

EXPRESSION OF miRNA-29a-3p AND IFN- γ

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EXPRESSION OF miRNA-29a-3p AND IFN- γ AS BIOMARKERS IN ACTIVE LUNG TUBERCULOSIS AND LATENT LUNG TUBERCULOSIS

ABSTRACT

Background: Diagnosis and management of latent pulmonary tuberculosis is one of the challenges of eradicating pulmonary. One of the important aspects to control the spread of pulmonary tuberculosis is to diagnose it at an early stage. One of the biological markers being evaluated for TB is microRNA (miRNA). Mycobacterium tuberculosis causes epigenetic changes during infection. The upregulation of miRNA-29a-3p can suppress the immune response by inhibiting the expression of IFN- γ by T cells at post-transcription so that it can increase susceptibility to pulmonary tuberculosis. This study aims to determine the expression of miRNA-29a as a biomarker of infection in active pulmonary tuberculosis and latent pulmonary tuberculosis.

Methods: This study used a case-control design involving 50 people with active TB, 33 household contacts with a positive Interferon-Gamma Release Assay (IGRA) test and 30 healthy controls. An ELISA-based IGRA was conducted to determine latent pulmonary tuberculosis infection in household contacts. MiRNA-29a-3p expression was examined using quantitative real-time PCR (q-PCR). Data analysis was carried out using the Anova and receiver operating characteristic (ROC) curve.

Results: The results showed that there was a significant difference in miRNA-29a-3p expression ($p = 0.000$) between active TB, latent TB, and healthy controls. ROC curve analysis showed that miRNA-29a-3p had 86% sensitivity and 73% specificity with an Area Under the Curve (AUC) of 0.763 (95% Confidence Interval 0.668 - 0.858). While the miRNA-29a-3p ROC curve in Latent Tuberculosis has a sensitivity of 84.8% and a specificity of 70% with an AUC of 0.808 (95% Confidence Interval 0.698 - 0.919). miRNA-29a-3p had a significant correlation with active pulmonary tuberculosis ($p = <0.0001$) and latent pulmonary tuberculosis ($p = <0.0001$). Based on the correlation test between miRNA-29a-3p expression and IFN- γ levels in active pulmonary TB ($R = 0.005$; $p = 0.62$), latent TB ($R = 0.010$; $p = 0.38$) and healthy controls ($R = 0.060$; $p = 0.19$).

Conclusion: There was an increase in miRNA-29a-3p expression in Active pulmonary TB and latent pulmonary TB. miRNA-29a-3p potential biomarker in latent pulmonary tuberculosis and active pulmonary tuberculosis. There is no significant correlation between IFN- γ levels and miR-29a-3p expression.

Keywords: *miRNA-29a-3p*, IFN- γ , Active pulmonary tuberculosis, Latent pulmonary tuberculosis.

Introduction

1 Diagnosis of TB is still a global problem with misdiagnoses that can increase morbidity and mortality. Therefore, infection biomarkers are needed to diagnose active pulmonary tuberculosis and latent tuberculosis. One of the biological markers being evaluated for TB is microRNA (miRNA). In several circumstances, including those involving infectious diseases, diabetes, heart disease, cancer, psoriasis, and pregnancy, miRNA has been offered as a new bio-diagnostic. Mycobacterium TB is one of many microbes that can alter epigenetic patterns when it infects a host. The epigenetic process is a change in the expression of an inherited gene but there is no change in the DNA sequence of the related gene. Epigenetic processes include DNA methylation, histone modification and microRNA (miRNA) [1,2].

As a biomarker for disease diagnosis, treatment outcome, and prognosis, miRNA affords the opportunity. Additionally demonstrating that miRNAs are related to a number of illnesses, such as immunological, cardiac, infectious diseases, and cancer [3]. Specific miRNAs from the organ that has been damaged may be released into the blood when illness occurs. Blood miRNA levels in several disorders are dramatically different from those in healthy people. MiRNAs have been investigated and used as molecular diagnostic markers for cancer, diabetes, psychiatric disorders, heart disease, and various infectious diseases up to this point [4,5].

In several previous studies, analyzes using microarrays have also been carried out to identify miRNAs, both in macrophages, PBMCs, serum, blood, and sputum of TB patients as candidate biomarkers that have the potential as TB biomarkers [2–9]. In a previous study related to circulating miRNA in patients with active pulmonary TB. The results showed that there were 6 59 miRNAs that were upregulated and 33 miRNAs that were downregulated compared to healthy controls [10]. One of them that is upregulated is miRNA-29a-3p, this happens because miRNA-29a-3p suppresses the immune response by inhibiting INF- γ expression by T cells in post-transcription so that it can increase susceptibility to TB [11–14].

2 In this study, we identified miRNA-29a-3p for the early diagnosis of both active and latent tuberculosis, and could prevent the spread of pulmonary tuberculosis by detecting it in the early stage [4].

Materials and methods

Study Design and Subjects

This research is an observational study using a case-control research design. The study population was all patients diagnosed with pulmonary TB who came for an examination at the Makassar Community Lung Health Center (BBKPM), which is a referral center for TB cases

from primary health services in the city of Makassar, as well as people who have household contact with these patients.

Sample Collection

The research subjects came from all pulmonary tuberculosis patients who came for examination to BBKPM Makassar and people who were household contacts, who met the selection criteria were included in the study until the required number of samples was met. The samples needed are sputum samples and blood samples for patients with pulmonary tuberculosis, and blood samples for household contacts.

