Analysis of the Indicating Value of Cardiac Troponin I, Tumor Necrosis Factor-α, Interleukin-18, Mir-1 and Mir-146b for Viral Myocarditis among Children

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Key Words
Viral myocarditis • cTnI • TNF-α • IL-18 • MiR-1 • MiR-146b

Abstract
Background/Aims: The primary objective of this study is to evaluate the diagnosis effect of serum protein factors and microRNAs for children suffering from viral myocarditis (VMC).

Methods: The expression levels of serum cardiac troponin I (cTnI), interleukin-18 (IL-18) and tumor necrosis factor-α (TNF-α) in both VMC and control groups were examined by using the Elisa Kit. The expression levels of miR-1 and miR-146b were measured through RT-PCR. Subsequently, the Receiver Operating Characteristic (ROC) curves were drawn based on the diagnostic results of VMC. Moreover, the Spearman correlation analysis was carried out to unveil the association between the indicator expression levels and the ultrasonic cardiogram results, including the left ventricular fractional shortening (FS) and left ventricular ejection fraction (EF).

Results: It is found that the expression levels between the VMC and control group portrait significant differences with respect to cTnI, IL-18, TNF-α, miR-1 and miR-146b (P < 0.05). The diagnostic thresholds for cTnI, IL-18, TNF-α, miR-1 and miR-146b were 160.00 ng/L, 2.26 ng/L and 1.14 ng/L, respectively. The diagnostic thresholds for miR-1 and miR-146b were 0.75 and 1.27, respectively. Results from the Spearman correlation analysis showed that levels of the miR-1 were negatively correlated with FS and EF, while levels of the cTnI, IL-18, TNF-α and miR-146b were positively correlated with FS and EF.

Conclusions: The expression levels of the TNF-α, IL-18 and cTnI and the expression levels of the miR-1 and miR-146b could be used to predict VMC among children and this approach may reinforce the diagnosis of VMC in clinical practices.

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Introduction

Viral myocarditis (VMC) is a common infection of the pediatric cardiovascular system. This infection could cause cardiomyocytes degeneration, necrosis, apoptosis, myocardial interstitial hyperplasia and an immune inflammatory response of the myocardial [1]. The Coxackie virus and the Adenovirus are common pathogens that cause VMC [2]. VMC could cause fatal arrhythmias and cardiogenic shock. In addition, some types of VMC can develop into dilated cardiomyopathy due to virus damage, immune, genetic predisposition and other factors [3]. The pathogenesis of VMC is not fully explained yet, whilst other factors such as direct virus damage cardiomyocytes, virus-induced autoimmune damage, genetic polymorphisms and individual susceptibility may be related to its pathogenesis. Most VMC patients showed no clinical symptoms or only mild symptoms. Some patients present non-specific ECG abnormalities, mild viral infection prodrome, acute hemodynamic compromise or even sudden death [3]. Current methods for VMC diagnosis include electrocardiogram, serology, echocardiography and endomyocardial biopsy [4].

Early diagnosis of VMC is critical to the prognosis of VMC patients and the use of serum markers such as cardiac troponin I (cTnI), interleukine-18 (IL-18) and tumor necrosis factor-α (TNF-α) has drawn increased attention for its accuracy in diagnosis of VMC [4]. cTnI is a cardiac-specific contractile protein in cardiomyocytes that involves the regulation of both systolic and diastolic functioning. The corresponding damage caused by cardiomyocytes is subject to physical, chemical and biological factors. It has been suggested that cardiomyocytes release cTnI into the blood stream promptly due to the integrity loss of the cardiomyocytes membrane, resulting in the abnormal elevation of the troponin serum [5]. The expression level of cTnI is peaked at 18-24 hours after cardiomyocytes being damaging, just before the disease progresses start [5]. The cTnI serum exhibits both high sensitivity and specificity for diagnosing the myocardial injury, suggesting it a potential specific marker for cardiomyocytes damage. Besides, the corresponding level of the cTnI serum is able to evaluate the severity of several diseases such as the myocarditis disease [6, 7].

