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Expression levels of plasma miRNA-21 and NT-proBNP in children with Kawasaki disease and their clinical significance

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Abstract. – OBJECTIVE: The purpose of this study was to detect the expression levels of plasma microRNA-21 (miRNA-21) and NT-proBNP (N-terminal prohormone of brain natriuretic peptide) in children with Kawasaki disease (KD), as well as their clinical significance.

PATIENTS AND METHODS: Children with KD (n=100) who were treated in our hospital from June 2017 to May 2019 were included. In the same period, non-KD children with febrile diseases were included as controls. Plasma levels of miRNA-21 and NT-proBNP were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and electrochemiluminescence, respectively. Then, the relationship between miRNA-21 and NT-proBNP in children with KD was analyzed by Pearson's correlation test. Potential factors influencing KD were analyzed by Multivariate logistic regression test. Finally, receiver operating characteristic (ROC) curves were depicted to assess the diagnostic potentials of miRNA-21 and NT-proBNP in KD.

RESULTS: The results showed that miRNA-21 and NT-proBNP levels were higher in children with KD. Plasma level of miRNA-21 was positively correlated with NT-proBNP level in children with KD. Besides, both miRNA-21 and NT-proBNP were risk factors influencing the onset of KD. According to ROC curves, the sensitivity and specificity of miRNA-21 in diagnosing KD was 83% and 89%, respectively (AUC=0.9212, 95% CI: 0.8809-0.9614, cut-off value=1.985). NT-proBNP also displayed diagnostic potential in KD (AUC=0.9788, 95% CI: 0.9630-0.9946, cut-off value=265.6, sensitivity=88%, specificity=95%).

CONCLUSIONS: MiRNA-21 and NT-proBNP are upregulated in plasma of children with KD. They are positively correlated with each other and serve as risk factors for KD. Both of them can be utilized as indicators of auxiliary diagnosis in KD.

Key Words:
MiRNA-21, NT-proBNP, Kawasaki disease.

Introduction

Kawasaki disease (KD) typically occurs in 6-month-old to 5-year-old children and it is characterized by highly activated immune system and immune-damaging vasculitis. Clinical symptoms of KD mainly include fever, rash and enlargement of cervical lymph nodes[1]. KD affects the coronary arteries, and about 20-25% of children with KD may develop coronary artery damage if active treatment is lacking. In severe cases, rupture of coronary artery aneurysms may lead to sudden cardiac death[2]. As KD lacks specific clinical manifestations, many children already have coronary artery damage at the time of diagnosis, which brings great challenges to the treatment of KD. Sensitive and specific indicators of KD are required to improve the early detective rate and its prognosis.

MicroRNAs (miRNAs) are non-coding NRAs with 18-24 nucleotides long. They are highly conserved and responsible for inhibiting mRNA translation or degrading mRNA by binding the 3′-untranslated region (3′-UTR)[3]. MiRNAs are extensively involved in regulating cell phenotypes, disease progression and life activities. Potential functions of miRNAs in disease diagnosis and treatment have been highlighted[4]. A recent report suggested that plasma level of miRNA-223 is markedly upregulated in patients with KD. In addition, plasma miRNA-223 subsequently translocates into endothelial cells and serves as

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an endocrine genetic signal that is responsible for vessel damage[5]. MiRNA-21 was previously reported to regulate neointima *in vivo*. Knockdown of miRNA-21 suppresses proliferative rate and accelerates apoptosis in cultured vascular smooth muscle cells by downregulating Bcl-2 and PTEN[6,7].

N-terminal prohormone of brain natriuretic peptide (NT-proBNP) is produced by ventricular myocytes under external stimuli. The half-life of NT-proBNP can last 120 min, and it is stably expressed *in vitro*. NT-proBNP is of significance in diagnosing acute heart failure and predicting its prognosis[8]. During the acute phase of KD, NT-proBNP sharply increases[9]. NT-proBNP, positively regulated by miRNA-21, influences the development of ischemic cardiomyopathy[10]. In this paper, the plasma levels of miRNA-21 and NT-proBNP in children with KD were detected, and their clinical significance in KD was further explored.

Patients and Methods

General Data of Included Subjects

A total of 100 children who were diagnosed with KD at the acute phase in our hospital from June 2017 to May 2019 were included. KD was diagnosed based on the Revised Diagnostic Guidelines for Kawasaki Disease (the 5th revised edition)[11]. In the same period, 100 non-KD children with febrile diseases were included as controls. Subjects with organic diseases were excluded. No significant differences in age and gender were observed between two groups (p>0.05). This investigation was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital. Signed written informed consents were obtained from all participants before the study.

