Diagnostic value of microRNA-125b in peripheral blood mononuclear cells for pulmonary tuberculosis

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Abstract. Pulmonary tuberculosis (TB) is a chronic respiratory infectious disease. Certain microRNAs (miRNAs or miRs) are reported to be involved in regulating TB progression. The present study aimed to evaluate the diagnostic potential of miR-125b in pulmonary TB. The expression levels of miR-125b and Raf1 proto-oncogene serine/threonine protein kinase (RAF1) were analyzed via reverse transcription-quantitative (RT-q)PCR in patients with TB. The correlation between miR-125b and the clinical indicators was investigated, and a receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of miR-125b. The relationship between miR-125b and RAF1 was examined using the dual luciferase reporter gene assay. IL-6, TNF-α, NF-κB and IFN-γ levels were detected using ELISA kits, and then the correlation between miR-125b expression and the levels of IL-6, TNF-α, NF-κB, IFN-γ and RAF1 in peripheral blood mononuclear cells (PBMCs) was analyzed. Moreover, RAF1 mRNA and protein expression levels were detected via RT-qPCR and western blotting. The results demonstrated that miR-125b expression was decreased in patients with TB, while RAF1 expression was increased. Furthermore, miR-125b expression was associated with sputum acid-fast bacillus smear. The area under the ROC curve of miR-125b was 0.9413, and the sensitivity and specificity of miR-125b expression for TB were 90 and 92.5%, respectively. IL-6, TNF-α, NF-κB and IFN-γ levels were negatively correlated with miR-125b expression, and were inhibited by miR-125b in PBMCs. Moreover, miR-125b targeted RAF1 to negatively regulate its expression levels. RAF1 reversed the role of miR-125b in attenuating IL-6, TNF-α, NF-κB and IFN-γ levels in PBMCs. The present study demonstrated that the levels of IL-6, TNF-α, NF-κB and IFN-γ were negatively correlated with miR-125b expression in PBMCs. Thus, it was suggested that miR-125b served important roles in the occurrence and development of TB by decreasing the levels of IL-6, TNF-α, NF-κB and IFN-γ by inhibiting RAF1.

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Introduction

Pulmonary tuberculosis (TB) is a chronic respiratory infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis)*, and has become a key public health issue worldwide (1). The World Health Organization reported 10 million new TB cases and 1.6 million TB-related mortalities worldwide in 2017 (2). TB is the ninth leading cause of mortality globally, second only to HIV/AIDS as the leading cause of mortality for a single infectious disease (3). An accurate, rapid and easy method for diagnosis is the key to controlling TB. At present, the gold standard for diagnosing TB still relies on old bacteriological tests, which are laborious and time-consuming, with a positive rate of only 30% (4,5). Therefore, it is important to identify novel diagnostic biomarkers with higher sensitivity and specificity.

MicroRNAs (miRNAs/miRs) are a class of small endogenous non-coding RNAs that are 18-22 nucleotides in length, which regulate gene expression by binding to the 3'-untranslated regions (3'-UTRs) of the target gene (6). miRNAs have been widely studied as sensitive diagnostic markers in the occurrence and development of various diseases, including cancer (7) and TB (8). Previous studies have reported that the expression levels of numerous host miRNAs are altered in the peripheral blood mononuclear cells (PBMCs) (9), serum (10) and macrophages (11) of patients with TB. For example, elevated miR-423-5p expression has been revealed to serve an important role in TB by inhibiting autophagosome-lysosome fusion (12). Furthermore, Fu *et al* (13) have shown that miR-206

could regulate the secretion of inflammatory cytokines and the expression of MMP9 by targeting TIMP metallopeptidase inhibitor 3 in *M. tuberculosis* infected THP-1 macrophages. Wang *et al* (14) have also confirmed that miR-31 could be a potential diagnostic marker in patients with TB by inhibiting the secretion of inflammatory cytokines. However, the specific regulatory role of miR-125b in TB remains unknown.

The present study investigated the expression levels of miR-125b and RAF1 in PBMCs from patients with TB, as well as the regulatory mechanism between miR-125b and RAF1 in TB progression *in vitro*. The present findings may provide new theoretical foundation for investigating novel diagnostic biomarkers with higher sensitivity and specificity for TB.

Materials and methods

Study subjects. A total of 40 patients with TB (23 women and 17 men) were recruited for the study in the Shanxi Provincial Institute for Tuberculosis Control and Prevention between December 2017 and June 2018. Among the patients with TB, 18 cases were under the age of 18 years and 22 cases were ≥18 years of age. In addition, 19 cases had a positive sputum smear and 21 were negative. The TB diagnostic criteria referred to were those of the 'Clinical diagnostic criteria and treatment guide for TB' (15). A total of 40 healthy volunteers (23 female and 17 male; age <18 years, n=18; age ≥18 years, n=22) with a background of Bacillus Calmette-Guérin vaccination were recruited in the same period. None of patients with TB or the healthy volunteers had other viral infections, autoimmune diseases, respiratory diseases or diseases of the vital organs, such as the kidney, heart and liver. The project was approved by the Ethics Committee of the Shanxi Provincial Institute for Tuberculosis Control and Prevention and it was performed in accordance with the Declaration of Helsinki. All the recruited patients and volunteers provided signed informed consent. The parents or guardians of patients who were <18 years old provided this consent.