TNF-α is a cytokine derived from monocytes and macrophages. TNF-α functions as anti-oncogene and enhances the phagocytosis of neutrophil. Moreover, the anti-infection effect of TNF-α further promotes cell proliferation and differentiation, which plays an important role in the immune process resulted from myocarditis or other diseases [8, 9]. Studies have shown that the expression levels of the myocarditis serum, TNF-α and relative mRNA are elevated in patients suffering from VMC [10]. Additionally, the serum IL-18, a cytokine involved in the inflammation and immune responses, could induce the secretion of the tumor necrosis factor. IL-18 plays a role in myocardial immune injury by acting on T-cells and stimulates the secretion of the tumor necrosis factor; although no antiviral activities have been shown on IL-18 [11, 12]. Fairweather et al. found that the expression level of IL-18 is positively correlated with myocarditis reactions in VMC patients [13].

MicroRNAs (miRNAs) are a conserved family of small non-coding RNA molecules, functioning as pivotal regulators of gene expressions [14]. MiRNAs modulate gene expressions via binding to the 3′ un-translated regions (3′UTR) of their target-mRNAs, resulting in either the repression of protein translation or mRNA decay [15, 16]. Numerous studies confirmed that miRNAs are involved in tumor cardiovascular diseases and regulate a variety of biological processes [17-19]. MiRNA chip technology enabled researchers to conclude that differentially expressed miRNAs were detected in both myocarditis mice and WT mice [20]. Studies have shown that miR-146b was involved in inflammatory responses and myocardial fibrosis, which influence the development and progression of VMC. For instance, Corsten et al. discovered that miR-146b was up-regulated in a mouse myocarditis model induced by CVB3 and such a trend was confirmed in myocarditis patients [21, 22]. Another study in VMC mice models suggested that miR-1 and its target protein Cx43 are jointly involved in VMC progress [23]. As a result, expression levels of miR-1 and miR-146b contained in the circulating blood may provide therapeutic value with respect to VMC.
Materials and Methods

Objects

In total, 119 children diagnosed with VMC were selected as subjects for this research. Subjects included 64 boys and 55 girls who were admitted to College of Veterinary Medicine, Northwest A&F University between January 2013 and December 2015. All patients are diagnosed in accordance with the VMC diagnostic criteria which is listed in the American Heart Association (AHA) guidelines [24]. The average age of patients is $6.7 \pm 2.9$ years and the detailed information is displayed in Table 1. In total, 120 healthy children were selected randomly as the control group. The control group included 65 boys and 55 girls with an average age of $7.2 \pm 3.0$ years. No statistically significant differences in gender or age were observed between the VMC and control groups ($P > 0.05$). This study was approved by the Ethics Committee of College of Veterinary Medicine, Northwest A&F University. Also all parents or guardians of the children involved in this research have signed informed consents.

Diagnostic criteria of VMC

The clinical diagnostic criteria of VMC included the follows: cardiac insufficiency, cardiac enlargement, cardio-cerebral syndrome, and cardiogenic shock. And the results of electrocardiography (ECG) showed that more than 2 ST-T wave changes in I, II, aVF, and V5 leads last longer than 4 days. Then the endomyocardial biopsy was used to make a certain diagnosis of myocarditis [24]. The biopsy sample sites (left, right ventricular, or wall segment) were chosen according to the results of magnetic resonance imaging (Siemens 1.5-T Magnetom Sonata) and echocardiography. And biopsies were taken with a biopome (H1518.02-A, Endoflex, Germany). The analysis of endomyocardial biopsies including three methods including: histological analysis according to the Dallas criteria [25], immunohistochemistry analysis according to scientific statements provided by World Health Organization [26], and molecular biological detection of viral genomes [27].