Blood Samples

Peripheral blood samples were collected prior to the treatment of intravenous immunoglobin G, and those in controls were collected as well. Then, these samples were subjected to ethylenediaminetetraacetic acid (EDTA) anticoagulation and 3000 r/min centrifugation for 5 min. After that, the supernatant was harvested and stored at -80°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Plasma miRNAs were extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), which were reversely transcribed using the TaqMan microRNA reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed using the 2×SYBR Green PCR Master Mix (TaKaRa, Otsu, Shiga, Japan). U6 was the internal reference. MiRNA-21: Forward: 5′-CGCGCTAGCTTATCAGACTGA-3′ and Reverse: 5′-GTGCAGGGTCCGAGGT-3′, and U6: Forward: 5′-GGTCGGGCAGGAAGAGGGC-3′ and Reverse: 5′-CTAATCTTCTCTGTATCGTTCC-3′. The relative expression of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

NT-ProBNP Determination

Plasma level of NT-proBNP was detected using the commercial kit (EDiagnosis, Wuhan, China) by enhanced chemiluminescence (ECL).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for all statistical analyses. Data were expressed as mean ± SD (standard deviation). The t-test was performed for analyzing differences between groups. The relationship between miRNA-21 and NT-proBNP in children with KD was analyzed by Pearson's correlation test. Moreover, potential factors influencing KD were analyzed by Multivariate logistic regression test, and receiver operating characteristic (ROC) curves were depicted to assess the diagnostic potentials of miRNA-21 and NT-proBNP in KD. p<0.05 indicated a statistically significant difference.

Results

Indexes Examinations

In KD group, there were 56 boys and 44 girls aging from 0.9 to 6.5 years old (average age: 2.8 years old). The control group involved 52 boys and 48 girls aging from 1.2 to 6.8 years old (average age: 2.9 years old). No significant differences in age and gender were observed between two groups (p>0.05). Compared with tested indexes, lower hematocrit and sodium levels, as well as higher CRP and ALT were seen in children with KD than controls (p<0.05; Table I). In addition, no significant differences in WBC, platelets and AST were found between groups (p>0.05).

Table I. Comparison of examined indexes.

Variables	Control (n = 100)	KD (n = 100)	χ^2/t	*p*
Male/Female	56/44	52/48	0.322	0.67
Age (year)	2.8 ± 0.68	2.9 ± 0.71	1.017	0.31
WBC (10^9/L)	13.65 ± 4.28	14.03 ± 4.51	0.611	0.542
Platelets (10^9/L)	340.79 ± 53.27	351.09 ± 55.33	1.341	0.181
Hematocrit (%)	32.81 ± 7.83	28.75 ± 6.82	3.91	< 0.001
CRP (mg/L)	74.58 ± 11.81	114.47 ± 21.51	16.256	< 0.001
ALT (U/L)	43.05 ± 3.72	50.84 ± 5.07	13.388	< 0.001
AST (U/L)	48.23 ± 6.21	47.39 ± 6.07	0.967	0.335
Sodium (mEq/L)	150.57 ± 30.84	135.17 ± 25.07	3.875	< 0.001

WBC: White blood cells; CRP: C-reactive protein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Relative Levels of MiRNA-21 and NT-proBNP In Plasma of Children With KD

Compared with those in controls, both miRNA-21 (Figure 1A) and NT-proBNP (Figure 1B) were upregulated in plasma of children with KD, suggesting their potential involvement in the development of KD.

Correlation Between MiRNA-21 and NT-proBNP

Pearson's correlation test was conducted to assess the relationship between levels of miRNA-21 and NT-proBNP in children with KD. It was shown that NT-proBNP level was increased with the elevation of miRNA-21 level in plasma of children with KD, displaying a positive correlation (r=0.4027, 95% CI: 0.2240-0.5553, p<0.001) (Figure 2). It is indicated that miRNA-21 stimulates NT-proBNP activity and thus influences the development of KD.

Risk Factors of KD

Multivariate logistic regression test was performed to evaluate potential factors influencing KD. As data showed, CRP, sodium level, miRNA-21 and NT-proBNP were potential risk factors of KD, with the calculated OR as 1.537, 0.628, 1.682 and 2.083, respectively (Table II). Collectively, highly expressed miRNA-21 and high level of NT-proBNP increased susceptibility to KD.

