum was diluted by buffer solution, 100 μL of buffer solution was added into each well, and then it was washed for 3 times (5 min per time) after it was in water bath for 1 h. Mouse anti-Human lgG was diluted by PBS buffer solution, the ratio was 1: 2×10. Then 100 μL of the mixture was added into each well, then it was in water bath for 1 h at 37 °C, next, it was washed for 5 times (5 min per time). One hundred μL of chromogenic reagent, tetramethylbenzidine, was added into each well, and then it was in water bath for 10 min at 37 °C, lastly 50 μL of stop solution was added into each well to stop color development. An enzyme-linked immunometric meter was used to measure the OD value at the main wavelength of 450 nm and reference wavelength of 630 nm. MiRNAs extraction kits were used to extract serum total RNA. The optical density value of RNA was measured by an ultraviolet-visible spectrophotometer and the concentration of RNA was calculated. Two μl of total RNA were collected to prepare cDNA according to the instructions of RNA reverse transcription kits. The reverse transcription reaction system: 42 °C for 60 min, 95 °C for 5 min. The synthesized cDNA sample was stored at -20 °C. U6 was used as an internal reference gene, the total volume was 20 μl. Reaction system: 10 μl of PCR Premix, 2 μl of upstream primers (10×), 2 μl of downstream primers (10×), and 5 μl of dd water (Rnase and Dnase free). PCR amplification cycle condition: 90 °C for 5 min, 90 °C for 5 s, 60 °C for 30 s, 72 °C for 5 s, a total of 40 cycles. Light Cycler real-time fluorescent quantitative PCR instrument manufacturer software was used to amplify and analyze the data. The results were expressed as $2^{-\triangle CT}$. The primer sequences are shown in Table 1.

Table 1: miR-146b primer sequences and U6 primer sequences

Gene	*Forward primer*	*Reverse primer*
miR-146b	5 ' -CCTGGCACTGAGAACTGAAT-3 '	5'-GCACCAGAACTGAGTCCACA-3'
U6	5 ' -CTCGCTTCGGCAGCACA-3 '	5 ' -AACGCTTCACGAATTTGCGT-3 '

Statistical methods

The statistical analysis of the data was performed by SPSS 20.0 (Chicago SPSS Co., Ltd.). The pictures of the data were drawn by GraphPad Prism 7 (San Diego Graphpad Software Co., Ltd.). The usage rate (%) of the count data was expressed by chi-square test and was expressed as χ^2. The measurement data were expressed as mean value ± standard deviation (x±sd). All measurement data were in accordance with normal distribution. Independent sample *t*-test was used in the comparison between two groups. The efficacy of

miR-146b and anti-cTnI in the diagnosis of viral myocarditis was evaluated by receiver operating characteristic (ROC) curve. Pearson test was used to analyze the correlation between miR-146b and anti-cTnI in serum of children with viral myocarditis. Logistic regression model was used for analysis of risk prognostic factors in children with viral myocarditis. When $P<0.05$, differences were statistically significant.

Results

The clinical data of children in two groups

The clinical data of patients in two groups were collected and compared. There was no obvious difference between clinical data of children in patient group and those of children in healthy group. The clinical data included gender, age, weight, height, the smoking history of parent, the drinking history of parent, and residence (Table 2).

The expression levels of miR-146b and anti-cTnI in serum

The expression level of miR-146b in serum of children in patient group was significantly higher than that of children in healthy group ($P<0.05$). The expression level of anti-cTnI in serum of children in patient group was significantly higher than that of children in healthy group ($P<0.05$) (Table 3 and Fig. 1).

Table 2: The clinical data of children in two groups [n(%)]/ ($\bar{x}\pm sd$)

Factor	*Patient group (n=48)*	*Healthy group (n=40)*	t/χ^2 *value*	P *value*
Gender			0.183	0.669
Male	25(52.08)	19(47.50)		
Female	23(47.92)	21(52.50)		
Age (yr)	6.46±2.17	6.37±2.26	0.190	0.850
Weight (kg)	19.58±2.37	20.03±2.54	0.858	0.393
BMI	12.39±2.37	12.42±3.14	0.051	0.959
Height (cm)	115.53±5.68	116.38±5.05	0.735	0.465
The smoking history of parent			0.040	0.842
Yes	19(39.58)	15(37.50)		
No	29(60.42)	25(62.50)		
The drinking history of parent			0.502	0.479
Yes	14(29.17)	9(22.50)		
No	34(70.83)	31(77.50)		
Residence			0.009	0.923
City	38(79.17)	32(80.00)		
Village	10(20.83)	8(20.00)		
Nationality			0.004	0.949
Minority	5(10.42)	4(10.00)		
Han	43(89.58)	36(90.00)		
The education level of mother			0.301	0.583
Below high school	8(16.67)	5(12.50)		
High school and above high school	40(83.33)	35(87.50)		