Mononuclear cell isolation. Peripheral venous blood (2 ml) from patients with TB and healthy volunteers was drawn and placed in a tube containing the anticoagulant EDTA. Then, PBMCs were isolated via Fycoll-Paque plus density gradient centrifugation (450 x g, at 20˚C for 20 min) according to the manufacturer's instructions. Following three washes with Hank's Balanced Salt Solution, PBMCs were collected, resuspended in PBS (1 ml) and stored at -80˚C until subsequent experimentation.

Cell transfection. The miR-125b mimics (forward, 5'-UCCCUGAGACCCUAACUUG-3' and reverse, 5'-ACAAGUUAGGGUCUCAGGGAUU-3'), miR-125b inhibitor (5'-UCACAAGUUAGGGUCUCAGGGA-3') and their corresponding negative control [miR-125b mimics NC (forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3') and miR-125b inhibitor NC (5'-CAGUACUUUUGUGUAGUACAA-3')] were purchased from Shanghai GenePharma Co., Ltd. RAF1 small interfering (si)RNA (si-RAF1-1 forward, 5'-CAUGGUAGUCACUAACAUA-3' and reverse, 5'-UAUGUUAGUGACUACCAUG-3'; si-RAF1-2 forward, 5'-GUCAAUAAAAUGCGGGUUU-3' and reverse, 5'-AUUAUCCUUUGGAUUCCCG-3'; si-RAF1-3 forward, 5'-GGGUAGCACCAUCUGAAA-3' and reverse, 5'-CAGUGCGUGUCCUGGAGU-3') and RAF1 siRNA NC (forward, 5'-UUCUCCGAACGUGUCACGU-3' and reverse, 5'-ACGUGACACGUUCGGAGAA-3') were supplied by Guangzhou RiboBio Co., Ltd. PBMCs were transfected with the different oligonucleotides (50 nM) using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. The transfected PBMCs were randomly divided into the following groups: Blank group (no-treatment group), miR-NC group (transfected with miR-125b mimics NC), miR-125b mimics group (transfected with miR-125b mimics), anti-miR-NC group (transfected with miR-125b inhibitor NC), miR-125b inhibitor group (transfected with miR-125b inhibitor), anti-miR-NC + si-NC group (transfected with miR-125b inhibitor NC and siRNA NC), anti-miR-NC + si-RAF1 group (transfected with miR-125b inhibitor NC and RAF1 siRNA), miR-125b inhibitor + si-NC group (transfected with miR-125b inhibitor and siRNA NC) and miR-125b inhibitor + si-RAF1 group (transfected with miR-125b inhibitor and RAF1 siRNA). All the cells were cultured at 37˚C in an incubator for 48 h.

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® reagent (Invitrogen, USA) was used to extract total RNA from the PBMCs. Then, total RNA was reverse-transcribed into cDNA at 42˚C for 45 min using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), and measured using a StepOne RealTime PCR (Thermo Fisher Scientific, Inc.) with SYBR green qPCR Master mix (Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 95˚C for 3 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 30 sec, and final extension at 72˚C for 1 min. The primers used for RT-qPCR analysis were as follows: miR-125b forward, 5'-GCCGTAAAGTGCTGACAGT-3' and reverse, 5'-GTGCAGGGTCCGAGGTAT-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-ACGCTTCACGAATTTGCGT-3'; RAF1 forward, 5'-CCTCCAGTCCCTCATCTGAA-3' and reverse, 5'-CTCAATCATCCTGCTGTCCA-3'; and GADPH forward, 5'-ATTGTCAGCAATGCATCCTG-3' and reverse, 5'-GTAGGCCATGAGGTCCACCA-3'. Expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (16). U6 was used as the internal control in the quantitative analysis of miR-125b, si-RAF1-1, si-RAF1-2, si-RAF1-3 and si-NC expression levels, and GADPH was used as the internal control in the quantitative analysis of RAF1 expression.

Western blot analysis. PBMCs were extracted using lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was measured using the BCA kit (Invitrogen; Thermo Fisher Scientific, Inc.). The total proteins (50 μg) were separated via SDS-PAGE on 10% polyacrylamide gels, and transferred to nitrocellulose membranes. Following blocking with 5% skimmed milk for 2 h at 25˚C, the membranes were incubated with the specific primary antibody, including RAF1 (1:1,000; cat. no. ab137435; Abcam) and GAPDH (1:1,000; cat. no. 100242-MM05; Sinopharm Chemical Reagent Co., Ltd.) at 4˚C overnight. Subsequently, the peroxidase-labeled secondary antibody (anti-rabbit IgG; 1:5,000; cat. no. 14708; Cell Signaling Technology, Inc.) was used for incubation for 1 h at 37˚C. The protein blots were visualized with an ECL