Diagnostic Potentials of MiRNA-21 and NT-proBNP in KD

ROC curves were depicted to assess the diagnostic potentials of miRNA-21 and NT-proBNP in KD. AUC of miRNA-21 and NT-proBNP in diagnosing KD was 0.9212 (95% CI: 0.8809-0.9614) and 0.9788 (95% CI: 0.9630-0.9946), respectively. The sensitivity and specificity of miRNA-21 in diagnosing KD were 83% and 89%, respectively (cut-off value=1.985), while those of NT-proBNP

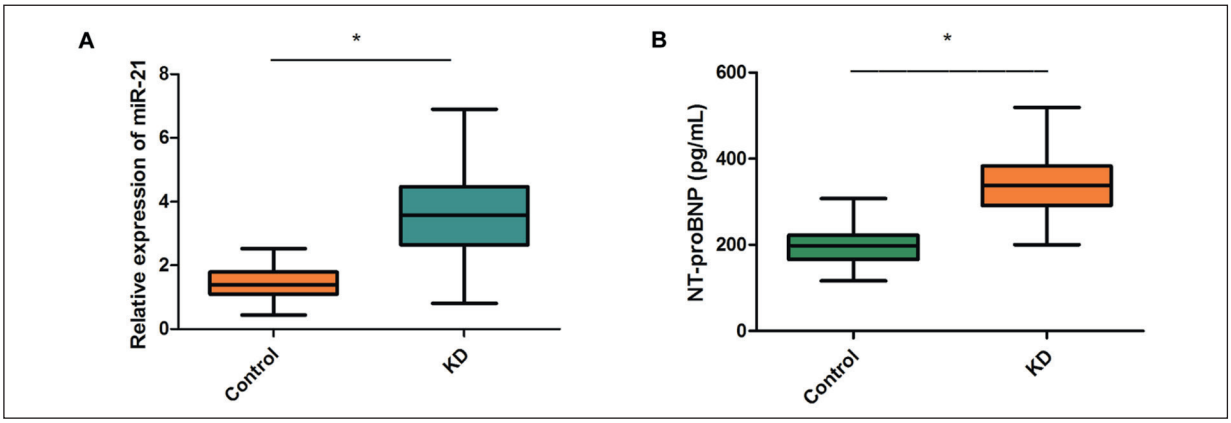

Figure 1. Relative levels of miRNA-21 and NT-proBNP in plasma of children with KD. **A, B,** Plasma levels of miRNA-21 (**A**) and NT-proBNP (**B**) in KD group and control group.

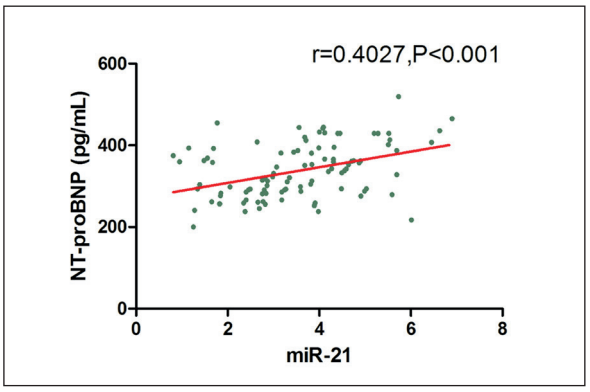

Figure 2. Correlation between miRNA-21 and NT-proBNP. MiRNA-21 level is positively related to NT-proBNP in plasma of children with KD (r=0.4027, 95% CI: 0.2240-0.5553, p<0.001).

were 88% and 95% (cut-off value=265.6 pg/mL) (Figure 3). MiRNA-21 and NT-proBNP were considered as potential diagnostic indicators in KD.

Discussion

KD, also known as mucocutaneous lymph node syndrome, is a pediatric disease manifested as fever and rash. Systemic vasculitis is the pathological change of KD, which is an important causative factor for acquired heart disease in children[12]. Epidemiological findings strongly suggest that infections of bacteria, viruses (adenoviruses, enteroviruses), and other microorganisms (mycoplasma, candida albicans) are involved in the onset of KD[13,14]. However, the cause of KD is largely unknown. Viral and/or bacterial infections produce an amplified cascade of immune responses, which act as stimulants[13]. Responding to certain stimuli, activated innate immunity in individuals with susceptibility to microbial biofilm-related molecules leads to severe inflammatory reactions[15].

