# Diagnostic values of microRNA-31 in peripheral blood mononuclear cells for pediatric pulmonary tuberculosis in Chinese patients

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**ABSTRACT.** We investigated the diagnostic values of microRNA-31 in peripheral blood mononuclear cells (PBMCs) for pediatric pulmonary tuberculosis in Chinese patients. Sixty-five children with TB were selected for this study, which was conducted at the Department of Infectious Diseases People's Hospital of Laiwu City between December 2013 and December 2014. Sixty healthy children, selected in parallel, served as the control group. Real-time PCR was used to detect miR-31 expression in PBMCs. Serum levels of IL-6, TNF-α, NF-κB, and IFN-γ was detected by ELISA. ROC curve was employed to evaluate the diagnostic value of miR-31 in pediatric TB. Results show that expression of miRNA-31 in pediatric TB patients was significantly lower than that in normal children (0.48 ± 0.15 *vs* 1.23 ± 0.36, $P < 0.05$). By contrast, serum levels of the innate immune response cytokines, IL-6, TNF-α, NF-κB, and IFN-γ, were significantly higher in pediatric TB patients compared with normal children ($P < 0.05$). Furthermore, miRNA-31 expression was negatively correlated with serum levels of IL-6 ($t = 69.91$, $P < 0.001$), TNF-α ($t = 10.96$, $P < 0.001$), NF-κB ($t = 39.94$, $P < 0.001$), and IFN -γ ($t = 37.94$, $P < 0.001$). The cut-off threshold of miR-31 for pediatric TB diagnosis is 0.835 with a sensitivity of 98.5% and a

 

specificity of 86.7%. Therefore, miR-31 has the potential to be a diagnostic marker in pediatric TB patients.

**Key words:** miR-31; Tuberculosis; Peripheral blood mononuclear cells; IL-6; TNF-α; NF-κB

## INTRODUCTION

Pulmonary tuberculosis (TB) is a major infectious disease that is second only to human immunodeficiency virus (HIV) in terms of disease-related deaths (Shaler et al., 2012). It is estimated that one third of the world's total population is currently infected with *Mycobacterium tuberculosis* (MtB), the causative bacterium of TB. Furthermore, new TB cases are occurring at an alarming rate of 9 million per year, with approximately 1.5 million TB-related deaths per year worldwide. While MtB poses significant risk to human health, fortunately, only 5-10% of those infected develop the active form of the disease (Dalgic et al., 2011). According to the World Health Organization (WHO) estimates, one million children develop TB worldwide, accounting for 11% of total annual TB cases (Dalgic et al., 2011). In 2011, half a million children (0-14 years) developed TB, and 64,000 children died from the disease. Risk of TB infection in children is 2-5% in developing countries (Veedu et al., 2013). At-risk groups in children include those of lower socioeconomic status, being in an immune-compromised state, having a history of incarceration, or living in countries with high TB incidence (Yousef et al., 2013). The host immune response to MtB determines the clinical outcome in the early phase of infection. MtB is an intracellular pathogen that is phagocytosed by antigen-presenting cells such as lung parenchyma macrophages, alveolar macrophages, and dendritic cells in the lungs (Torun et al., 2014). Pediatric TB is often treated as a low-priority disease by national TB control programs owing to limited resources, which are reserved for sputum smear-positive cases that contribute to active TB transmission (Wang et al., 2013). Furthermore, diagnosis of pediatric TB is challenging, and children infected with TB are major reservoirs for adulthood tuberculosis and disease transmission (Bolursaz et al., 2014). Recent studies have focused on the role of microRNAs (miRs) as sensitive diagnostic markers in various diseases, including pediatric TB.