Detection of serum protein factor cTnI, IL-18 and TNF-α

All the patients included in the study didn’t receive any treatment before admission and the venous blood was collected after diagnosis at once. Venous blood samples (2ml) were collected from fasting subjects in both groups. Then samples were centrifuged by 3000 r/min for 10 minutes at room temperature for serum collection and were subsequently stored at -80°C. Instruments including cTnI, IL-18 and TNF-α Elisa Kit (Beijing An Biqi biotechnology company, Shanghai Yanhui Biotechnology Company and Nanjing Haiker biotechnology company, respectively) were manipulated to carry out the above mentioned procedures.

RT-PCR for serum microRNA

A TRIZOL Kit (Invitrogen, United States) was used to extract total RNA in serum (1 ml) and then the extracted RNA was reversely transcribed into cDNA by using the TaqMan® MicroRNA Reverse Transcription Kit (Fermentas, United States). MiR-1, miR-146b and β-actin were amplified with TaqMan® Gene Expression Assay Kit (Fermentas,
The amplification reaction conditions are as follows: (1) pre-degenerated at 95°C for 10 minutes, (2) then subject to 95°C for 15 seconds, (3) 60°C for 30 seconds and (4) 72°C for 15 seconds. This process is repeated 40 times. Each sample was conducted for three wells from which the average value was obtained. Next, the corresponding cycle threshold value of Ct was obtained and the expression levels of target genes were analyzed by using the approach of $2^{-\Delta\Delta Ct}$. All primer sequences are listed in Table 2.

### Ultrasonic cardiology

Left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) of the children in both groups were obtained by using the color ultrasonic echocardiography (HP, United States). Subsequently, the corresponding rate of left ventricular fractional shortening (FS) was calculated by the following formula: $\text{FS}\% = (\text{LVEDD} - \text{LVESD})/\text{LVEDD} \times 100\%$ and the normal FS should range from 28% to 38%. Left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) were obtained in the same way and the left ventricular ejection fraction (EF) were calculated by the formula: $\text{EF}\% = (\text{LVEDV} - \text{LVESV})/\text{LVEDV} \times 100\%$ with the normal reference of EF ranging from 45% to 75%. All procedures were conducted by an ultrasound practitioner with over five years’ experience and the average value of three independent tests was obtained finally.

### Statistical analysis

All data was analyzed by SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad software 5.0 (Graphpad software, La Jolla, CA, USA). Measurement variables were demonstrated as counted data and compared using the chi-square test whereas continuous variables were presented as mean ± standard error and compared using the unpaired t-test. The ROC curve was derived and the area under the ROC curve as well as the diagnosis threshold corresponding to the sensitivity and specificity were also calculated. These measures were used to evaluate the diagnostic precision and the Spearman correlation analysis is carried out to evaluate the association between the molecules and echocardiogram parameters. Differences are regarded significant if $P < 0.05$.

### Results

#### Serum levels of cTnI, IL-18 and TNF-α

The expression levels of certain serum protein factors were identified for the VMC and control group. The corresponding concentration of cTnI, IL-18 and TNF-α serums in the VMC group were 0.45 ± 0.17 ng/mL (Fig. 1A), 3.06 ± 0.83 ng/L (Fig. 1B) and 1.53 ± 0.60 ng/L (Fig. 1C), respectively. These concentrations were significantly higher than those in the control group (0.08 ± 0.03 ng/ml, 1.62 ± 0.34 ng/L and 0.88 ± 0.15 ng/L, respectively, $P < 0.05$).

Besides that, the corresponding Receiver Operating Characteristic (ROC) curve was drawn in order to assess the diagnostic accuracy in relation to these serum levels above. The area under the ROC curve of cTnI is 0.982 and the corresponding cut-off value is 0.16 ng/mL. Diagnosing VMC by cTnI exhibits high sensitivity and specificity (sensitivity = 95%, specificity = 99.2%) (Fig. 2A). Likewise, both IL-18 and TNF-α exhibit high diagnostic accuracy with respect to VMC (IL-18: AUC = 0.943, cut-off value = 2.26 ng/L, sensitivity = 83.2%, specificity = 98.3%; TNF-α: AUC = 0.867, cut-off value = 1.14 ng/L, sensitivity = 76.5%, specificity = 95%) (Fig. 2B, 2C).