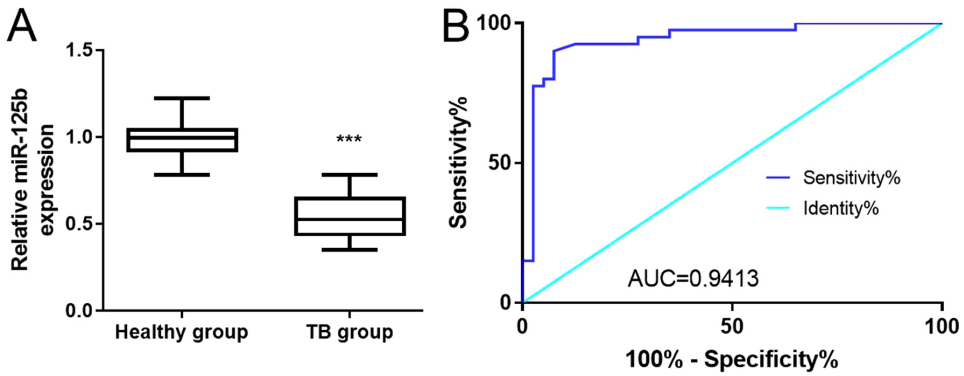

Figure 1. miR-125b is downregulated in peripheral blood mononuclear cells of patients with TB and has the potential as a biomarker for TB. (A) Expression of miR-125b was detected via reverse transcription-quantitative PCR. (B) Receiver operating characteristic curve was used to evaluate the diagnostic value of miR-125b in TB. Data are presented as mean ± SD of ≥3 independent repeats. ***P<0.001 vs. Healthy group. miR, microRNA; TB, pulmonary tuberculosis; AUC, area under the curve.

kit (Thermo Fisher Scientific, Inc.). Finally, the density of western blotting bands was analyzed using a Gel-Pro analyzer (version 4.0; Media Cybernetics, Inc.).

ELISA. The levels of TNF-α (cat. no. PDTA00D; R&D Systems), IL-6 (cat. no. PD6050; R&D Systems), NF-κB (cat. no. ab176647; Abcam) and IFN-γ (cat. no. ab174443; Abcam) were measured using ELISA kits (according to manufacturer's instructions. The absorbance of each well was measured at 450 nm using a microplate reader (Molecular Devices LLC).

Dual luciferase reporter gene assay. The targeted relationships between miR-125b and RAF1 was analysed using the TargetScan software (version 5.2; targetscan.org). The 3'-UTR fragment of RAF1 was cloned and ligated into Psi-CHECK2 reporter vector (Promega Corporation) to construct wild-type (WT) Psi-CHECK2-WT-RAF1-3'-UTR (RAF1-WT) and mutant (MUT) Psi-CHECK2-MUT-RAF1-3'-UTR (RAF1-MUT). Subsequently, miR-125b mimics or miR-125b mimics NC (80 ng) were co-transfected with the reporter plasmids into PBMCs ($2x10^5$ cells/well) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Based on the differences in the transfected sequences, the PBMCs were grouped as follows: MUT + miR-125b mimics group (transfected with RAF1-MUT and miR-125b mimics), MUT + NC group (transfected with RAF1-MUT and miR-125b mimics NC), WT + miR-125b mimics group (transfected with RAF1-WT and miR-125b mimics) and WT + NC group (transfected with RAF1-WT and miR-125b mimics NC). Following 48 h transfection at 37˚C, *Renilla* and firefly luciferase activities were detected using a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. The activity of firefly luciferase was normalized to the activity of *Renilla* luciferase.

Statistical analysis. All statistical analyses were performed using the SPSS 22.0 statistical software (IBM Corp.). Data are presented as the mean ± SD. The two-tailed t-test was used for comparison between two groups, while one-way ANOVA followed by followed by Tukey's post hoc test was used for comparison among multiple groups. Pearson's correlation analysis was used to determine the correlations between the expression levels of miR-125b and IL-6/TNF-α/NF-κB/IFN-γ in/RAF1 in patients with TB. The diagnostic analysis was performed via receiver operating characteristic (ROC) curve analysis with healthy controls as true negative cases and patients with TB as true positive cases. All experiments were repeated three times in this study. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-125b is downregulated in the PBMCs of patients with TB, and is a potential biomarker for TB. The expression of miR-125b in the PBMCs of patients with TB and healthy volunteers was analyzed using RT-qPCR (Fig. 1A). When compared with healthy volunteers, miR-125b expression was significantly decreased in the patients with TB (P<0.001). Subsequently, it was assessed whether miR-125b was a potential biomarker for TB via ROC curve analysis (Fig. 1B). The area under the curve (AUC) of miR-125b was 0.9413 (95% CI=88.55-99.7%). The sensitivity and specificity of miR-125b expression for TB were 90 and 92.5%, respectively, suggesting that it possessed a high diagnostic value. In addition, the correlation between the expression of miR-125b and the clinical indicators in TB patients was investigated (Table I). The results demonstrated that the expression of miR-125b was significantly decreased in sputum smear positive group compared with sputum smear negative group (P<0.01). However, other clinical factors such as age, sex and clinical classification had no significant association with the miR-125b expression (P>0.05). All these results suggested that miR-125b was downregulated in the PBMCs of patients with TB, and thus, may be a potential biomarker for TB.

Levels of IL-6, TNF-α, NF-κB and IFN-γ are negatively correlated with the expression of miR-125b in PBMCs. The results of ELISA demonstrated that the levels of IL-6, TNF-α, NF-κB and IFN-γ in the TB group were significantly increased compared with those in the healthy group (P<0.001; Fig. 2A-D). Very strong negative correlations were identified between miR-125b expression and the levels of IL-6 (r=-0.9780; P<0.001), TNF-α (r=-0.9823; P<0.001), NF-κB (r=-0.9853; P<0.001) and IFN-γ (r=-0.9771; P<0.001) in patients with TB (Fig. 2E-H).

Table I. Analysis of expression of miR-125b and clinical parameters in patients with TB.