Table 3: The expression levels of miR-146b and anti-cTnI in serum of children in patient group and healthy group ($\bar{x}\pm sd$)

Group	*n*	*miR-146b*	*anti-cTnI(μg /L)*
Patient group	48	1.677±0.373	0.188±0.096
Healthy group	40	1.291±0.328	0.137±0.047
t		5.103	3.066
P		<0.001	0.003

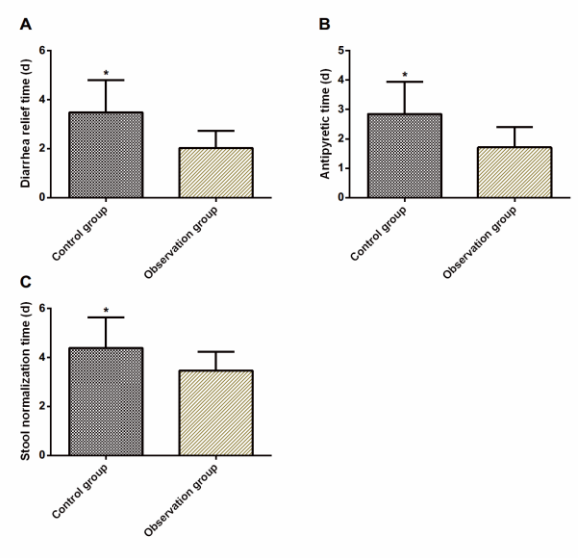

Fig. 1: The expressions of miR-146b and anti-cTnI of children in patient group and healthy group
The expression level of miR-146b in serum of children in patient group was significantly higher than that of children in healthy group (P<0.05) (A). The expression level of anti-cTnI in serum of children in patient group was significantly higher than that of children in healthy group (P<0.05) (B). Note: compared to healthy group, *P<0.05

The diagnostic value of miR-146b and anti-cTnI for children with viral myocarditis

The ROC curve of the expression levels of miR-146b and anti-cTnI in the diagnosis of children with viral myocarditis was drawn. The AUC of miR-146b was 0.741, (95% CI: 0.638-0.843), the specificity was 62.50%, the sensitivity was 82.50%, Cut-off value was 1.552, and that the AUC of anti-cTnI was 0.720 (95% CI: 0.608-0.832), the specificity was 64.58%, the sensitivity was 92.50%, Cut-off value was 0.176 (μg /L) (Table 4 and Fig. 2).

Table 4: The diagnostic value of miR-146b and anti-cTnI for children with viral myocarditis

Indicator	*AUC*	*95%CI*	*Specificity (%)*	*Sensitivity (%)*	*Cut-off*
miR-146b	0.741	0.638~0.843	62.50%	82.50%	1.552
anti-cTnI	0.720	0.608~0.832	64.58%	92.50%	0.176 (μg /L)

Note: AUC: area under the curve, Cut-off: cut-point

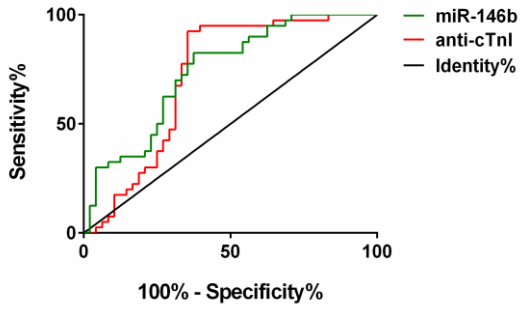

Fig. 2: The diagnostic value of miR-146b and anti-cTnI in the death status of children with viral myocarditis
The AUC of miR-146b was 0.741, 95% CI: 0.638-0.843, the specificity was 62.50%, the sensitivity was 82.50%, Cut-off value was 1.552. The AUC of anti-cTnI was 0.720, 95% CI: 0.608-0.832, the specificity was 64.58%, the sensitivity was 92.50%, Cut-off value was 0.176

The correlation analysis of miR-146b and anti-cTnI in serum of children with viral myocarditis

The relationship between miR-146b and anti-cTnI in serum was analyzed by Pearson correlation analysis. The expression of miR-146b was positively correlated with the expression of anti-cTnI (r=0.607, *P*<0.001). The expression level of anti-cTnI increased as the expression level of miR-146b increased (Fig. 3).