MiRs are short RNA molecules of 21-25 nt in length, and are important regulators of post-transcriptional gene expression. Upon binding to their target mRNAs, miRs either inhibit translation or promote mRNA degradation, thereby controlling protein synthesis (Spinelli et al., 2013). MiRs regulate diverse cellular processes such as cell differentiation, proliferation, organ development and act as biomarkers for infectious diseases (Fu et al., 2011). Several miRs have been found to regulate differentiation and function of T cells, and have critical roles in regulating macrophages, dendritic cells, and innate functions of NK cells (Liu et al., 2011; Harapan et al., 2013). For example, miRNA-155 is essential for T cell-mediated *Helicobacter pylori* infection, and miR-147 attenuates TLR-induced inflammatory responses (Yi et al., 2012). In addition, Wu et al. found that miR-155 and miR-155* are potential diagnostic markers that are up-regulated during immune challenge with MtB antigens (Wu et al., 2012). Liu et al. also demonstrated that miR-582-5p plays an essential role in regulating anti-MtB directed immune responses by inhibiting monocyte apoptosis as well as down-regulating FOXO1 expression (Liu et al., 2013).

In this study, we investigated the diagnostic values of microRNA-31 in peripheral blood mononuclear cells for pediatric pulmonary tuberculosis patients in a Chinese population. The correlation between miR-31 expression and serum levels of IL-6, TNF-α, NF-κB, and IFN-γ was determined to understand the pathways involved in TB development.

## MATERIAL AND METHODS

### Subjects

Sixty-five pediatric TB patients were enrolled for the study at the Department of Infectious Diseases in People's Hospital of Laiwu City, Shandong Province, between December 2013 and December 2014. The TB group consisted of 38 males and 27 females and ranged between 1 and 10 years of age. Among these, 21 and 44 patients were under and over the age of 3, respectively. All pediatric TB subjects met the "Clinical diagnostic criteria and treatment plan for pediatric tuberculosis" (Pan et al., 2014). Sixty healthy children were also recruited within the same time-period, and were defined as the control group. The control group consisted of 35 males and 25 females, and ranged between 2 and 11 years of age. Among the healthy subjects, 20 and 40 were under and over the age of 3, respectively. The control group had no history of recent exposure to TB, other viral infections, and disease of the vital organs such as heart, liver, lung, and kidney. There were no significant differences in age and gender between the TB group and the control group ($P > 0.05$). This study was approved by the Ethics Committee of People's Hospital of Laiwu City, Shandong Province, and parents of all the children in this study signed written informed consents. This study conformed to the Declaration of Helsinki.

### PBMC collection and RNA extraction and detection

Peripheral venous blood (2 mL) from each subject was collected and placed into tubes containing the anticoagulant EDTA. All plasticware was RNase-free. The blood was briefly mixed, and transferred into 15mL centrifuge tubes. Next, 1X erythrocyte lysis buffer (6 mL) was added to the tubes, the contents were mixed and placed on ice for 5 min. The tubes were centrifuged at 3000 rpm for 5 min, and the supernatant was carefully aspirated and discarded. If the red cell mass remained at the bottom of the tube, indicating insufficient lysis, 1 mL 1X erythrocyte lysis buffer was added again, mixed and centrifuged to isolate the white cell pellet, which was defined as the PBMC. Following isolation of the PBMC, 1 mL Trizol reagent was added and mixed on ice for 5 min. Chloroform (200 µL) was then added, mixed, and the samples were left on ice to resolved the two phases. Following this, samples were centrifuged for 15 min at 4°C and 12,000 rpm. Three layers were visualized: the upper layer (aqueous phase) contained RNA, the intermediate layer contained proteins and DNA, and the lower layer consisted of the organic phase. The supernatant (550 µL) was carefully transferred to a 1.5 mL EP tubes and 550 µL isopropanol was added. The sample was mixed and incubated at -20°C for 20 min. After centrifuging at for 15 min at 12,000 rpm and 4°C, visible RNA pellet was observed at the bottom of the tube. The isolated total RNA was dissolved in 50 µL diethylpyrocarbonate-treated water at 55°C for 5 min. Nano-Drop 1000 UV-vis spectrophotometer was used to detect RNA concentration and purity. Purity was indicated by OD260/OD280, and a value between 1.8 and 2.1 was regarded as acceptable for the study.