Parameter	Cases	miR-125b expression	P-value
Age, years			
<18	18	0.558±0.139	0.9488
≥18	22	0.545±0.142	
Sex			
Male	17	0.580±0.141	0.7887
Female	23	0.527±0.135	
Sputum acid-fast bacillus smear			
Negative	21	0.657±0.066	0.0078[a]
Positive	19	0.428±0.045	
Clinical classification			
Primary pulmonary tuberculosis	22	0.510±0.110	0.7711
Hematogenous tuberculosis	7	0.656±0.100	
Secondary pulmonary tuberculosis	11	0.549±0.149	

The two-tailed t-test was used for comparison between two groups, while one-way ANOVA followed by followed by Tukey's post hoc test was used for comparison among multiple groups. [a]P<0.01 vs. positive bacillus smear group. miR, microRNA.

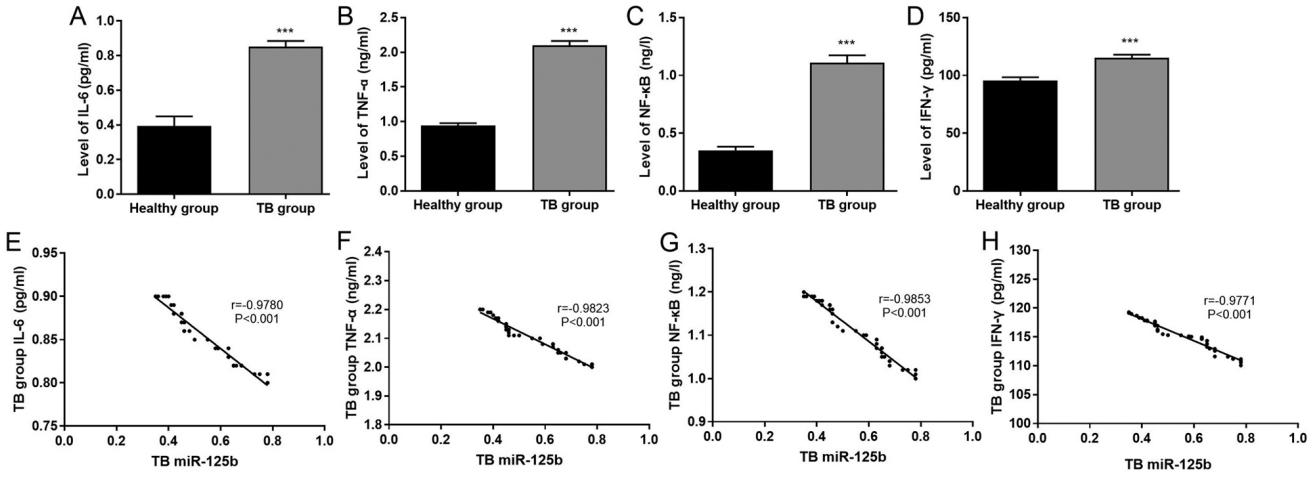

Figure 2. Correlation between miR-125b expression and the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs. Levels of (A) IL-6, (B) TNF-α, (C) NF-κB and (D) IFN-γ were measured using ELISA. Correlation between miR-125b expression and levels of (E) IL-6, (F) TNF-α, (G) NF-κB and (H) IFN-γ, respectively. Data are presented as mean ± SD of ≥3 independent repeats. ***P<0.001 vs. Healthy group. miR, microRNA; TB, pulmonary tuberculosis.

miR-125b decreases the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs. As presented in Fig. 3A, the expression of miR-125b was significantly increased in the miR-125b mimics group compared with the miR-NC group (P<0.001). On the contrary, miR-125b expression in miR-125b inhibitor group was significantly lower compared with that in the anti-miR-NC group (P<0.001), suggesting that the transfection method was successful. The results of ELISA demonstrated that the levels of IL-6, TNF-α, NF-κB and IFN-γ were significantly decreased in the miR-125b mimics group compared with the miR-NC group (P<0.01 or P<0.001). Moreover, IL-6, TNF-α, NF-κB and IFN-γ levels in the miR-125b inhibitor group were significantly increased compared with the anti-miR-NC group (P<0.01 or P<0.001) (Fig. 3B-E). These results indicated that miR-125b could decrease the levels of IL-6, TNF-α, NF-κB, and IFN-γ in the PBMCs.

miR-125b targets the inhibition of the expression of RAF1 in PBMCs. The results of both RT-qPCR and western blotting indicated that the expression of RAF1 in the TB group was significantly increased compared with the healthy group (P<0.001; Fig. 4A and B). Moreover, Pearson's correlation analysis demonstrated that the expression of miR-125b in the PBMCs was strongly negatively correlated with the expression of RAF1 (r=-0.8951; P<0.001; Fig. 4C). TargetScan predicted that the binding site of RAF1 to miR-125b was in the 3'-UTR region (Fig. 4D). According to the luciferase reporter assay, the luciferase activity in the WT + miR-125b mimics group was significantly lower compared with that in WT + NC group (P<0.001), while the difference in luciferase activity between MUT + NC and MUT + miR-125b mimics group was not statistically significant (Fig. 4E). Therefore, it was suggested that miR-125b directly regulated RAF1 expression.