MiRNAs negatively regulate downstream gene expressions owing to the stem-loop structure. They are widely involved in innate immunity and adaptive immunity[16-18]. Shimizu et al[19] identified six miRNAs that are remarkably upregulated in the acute phase of KD. Yun et al[20] showed that miRNA-200C and miRNA-371-5p are highly expressed in plasma of children with KD. MiRNA-200C influences host defense against microbial pathogens by the TLR4-MyD88 pathway and inhibits apoptosis and senescence in endothelial cells by targeting ZEB1. MiRNA-371-5p is a vital regulator in inflammatory response. He et al[21] reported that downregulated miRNA-483 results

Table II. Multivariate logistic regression test on risk factors of KD.

Variables	OR	95% CI	p
Hematocrit (%)	0.529	0.266-0.744	0.027
CRP (mg/L)	1.537	1.192-2.162	< 0.001
Sodium (mEq/L)	0.628	0.321-0.755	0.018
MiR-21	1.682	1.225-3.283	0.025
NT-proBNP (pg/mL)	2.083	1.728-2.331	0.006

CRP: C-reactive protein; NT-proBNP: N-terminal prohormone of brain natriuretic peptide.

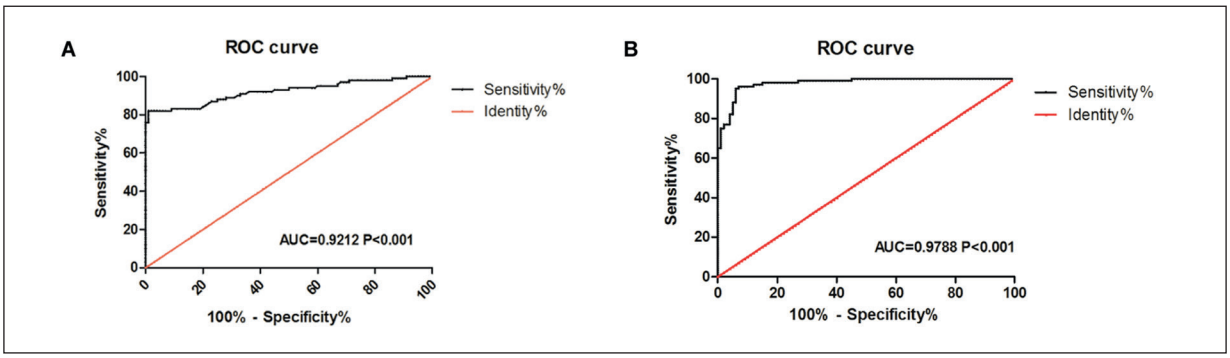

Figure 3. Diagnostic potentials of miRNA-21 and NT-proBNP in KD. **A,** Diagnostic potential of miRNA-21 in KD (AUC=0.9212, 95% CI: 0.8809-0.9614, cut-off value=1.985, sensitivity=83%, specificity=89%). **B,** Diagnostic potential of NT-proBNP in KD (AUC=0.9788, 95% CI: 0.9630-0.9946, cut-off value=265.6 pg/mL, sensitivity=88%, specificity=95%).

in CTGF elevation in the plasma of children with acute phase of KD, which facilitates EMT and thus causes vessel damage and formation of coronary aneurysm.

Myocardial ischemia, necrosis, injury, ventricular wall tension and pressure overload all stimulate abundant release of pre-proBNP in ventricular myocytes. It is cleaved into proBNP and a signal peptide. The former one translocates into blood and it is further cleaved into NT-proBNP and BNP equivalently. NT-proBNP is optimal to be used in clinical examination due to its longer half-life and higher plasma concentration stability[22,23]. Jiang et al[24] showed that NT-proBNP level is positively related to miRNA-21 level, and both of them are capable of regulating the development of atherosclerosis. The findings of this research identically revealed that miRNA-21 and NT-proBNP were positively related to each other, and they were upregulated in plasma of children with KD. Besides, both of them were risk factors for KD, presenting certain diagnostic potentials.

For the first time we found that miR-21 could regulate the expression of NT-proBNP and could be used as an auxiliary diagnostic indicator for KD with good clinical application value. This provides new ideas for the clinical diagnosis and treatment of KD.

Conclusions

In summary, miRNA-21 and NT-proBNP are upregulated in plasma of children with KD. They are positively correlated with each other and serve as risk factors for KD. Besides, they can be utilized as indicators for auxiliary diagnosis in KD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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