### Real-time PCR

The miScript Reverse Transcription kit (Qiagen) was used for cDNA synthesis. Quantitative PCR was performed using the miScript SYBR Green PCR kit (Qiagen), and was conducted on the Applied Biosystems 7900HT Sequence Detection System. The reaction conditions were as follows:

initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 20s, annealing at 60°C for 34s, and extension at 95°C for 15 s, followed by 60°C for 15 s and 95°C for 15 s. Primers were synthesized by Shanghai Biotechnology Co., Ltd. Sequences of the primers used are as follows: miR-31: forward: 5'-AGGCAAGATGCTGGCATAG-3', reverse: 5'-AUUACGUUCUACGACCGUAUC-3'; U6: forward: 5'-TTCGTGAAGCGTTCCATATTTT3', reverse: 5'-GTGCAGGGTCCGAGGT-3'. U6 was used as internal control, and qPCR was formed in triplicates. Relative quantitation method was used to detect miR-31 expression in both the TB group and the control group. The relative miR-31 expression was calculated as $2^{-\Delta\Delta Ct}$. As shown in Figure 1A and B, the peak value of the melting curves for miR-31 and U6 was defined as Tm, which represents the temperature at which 50% of the double-stranded DNA denatures. It was determined that Tm of miR-31 was 79°-80°C, and as illustrated by the single melting peak, the primers used were specific for said gene. The x-axis in the amplification curve represents fluorescence intensity, and the y-axis represents the number of PCR cycles. The intersection point of the green line and the amplification curve denotes the Ct value, i.e., the cycle number at which fluorescence intensity reached the threshold value.