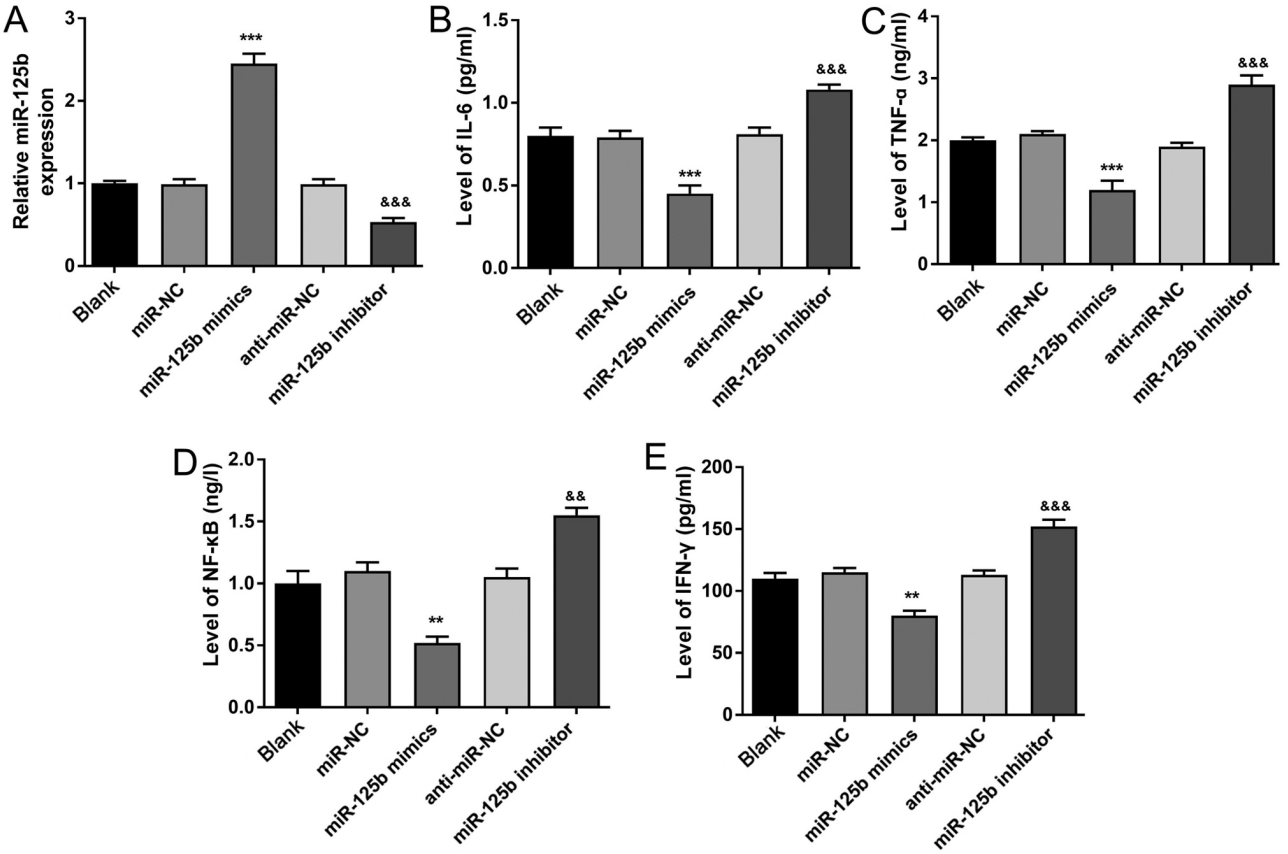

Figure 3. miR-125b decreases the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs. (A) Expression of miR-125b was detected via reverse transcription-quantitative PCR after transfection. Levels of (B) IL-6, (C) TNF-α, (D) NF-κB and (E) IFN-γ were measured using ELISA after transfection. Data are presented as mean ± SD of ≥3 independent repeats **P<0.01, ***P<0.001 vs. miR-NC group; $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. anti-miR-NC group. miR, microRNA; NC, negative control.

To further investigate whether miR-125b regulated the expression of RAF1, RT-qPCR and western blotting were performed (Fig. 4F and G). Both the mRNA and protein expression levels of RAF1 in the miR-125b mimics group were significantly lower compared with those in the miR-NC group (P<0.01). When compared with the anti-miR-NC group, RAF1 mRNA and protein expression levels were significantly increased in the miR-125b inhibitor group (P<0.001). All these results indicated that miR-125b could target the inhibition of the expression of RAF1 in PBMCs.

RAF1 reverses the roles of miR-125b in attenuating the levels of IL-6, TNF-α, NF-κB, and IFN-γ in PBMCs. The protein expression of RAF1 was detected via western blotting (Fig. 5A). Compared with the si-NC group, the protein expression of RAF1 was significantly decreased in the si-RAF1-2, si-RAF1-3 and, especially in the si-RAF1-1 group (P<0.001). Therefore, si-RAF1 could successfully interfere with RAF1 expression, and si-RAF1-1 was selected for subsequent experiments.

The ELISA results (Fig. 5B-E) demonstrated that, while the levels of IL-6, TNF-α, NF-κB and IFN-γ were significantly decreased in the anti-miR-NC + si-RAF1-1 group compared with those in the anti-miR-NC + si-NC group (P<0.01), their levels were significantly increased in miR-125b inhibitor + si-NC group (P<0.01). In addition, the levels of IL-6, TNF-α, NF-κB, and IFN-γ in the miR-125b inhibitor + si-RAF1-1 group were significantly higher compared with those in the anti-miR-NC + si-RAF1-1 group (P<0.01). The levels of IL-6, TNF-α, NF-κB and IFN-γ in the miR-125b inhibitor + si-RAF1-1 group were significantly lower compared with those in the miR-125b inhibitor + si-NC group (P<0.01), suggesting that RAF1 could reverse the roles of miR-125b in attenuating the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs.