Univariate analysis of viral myocarditis prognosis

According to the efficacy on patients in the observation group after 1 yr of treatment, the uncured children were classified as poor prognosis group (21 cases), and the fully recovered children were classified as good prognosis group (27 cases). According to univariate analysis, there were no differences in gender, age, body mass index, history of upper respiratory tract infection, attention to rest, and place of residence between the two groups of patients, while there were statistical differences in CK-MB, LDH1, TNF-α, IL-1β, CD3 (%), CD4 (%), CD8 (%), LVEF (%) (*P* <0.05) (Table 5).

Multivariate analysis of viral myocarditis prognosis

The indicators with differences in univariate analysis were analyzed. Multivariate logistic proportional hazard regression model analysis showed that CK-MB, LVEF, miR−146b and anti-cTnI expression were independent risk factors affecting the prognosis of viral myocarditis (*P* <0.05) (Table 6).

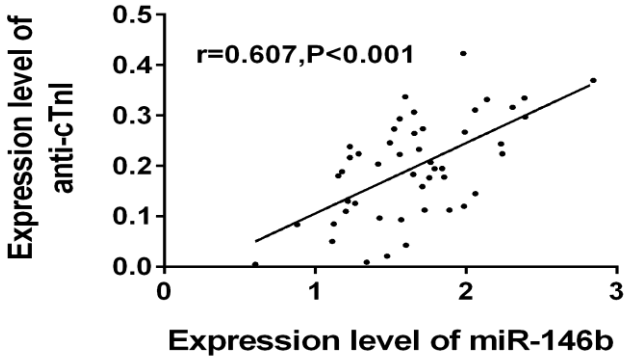

Fig. 3: The correlation analysis of miR-146b and anti-cTnI in serum of children with viral myocarditis
The expression of miR-146b was positively correlated with the expression of anti-cTnI in serum of children with viral myocarditis (r=0.607, *P*<0.001)

Table 5: Univariate analysis of viral myocarditis prognosis

Factors	*Good prognosis group (n=27)*	*Poor prognosis group (n=21)*	*t/c2*	P
Gender			0.383	0.536
Male	13(48.15)	12(57.14)		
Female	14(51.85)	9(42.86)		
Age (yr)	6.38±2.23	6.48±2.62	0.143	0.887
BMI	12.46±2.51	12.35±3.08	0.136	0.892
CK-MB (U/L)	28.56±4.33	36.54±5.21	5.795	<0.01
LDH1 (U/L)	77.94±7.32	89.64±9.37	4.860	<0.01
TNF-α (ng/L)	164.23±9.87	171.44±14.89	2.014	0.049
History of upper respiratory infection			1.383	0.240

Yes	5(18.52)	7(33.33)		
No	22(81.48)	14(66.67)		
Attention to rest			2.364	0.124
Yes	24(88.89)	15(71.43)		
No	3(11.11)	6(28.57)		
IL-1β (ng/L)	17.64±4.38	21.44±3.67	3.196	0.003
Place of residence			0.196	0.658
Urban	12(44.44)	8(38.1)		
Rural	15(55.56)	13(61.9)		
CD3 (%)	54.77±6.89	49.62±4.57		
CD4 (%)	39.28±4.51	35.16±4.26	3.216	0.002
CD8 (%)	23.34±3.54	25.87±2.43	2.799	0.008
LVEF (%)	58.32±3.98	48.95±4.76	7.246	<0.01
miR-146b	1.503±0.384	1.841±0.412	2.930	0.005
Anti-cTnI	0.154±0.051	0.197±0.077	2.323	0.025