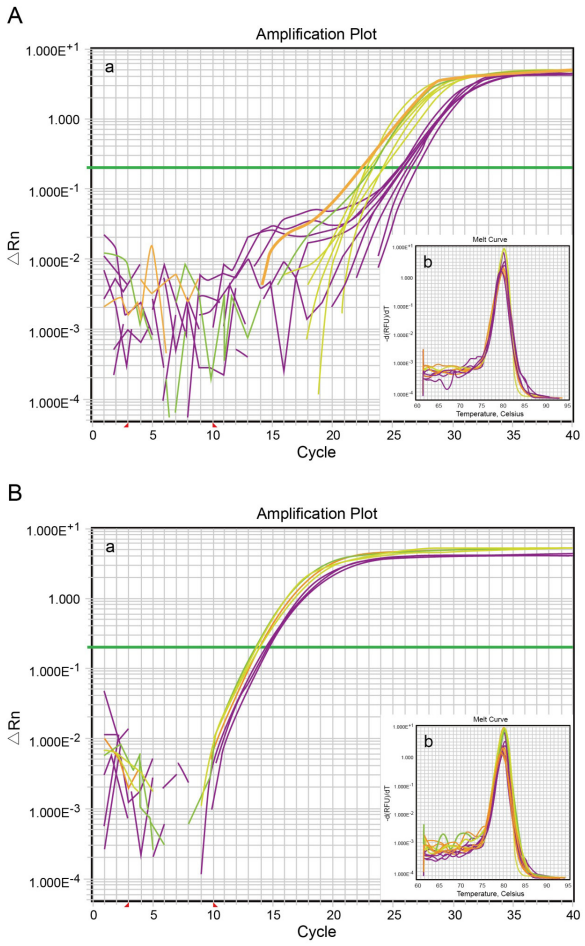


**Figure 1**. Amplification plot (a) and melting curve (b) of (**A**) miRNA-31 and (**B**) U6 assay.

## Serum IL-6, TNF-α, NF-κB and IFN-γ levels detection

Serum levels of IL-6, TNF-α, NF-κB, and IFN-γ were detected by Enzyme-linked immunoassay (ELISA). Peripheral venous blood (1 mL) from each subject was collected and centrifuged at 3000 rpm for 10 min. The supernatant was collected in EP tube and stored at -70°C. IL-6, TNF-α, NF-κB and IFN-γ detection kits were purchased from Thermo Electron Corporation (US), and assays were performed according to manufacturer protocols. Briefly, 100 µL standards, controls, and test samples at different concentrations were added to the wells of a microplate. The plate was sealed and incubated at room temperature for 2 hours. Next, wells were washed four times with 200 µL cleaning mixture using an automatic washer. All samples were incubated with 200 µL horseradish peroxidase (HRP) (200 µL) at room temperature for 2 hours. Following standard washing procedures, chromogenic substrate (200 µL) was added to the samples and incubated for 30 min at room temperature in the dark. Finally, 50 µL of stop solution was added in each well, and absorbance at 450 nm was measured using a microplate reader within 30 minutes of reaction termination. All OD values were normalized to control values. A standard curve was plotted with concentration of standards as the x-axis and the OD value as the y-axis. Concentrations of individual samples were extrapolated from the standard curve.

## Statistical analysis

The SPSS18.0 software was used for data processing. Measurement data was presented as mean ± standard deviation. The *t*-test was used for statistical analysis of measured data. Pearson's correlation method was used for correlation analysis. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of miR-31 expression in pediatric TB. $P < 0.05$ represents statistical significant difference.

# RESULTS

## Comparison of miR-31 expression in the TB group and the control group

Expression of miR-31 in the TB group was significantly lower than the control group (0.48 ± 0.15 *vs* 1.23 ± 0.36, $P < 0.05$) (Figure 2). However, miR-31 showed no significant differences with age, gender, and clinical classification of pediatric TB (all $P > 0.05$) (Table 1).

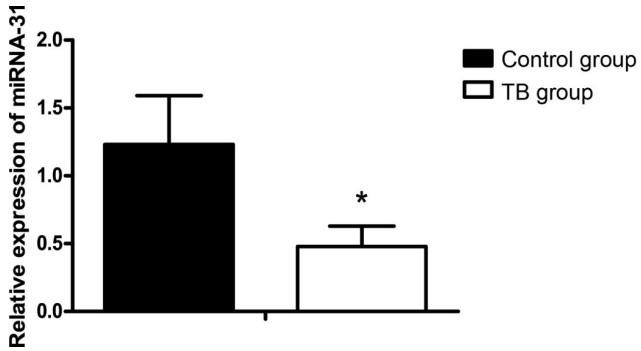


**Figure 2.** RT-PCR detection of miR-31 expression in the control TB group. *Compared with the control group, $P < 0.05$.

**Table 1.** Relationship between miR-31 expression and clinical features of TB patients.

| Parameter | Cases | miR-31 expression | P value |
|---|---|---|---|
| Age (years) | | | |
| <3 | 21 | 0.48 ± 0.16 | 0.621 |
| ≥3 | 44 | 0.46 ± 0.13 | |
| Gender | | | |
| Male | 38 | 0.49 ± 0.16 | 0.254 |
| Female | 27 | 0.45 ± 0.12 | |
| Clinical classification | | | |
| Primary pulmonary tuberculosis | 50 | 0.46 ± 0.16 | 0.155 |
| Hematogenous tuberculosis | 8 | 0.57 ± 0.18 | |
| Secondary pulmonary tuberculosis | 7 | 0.54 ± 0.22 | |

## Comparison of serum IL-6, TNF-α, NF-κB, and IFN-γ levels in the TB and control groups

Serum levels of IL-6, TNF-α, NF-κB, and IFN-γ were significantly higher in the TB group as compared with the control group (IL-6: 0.87 ± 0.04 *vs* 0.42 ± 0.03, t = 69.91, P < 0.001; TNF-α: 2.13 ± 0.82 *vs* 0.96 ± 0.25, t = 10.96, P < 0.001; NF-κB: 1.18 ± 0.15 *vs* 0.33 ± 0.08, t = 39.94, P < 0.001; IFN-γ: 116.21 ± 4.96 *vs* 90.78 ± 2.06, t = 37.94, P < 0.001) (Table 2).