Discussion

China has the second largest TB incidence rate in the world, with 4.99 million active TB cases (15). Currently, in the absence of a quick and effective method to detect TB, 14% of patients are discharged without complete treatment (17). Therefore, the identification of novel diagnostic biomarkers for TB is a priority. The present study demonstrated that miR-125b could serve an important role in the occurrence and development of TB by inhibiting RAF1.

Some miRNAs have been previously investigated as potential TB biomarkers (10,18). Peripheral venous blood is a commonly used sample in clinical practice, and there is low risk of infection in using the peripheral venous blood of patients with TB (19). Thus, miRNAs in the PBMCs could be used as effective biomarkers for the diagnosis of TB (14). In addition, the expression levels of miRNAs in the PBMCs have revealed differences between patients with TB and

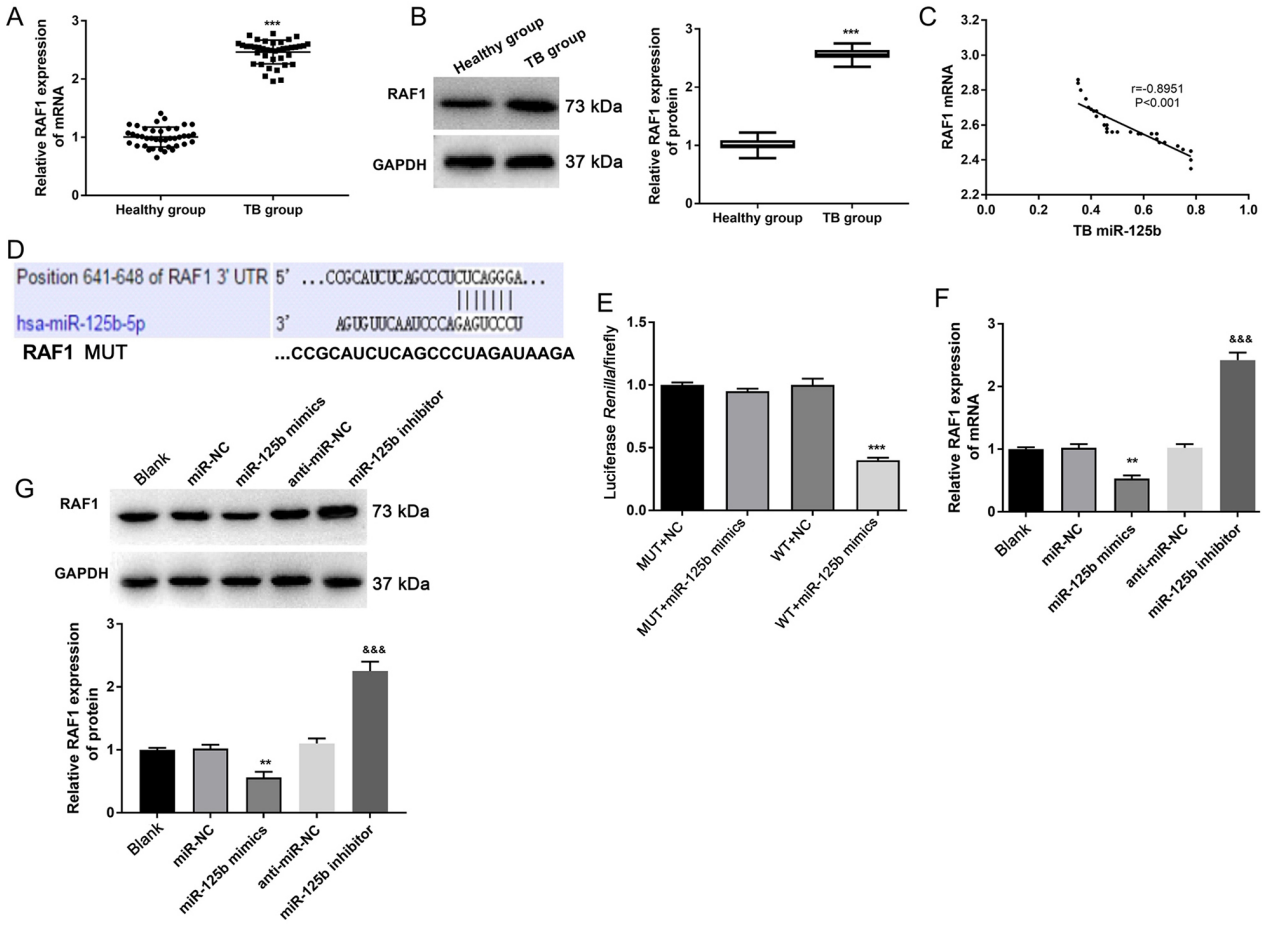

Figure 4. miR-125b targets the inhibition of the expression of RAF1 in PBMCs. (A) mRNA expression of RAF1 was detected via RT-qPCR. (B) Protein expression of RAF1 was measured via western blotting. ***P<0.001 vs. Healthy group. (C) Correlation between the expression levels of miR-125b and RAF1. (D) Binding target of RAF1 and miR-125b was predicted using TargetScan software. (E) Luciferase activity was measured using a dual luciferase reporter gene assay. ***P<0.001 vs. WT + NC group. (F) mRNA expression of RAF1 was detected via RT-qPCR after transfection. (G) Protein expression of RAF1 was measured using western blotting after transfection. Data are presented as mean ± SD of ≥3 independent repeats. **P<0.01 vs. miR-NC group; &&&P<0.001 vs. anti-miR-NC group. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; NC, negative control; WT, wild-type; MUT, mutant; UTR, untranslated region; TB, pulmonary tuberculosis; RAF1, Raf1 proto-oncogene serine/threonine protein kinase.