#### MiR-1 down-regulated while miR-146b up-regulated in VMC children

Compared to the control group, the expression levels of miR-1 in the VMC group decreased significantly (Fig. 3A, $P < 0.05$), with 0.48 ± 0.19 for the VMC group compared to 1.00 ± 0.32 in the control group. Unlike miR-1, the expression level of miR-146b serum
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increased significantly in the VMC group in comparison to the control group (Fig. 3B, P < 0.05), with 1.46 ± 0.53 for the VMC group and 1.00 ± 0.38 for the control group. As suggested by the ROC curve of miR-1, the area under the curve is 0.921 which corresponds to a cut-off value of 0.75 and the corresponding sensitivity and specificity are 94.1% and 78.3%, respectively (Fig. 4A). The area under the ROC curve of miR-146b is 0.752, corresponding to a sensitivity and specificity of 63% and 77.5%, respectively (Fig. 4B).

**Decreased FS and ES in the VMC group**

The results of ultrasonic echocardiography showed that VMC patients always had enlarged left atrium and left ventricle, mural thrombus in left ventricle, impaired left ventricular systolic function, and pericardial effusion.

Results in Table 3 reveals that a lower average FS was observed in the VMC group in comparison to the control group (VMC group: FS = 24.66 ± 5.07%; control group: FS = 32.87 ± 3.08%, P < 0.05). Likewise, a significantly lower average EF value was observed in VMC group in comparison to the control group (VMC group: 39.41 ± 8.19%; control group: 58.83 ± 7.75%, P < 0.05).

**Fig. 1.** Comparison of serum protein factor levels between the VMC (n = 119) and control (n = 120) groups, including cTnl (A) IL-18 (B) and TNF-α (C).

**Fig. 2.** The corresponding Receiver Operating Characteristic (ROC) curves for serum protein factors including cTnl (A), IL-18 (B) and TNF-α (C).

**Fig. 3.** Comparison of the expression levels of microRNA miR-1 (A) and miR-146b (B) between the VMC (n = 119) and control (n = 120) groups.
Results from the Spearman correlation analysis are shown in Table 4. The miR-1 serum level was negatively correlated with the FS \( (r = -0.313, P < 0.001) \), while the levels of cTnI, IL-18, TNF-α and miR-146b serums were positively correlated with the FS \( (r = 0.468, P < 0.001; r = 0.324, P < 0.001; r = 0.162, P = 0.012; r = 0.145, P = 0.025, \text{ respectively}) \). Likewise, it appears that the miR-1 expression levels are negatively correlated with the EF \( (r = -0.392, P < 0.001) \), while the expression levels of the cTnI, IL-18, TNF-α and miR-146b serums are positively correlated with the EF \( (r = 0.517, P < 0.0001; r = 0.394, P < 0.001; r = 0.235, P < 0.001; r = 0.169, P = 0.009, \text{ respectively}) \).

### Discussion

Misdiagnosis of VMC is a common issue in clinical practices as symptoms of VMC vary significantly among patients. In addition to the virology pathogen examination, VMC diagnosis also includes electrocardiogram, echocardiogram or detection of any significant changes in serum levels [13]. These approaches may have equivalent diagnostic accuracy and they may be used simultaneously to enhance the diagnostic precision.