**Table 2.** Comparison of serum IL-6, TNF-α, NF-κB, and IFN-γ levels between the control and TB groups.

| | Control group | TB group | *t* | P |
|---|---|---|---|---|
| IL-6 (pg/mL) | 0.42 ± 0.03 | 0.87 ± 0.04 | 69.91 | <0.001 |
| TNF-α (ng/mL) | 0.96 ± 0.25 | 2.13 ± 0.82 | 10.96 | <0.001 |
| NF-κB (ng/mL) | 0.33 ± 0.08 | 1.18 ± 0.15 | 39.94 | <0.001 |
| IFN-γ (pg/mL) | 90.78 ± 2.06 | 116.21 ± 4.96 | 37.94 | <0.001 |

## Correlation analysis

Significant negative correlation was found between miR-31 expression and serum levels of IL-6, TNF-α, NF-κB, and IFN -γ (IL-6: r = -0.834, P < 0.001; TNF-α: r = -0.941, P < 0.001; NF-κB: r = -0.926, P < 0.001; IFN-γ: r = -0.866, P < 0.001) (Figure 3A, B, C and D).

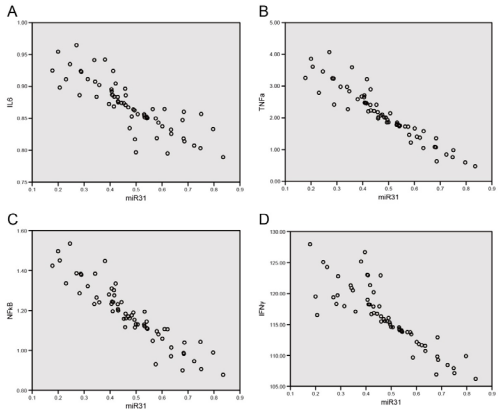


**Figure 3.** Negative correlation between miR-31 expression and (**A**) IL-6, (**B**) TNF-α, (**C**) NF-κB, and (**D**) IFN-γ in children with TB.

## Diagnostic value of miR-31 expression levels in pediatric TB

The area under the ROC curve (AUC) was 0.966 (95%CI = 0.934-0.998). MiR-31 expression at a cut-off value of 0.835 in pediatric TB patients was clearly distinguishable from the healthy controls. With this cutoff value, sensitivity and specificity of the miR-31 expression for pediatric TB were 98.5 and 86.7%, respectively, indicating its high diagnostic value (Figure 4).