healthy participants, which supports the potential application of miRNAs in TB diagnosis (20). For instance, miR-21 and miR-223 are differentially expressed in patients with TB compared with healthy participants (21,22). miRNAs have been demonstrated to serve a critical role in TB. For example, miR-423-5p serves a vital role in the occurrence of TB by inhibiting the fusion of autophagosomes and lysosomes (12). In the present study, miR-125b expression was significantly decreased in patients with TB compared with healthy volunteers, which was consistent with previous research (21,22). The potential of a biomarker can be assessed by analyzing the AUC value for differentiating the test subjects as compared with the controls (23). Previous research has evaluated the AUC values of some miRNAs and found that miR-155 has an AUC value of 0.970 (23), while miR-29a-3p and miR-889 have AUC values of 0.814 and 0.765, respectively (3,10). In the present study, the ROC analysis identified that miR-125b has an AUC value of 0.9413. However, it is acknowledged that the sample size of the present study was a limitation and that the sampling was not been adjusted for confounding factors, such as age and sex. Therefore, it can only be concluded that miR-125b has the potential as a biomarker for TB.

NF-κB is the primarily intracellular signaling pathway involved in inflammatory responses (24). Inflammatory cytokines including TNF-α and IL-6 can be induced via the NF-κB signaling pathway (25). In addition, IL-6 is secreted during early *M. tuberculosis* infection, and is also involved in anti-tuberculosis immunity (26). Yang *et al* (27) reported that *M. tuberculosis* activates inflammatory mediators, including TNF-α. IFN-γ is a pivotal factor in the control of *M. tuberculosis* infection in murine models, and IFN-γ gene knockout mice are highly sensitive to *M. tuberculosis* (28). However, the function of IFN-γ remains unknown in humans. Previous studies have confirmed that IFN-γ is highly expressed in the pleural effusion of patients with TB (29). IFN-γ induces autophagy in mycobacteria-infected cells, which is associated with protection against *M. tuberculosis* (30). In the present study, the levels of IL-6, TNF-α, NF-κB and IFN-γ were significantly increased in patients with TB compared with healthy participants.

Accumulating evidence supports the notion that miRNAs serve an important role in the host response to mycobacterium-induced inflammation and immune responses, and that they could also affect the inflammatory