Serum cTnI is particularly effective for diagnosing VMC in clinical practices. Although cTnI is rarely discovered in peripheral blood, it can be detected in myocardial cells. Our study suggests that the average cTnI serum concentration among VMC children is significantly higher than that of the control group and this trend perhaps is explained by the small molecular weight of cTnI. Since small molecules are more likely to penetrate the cell membrane of VMC subjects, the cTnI serum concentration is expected to increase substantially. A recently published meta-analysis study concluded that elevation of cardiac troponin (cTn) on admission due to acute aortic dissection (AAD) is associated with an increased in-hospital mortality [28]. IL-18 is a type of cell protein factor produced by macrophages which is involved in a variety of human immune responses. IL-18 is also known to participate in both
innate and adaptive immune responses [29]. Dinarello et al. demonstrated that the relief of VMC symptoms was associated with a decrease in the IL-18 concentration [29]. Yoshida et al. also found that exogenous IL-18 suppresses the expression of cardiac TNF-α and alleviates viral myocarditis in mice when exogenous IL-18 is injected with the viral inoculation [12]. Another study conducted by Gao et al. suggests that IL-18 is able to predict adverse event in patients with ST-segment elevation acute myocardial infarction (STEMI) over a 60-day period [30]. In our study, the average IL-18 serum expression level in the VMC group is significantly higher than that of the control group, which indicates that expression of IL-18 may be related to the pathogenesis of VMC. On the other hand, TNF-α is also a cellular protein factor produced by macrophages. TNF-α not only aggravates myocardial inflammation reaction but also stimulates macrophages to release excessive inflammatory factors such as IL-18 and thereby cardiac functions are likely to be affected [10]. This study shows that the average TNF-α serum in the VMC group was significantly higher than that in the control group, which provides potential link between TNF-α and VMC. More importantly, the corresponding ROC curve of cTnI, IL-18 and TNF-α indicates that these serum concentrations are able to provide accurate VMC diagnosis. Finally, the Spearman correlation analysis suggests that these serums may explain the association between decreased left ventricular systolic function and cardiac damage in VMC children.

A major challenge of cardiovascular disease is the identification of effective biomarkers that could be routinely detected in plasma. MicroRNAs (miRNAs) were recently put forward as a convincible marker that circulates in the bloodstream [31]. MiRNA is involved in many biological processes in cells and its expression is associated with cancers and other diseases, such as enteroviral infections [32]. Furthermore, miRNA has been considered as a biomarker for diagnosing various diseases in clinical practices [33, 34]. Since miRNAs are much shorter than proteins and mRNAs, detection of the miRNA concentration in serum is achieved by using techniques like RT-PCR. As suggested by previous studies, abnormal expression levels of miR-1 are closely related to a variety of cardiovascular diseases such as cardiac arrhythmia, acute myocardial infarction and cardiac hypertrophy [35-37]. Our experiments suggest that children in the VMC group exhibit significantly lower average expression of miR-1 compared to those in the control group and our results are consistent with the conclusions from Zhang et al. [38]. Moreover, miR-146b not only is related to the pathogenesis of VMC but also have a positive association with the TNF-α serum concentration [39]. In our study, the average expression level of miR-146b in the VMC group is significantly elevated in comparison to the control group, suggesting a potential connection between miR-146b and VMC. More importantly, both miR-1 and miR-146b can be potential diagnosis biomarkers for VMC based on their corresponding ROC curves, sensitivity and specificity. On top of that, the Spearman correlation analysis reveals that changes in the expression level of miRNA may reflect the degradation of both cardiac functions and left ventricular systolic functions among VMC patients. A few studies also concluded that miRNA is capable of restoring cardiac functions through influencing the NF-kB pathway among acute viral myocarditis patients [40]. Nevertheless, further research should be carried out to reinforce the corresponding conclusions provided by our experiments.

There are still several limitations in our study. Our study only contains a small sample size due to the resource constraint and we motivate future researchers to step further in order to verify the above conclusions. Besides, the mechanism of how serum cTnI, IL-18, TNF-α, miR-1 and miR-146b influence the process of VMV is not revealed. There may be certain association between such inflammatory cytokines and miRNAs. Further study will pay more attention on it.

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The authors declare no commercial or financial conflict of interest.

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