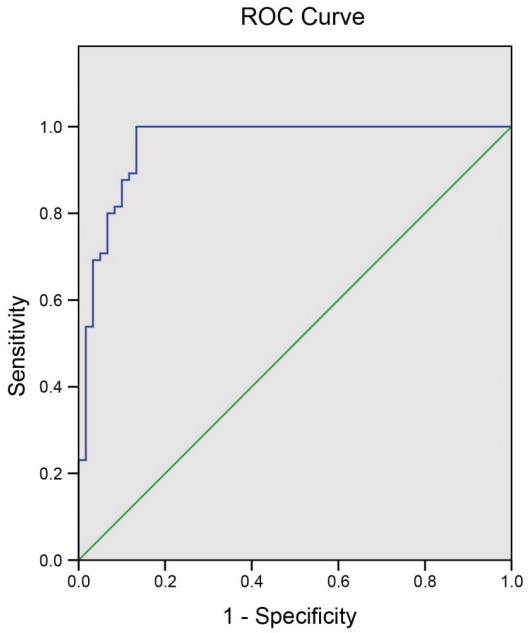


**Figure 4.** Receiver operating characteristic (ROC) plot of miR-31 for assessment of accuracy.

## DISCUSSION

Our study examined the role of miR-31 in the development of pediatric TB. MtB can cause TB, and the host cell-mediated immune responses play a fundamental role against MtB infection (Wu et al., 2012). Many studies support the view that miRs have important roles in viral replication and can control viral infections including retroviruses, herpes viruses and small DNA viruses (Ha, 2011). Moreover, miRNAs are essential in both adaptive and innate immunity by influencing immune cell differentiation and mediating their immunological functions (Spinelli et al., 2013). For example, miR-31 regulates the expression of multiple pro-metastatic genes, and inhibits cellular processes involved in tumor invasion and metastasis (Valastyan et al., 2009; Schmittgen, 2010). In addition, miR-31 inhibits noncanonical NF-kB pathways by targeting NF-kB inducing kinase, and the loss of miR-31 triggers oncogenic signaling (Yamagishi et al., 2012). Furthermore, miR-31 also participates in inflammatory responses, and it is found that TNF-α enhances miR-31 expression in endothelial cells to suppress E-selectin, a endothelial adhesion molecule that triggers inflammatory signaling (Valastyan and Weinberg, 2010). However, few studies have addressed the role of miR-31 in infectious diseases, especially pediatric TB.

Our result showed that expression of miR-31 in pediatric TB patients was significantly lower as compared to the control group, suggesting the miR-31 found in PBMC may have protective roles. Consistent with this observation, miR-31 expression at 0.835 was the clear cut-off value to distinguish pediatric TB patients from healthy controls. In terms of diagnostics, sensitivity and specificity of miR-31 expression for pediatric TB were 98.5 and 86.7%, respectively, suggesting high diagnostic potential for miR-31. Due to the immature nature of the immune system in neonates and children under the age of 3, diagnosis of TB based on immune responses is difficult. Children, unlike the adult patients, generally do not mount a measurable immune response to MtB. Thus, mi-31 offers a sensitive and specific alternative for TB detection in children. Bioinformatic analyses showed that 59 miRs were down-regulated and 33 miRs were up-regulated in the TB serum compared to normal control serum, suggesting potential roles for miRs in active TB infections (Fu et al., 2011). Qi et al. (2012) also found that levels of miR-361-5p, miR-889, and miR-576-3p distinguished TB patients from healthy controls with moderate sensitivity and specificity (AUC range, 0.711-0.848).

Another aim of our study focused on the mechanism of miR-31 during the development of pediatric TB. Serum levels of IL-6, TNF-α, NF-κB and IFN-γ were found to be significantly higher in the TB group compared with the control group. In addition, expression of miR-31 expression was negatively correlated with serum levels of the innate immunity cytokines IL-6, TNF-α, NF-κB, and IFN-γ. IL-6 is secreted by Toll-like receptor 2-expressing cells during early MtB infection, and is involved in anti-tuberculosis immunity (Feng et al., 2014). TNF-α increases the capacity of macrophages to phagocytose and stimulates macrophages apoptosis, which leads to increased presentation of mycobacterial antigens by dendritic cells (Cavalcanti et al., 2012). Infection of pulmonary epithelial cells with MtB may indirectly lead to NF-kB activation, resulting in IL-1 release and activation of the classical NF-kB pathway (Bonizzi and Karin, 2004). IFN-γ induces autophagy in mycobacteria-infected cells, which is associated with protection against MtB (Dutta et al., 2012).

Taken together, miR-31 expression in PBMC is down-regulated in children with TB, and could be a potential biomarker for TB diagnosis. Furthermore, miR-31 expression levels negatively correlate with serum levels of innate immune cytokines, suggesting that miR-31 may play a role in early immune response against MtB in pediatric TB patients.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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