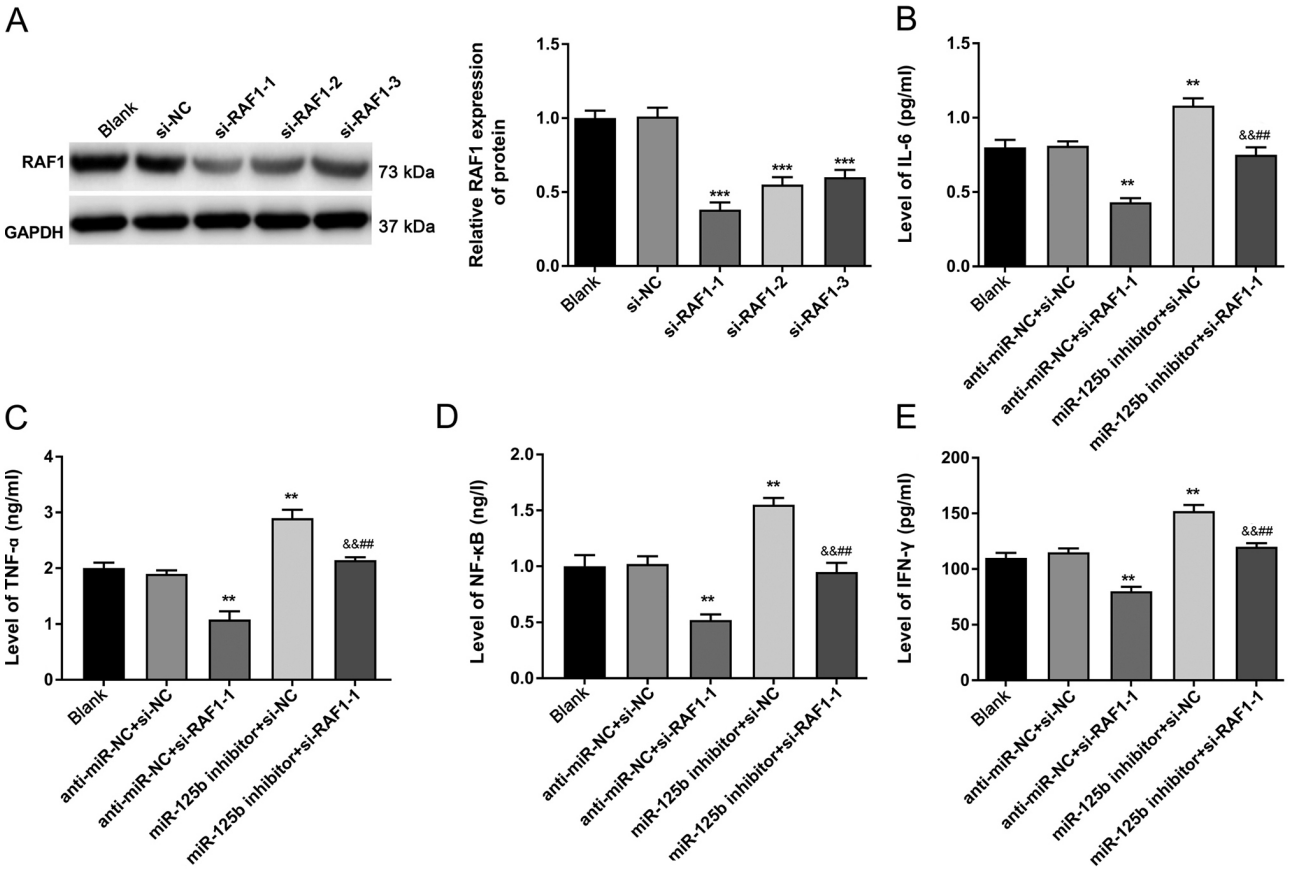

Figure 5. RAF1 reverses the roles of miR-125b on weakening the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs. (A) Protein expression of RAF1 was measured via western blotting after transfection. ***P<0.001 vs. si-NC group. Levels of (B) IL-6, (C) TNF-α, (D) NF-κB and (E) IFN-γ were measured using ELISA after transfection. Data are presented as mean ± SD, with experiments repeated three times. **P<0.01 vs. anti-miR-NC + si-NC group; &&P<0.01 vs. anti-miR-NC + si-RAF1-1 group; ##P<0.01 vs. miR-125b inhibitor + si-NC group. miR, microRNA; NC, negative control; siRNA, small interfering RNA; RAF1, Raf1 proto-oncogene serine/threonine protein kinase.

function in patients with TB (31,32). Previous studies have reported that miR-125b could protect the liver from hepatic ischemia and reperfusion injury by inhibiting the NF-κB signaling pathway (33). Xiao *et al* (34) have revealed that miR-125b could suppress the carcinogenesis of osteosarcoma cells via the MAPK/STAT3 signaling pathway. miR-206 has also been reported to regulate the secretion of the inflammatory cytokines, such as IL-6, IFN-γ, IL-1β and TNF-α, and MMP9 in TB (13). Furthermore, Wang *et al* (14) observed that the expression of miR-31 was negatively correlated with the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs. However, whether miR-125b regulates mycobacterium-induced inflammatory responses in TB is yet to be elucidated. The present results suggested that the levels of IL-6, TNF-α, NF-κB, and IFN-γ were negatively correlated with the expression of miR-125b in the PBMCs of patients with TB. Moreover, the high levels of IL-6, TNF-α, NF-κB, and IFN-γ in patients with TB were reversed by miR-125b. These aforementioned findings are consistent with those of previous studies (13,33).

RAF1 acts as a part of the RAF/MEK/ERK pathway, and regulates cell cycle, migration, apoptosis and proliferation (35). In addition, a previous study demonstrated that RAF1 serves an important role in regulating the transactivation property of NF-κB (36). Both the NF-κB and ERK pathways are crucial to the expression of the inflammatory mediators, including IL-6 and TNF-α (24,27). In the current study, the dual luciferase reporter gene assay demonstrated that RAF1 was the target gene of miR-125b. Furthermore, it was identified that miR-125b targeted the inhibition of the expression of RAF1 in PBMCs. It was also demonstrated that RAF1 reversed the roles of miR-125b in attenuating the levels of IL-6, TNF-α, NF-κB and IFN-γ in PBMCs.

There are, however, some limitations to the present study. Firstly, the sample size of the study was relatively small and limited. Next, the levels of miR-125b between the serum, plasma and the PBMCs, and the diagnostic values of miR-125b from serum and plasma for TB were not investigated. The experiments were conducted with PBMC from patients with TB, followed by detection of inflammatory factors after transfection of miR-125b mimics and miR-125b inhibitor *in vitro*. However, detection of the expression levels of the inflammatory factors of transfected PBMC with *M. tuberculosis* was not performed. These limitations will be addressed in additional investigations in the future.

In conclusion, the present study demonstrated that miR-125b was downregulated and RAF1 was upregulated in patients with TB. Moreover, the levels of IL-6, TNF-α, NF-κB and IFN-γ were negatively correlated with the expression of miR-125b in the PBMCs. The results indicated that miR-125b

served an important role in the occurrence and development of TB by decreasing the levels of IL-6, TNF-α, NF-κB and IFN-γ via the inhibition of RAF1. Collectively, the present study provides a new theoretical foundation for investigating novel diagnostic biomarkers with higher sensitivity and specificity for TB.

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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

XS and KL were involved in the conception, design and analysis of data, as well as performed the data analyses and wrote the manuscript. XW and TZ contributed to the conception of the study. XL and YZ contributed significantly to data analysis and manuscript preparation. All authors performed the experiments and read and approved the final manuscript.

Ethics approval and consent to participate

The current study was conducted after obtaining approval from the Shanxi Provincial Institute for Tuberculosis Control and Prevention's Ethical Committee (approval no. 202008). Written informed consent was provided by all subjects. The parents or guardians of patients <18 years of age provided this consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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