Table 6: Multivariate analysis of viral myocarditis prognosis

Variables	*β*	*SE*	*Waldχ^2*	*OR (95%CI)*	P
CK-MB	0.064	0.418	7.516	3.42(2.023~2.578)	0.007
LVEF (%)	0.058	0.317	14.134	4.563(1.668~4.782)	< 0.01
miR-146b	0.035	0.274	6.532	1.321(1.253-1.397)	0.023
Anti-cTnI	0.048	0.197	3.272	3.54(3.196~3.943)	0.003

Discussion

Viral myocarditis is a myocardial inflammation caused by viral infection or autoimmune dysfunction. Coxsackie virus and adenovirus are pathogens that lead to viral myocarditis (20). The pathological changes of viral myocarditis can manifest as focal infiltration, scattered infiltration or diffuse infiltration of myocardial interstitial tissues and inflammatory cells around blood vessels (21). However, as obtaining specimens is hard and some patients may be accompanied with pericarditis or endocarditis, thus, their clinical symptoms are different, and their pathogenesis cannot be diagnosed timely (22). Therefore, finding good markers that affect early prediction of viral myocarditis is significant for improving patients' life quality and survival rate.

As a single-stranded, non-coding, and endogenous, miRNAs can complement and combine with 3' non-coding region of target mRNA, directly degrading target mRNA or inhibiting translation processes of proteins after transcribed (23, 24). Although currently the functions of miRNAs are unknown, some studies have shown that the abnormal expression or mutation of miRNAs is closely related to the occurrence and development of many cardiomyopathy (25, 26). MiRNA is a new focus in the research field of cardiology biotherapy (27). In the study of Goldberg et al (28), miRNA was a potential biomarker of cardiac injury, inflammatory response and the recovery of left ventricular function of children with viral myocarditis. In this study, the expression level of miR-146b in serum of children with viral myocarditis was significantly up regulated. Reducing the expression level of miR-146b could improve the cellular immune imbalance of children with viral myocarditis (29). This result indicates that miR-146b may be involved in the occurrence and development of viral myocarditis. CTnI is one of the three subunits of troponin and plays an important role in regulating myocardial contraction and relaxation. It produces cTnI antibody and can exist in the blood for a long time. It has some characteristics, for example, its window phase is long in the diagnosis time (30). Anti-cTnI is abnormally expressed in patients with

myocardial injury. For example, Tang et al (31) detected some anti-cTnI in serum of patients with acute myocardial infarction. Fan et al (32) researched anti-cTnI in serum of patients with ST segment elevation acute myocardial infarction and found that anti-cTnI was an independent predictor of the remodeling of the left ventricle.

In this study, the expression level of anti-cTnI in serum of children with viral myocarditis was significantly higher than that of healthy children. Anti-cTnI may play an important role in myocardial injury and myocardial recovery. Besides, the relationship between the expression level of anti-cTnI in serum and viral myocarditis was elucidated specifically, which was important for anti-cTnI to become a new biomarker or therapeutic target. This result indicates that miR-146b and anti-cTnI can be used as biological indicators for diagnosing the sensitivity and specificity of viral myocarditis. The result indicates that there may be a close relationship between miR-146b and anti-cTnI. However, this indication was not researched deeply. At the end of the study, we analyzed the factors affecting the prognosis of children with viral myocarditis. CK-MB, LVEF, miR-146b and anti-cTnI expression were independent risk factors affecting the prognosis of viral myocarditis, which indicated that miR-146 and anti-cTnI could be used as clinical indicators to evaluate the prognosis of children with viral myocarditis.

Although this study confirmed effects of miR-146b and anti-cTnI in viral myocarditis, there are still some defects in this study due to the limited experimental conditions. For example, the experiment period was short. Currently there were some limitations in the relevant clinical experiments. The specific relationship among miR-146b and anti-cTnI and the pathogenetic process of viral myocarditis cannot be explained completely. In later studies, the effects and mechanism of miR-146b and anti-cTnI in viral myocarditis will be deeply investigated, to learn the correlation among miR-146b, anti-cTnI and the pathogenetic process of viral myocarditis and provide help for the early diagnosis and treatment of viral myocarditis later.

Conclusion

MiR-146b and anti-cTnI are highly expressed in children with viral myocarditis, suggesting that miR-146b and anti-cTnI may be involved in the occurrence and development of children viral myocarditis. They are expected to become good indicators to diagnose and treat viral myocarditis as well as guide clinical diagnosis and treatment.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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No funding was received in this study.

Conflict of interest

The authors declare that there is no conflict of interest.

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