This research was carried out in the Hasanuddin University Medical Research Center (HUM-RC) laboratory, Hasanuddin University Hospital. The Center for Community Lung Health conducted a sampling of patients with active and latent pulmonary tuberculosis. The Hasanuddin University's Health Research Ethics Committee, Makassar, Indonesia, issued the permission under the following number: 243/UN4.6.4.5.31/PP36/2021. The number of samples in this study was 113 samples consisting of 50 samples of patients with positive AFB smear results and a positive MGIT confirmation test were designated as active pulmonary tuberculosis group. Meanwhile, there were 63 samples of household contacts with an ELISA-based Interferon-Gamma Release Assay (IGRA) with positive results as many as 33 people were designated as the latent TB group and 30 people with negative Interferon-Gamma Release Assay (IGRA) results were designated as healthy groups.

Sample Processing and Assessments

Sputum Collection

Research subjects were interviewed with standardized questionnaires. The study participants' sputum was collected for Ziehl-Neelsen staining and confirmation using a culture on liquid medium and a Mycobacteria Growth Indicator Tube (MGIT) (BD BACTEC MGIT 960, BD, UK).

Blood Collection

A total of 5-10 ml of venous blood samples from patients with active pulmonary TB were put into a blood tube and 5-10 ml of household contact venous blood was put into a QuantiFERON-TB Gold plus ELISA (QFT-Plus) tube (Qiagen GmbH, Qiagen Strasse 1, 40724 Hilden, Germany) and then centrifuged at 3000 rpm for total RNA extraction.

Total RNA Extractions

RNA was extracted and purified from blood using the QIAamp RNA Blood mini kit (Qiagen, USA, Cat No. 52304). A total of 200 l of blood samples were lysed and then RNA binding was performed. The washing process was then carried out with wash buffer. Finally, RNA elution was carried out with RNase free water to increase the RNA concentration.

cDNA Synthesis

The amplification of cDNA from the RNA extraction was carried out by the Reverse Transcription PCR method. This process is carried out using the iScript™ cDNA Synthesis Kit Master No. Catalog 178890 (Bio-Rad, California, USA). The cDNA results were quantified using real time-PCR (qPCR).

Quantification of miRNA-29a-3p with *Real-time PCR* (qPCR)

To quantify miRNA-29a-3p, a real-time PCR (q-PCR) process was performed using specific primers. The principle of this examination is that the specific sequence of miRNA-29a-3p contained in cDNA will experience amplification in the fragment that is limited by a specific primer pair. Amplification was carried out with a commercial kit (miRCURY LNA miRNA PCR assay GeneGlobe ID: YP00204698 Qiagen-Cat No: 339306 MIMAT0000086). The specific sequence used for the miRNA-29a primer was 5'UAGCACCAUCUGAAAUCGGUUA. The amplification results are detected in real time and reported as a cycle threshold (CT), which is the number of amplification cycles when the detected amplicon reaches the detection threshold (threshold).

Statistical Analysis

Results were analyzed using SPSS Statistics version 23.0 (IBM Co., Armonk, NY, USA). miRNA-29a-3p expression in the groups of individuals with active pulmonary TB, latent pulmonary TB, and healthy individuals was compared in an analysis of variance (ANOVA). All analyses required a significant P value of less than or equal to 0.05 [9].

Results

Data Characteristics

The characteristics of the sample data in this study included age, gender, relationship with active TB patients, and smoking history. Based on table 1 of 50 samples of active pulmonary TB patients, 34 of them were male and 16 female patients, while in household contacts with

positive IGRA results or latent pulmonary TB group, 9 of them were male and 24 female. Meanwhile, in the household contact group with negative IGRA results or in the healthy group, 7 were men and 23 were women. Based on IGRA results, there was a strong association between gender and the incidence of TB, as well as the incidence of latent and healthy TB ($p = 0.000$). Based on age, all samples were homogeneous ($p = 0.562$).

Based on the relationship of household contacts with active TB patients, it was found that there were significantly more ($p = 0.266$) household contacts who were partners (husband or wife) which was 25.4% compared to other household contacts such as children, parents, and other family relationships (such as relatives, cousins, in-laws) or other relationships such as friends/friends or neighbours. While the sample is homogeneous based on smoking history ($p = 0.000$).

Table 1. Characteristics of the study participants in the groups with Active TB, Latent TB, and Healthy TB.

Characteristics	Active TB n= 50	Groups latent TB n= 33	Healthy n= 30	P- Value
Sex				
• Male	34 (30.1%)	9 (8.0%)	7 (6.2%)	0.000
• Female	16 (14.2%)	24 (21.2%)	23 (20.4%)	
Age	41.04±12.32	39.03 ± 12.66	37.83±15.74	0.562
Relationship				
• Parents		6 (9.5%)	3 (4.8%)	0.266
• Children		7 (11.1%)	7 (11.1%)	
• Spouse (Husband/Wife)		16 (25.4%)	12 (19.0%)	
• Sibling		3 (4.8%)	2 (3.2%)	
• Others		1 (1.6%)	6 (9.5%)	
Smoking History				
• Smoking	33 (29.2%)	9 (8.0%)	6 (5.3%)	0.000
• Do Not Smoke	17 (15.0%)	24 (21.2%)	24 (21.2%)	

miRNA-29a-3p in Active, Latent and Healthy TB Patients

Based on the findings of the miRNA-29a-3p analysis in the group of people with active TB, latent TB, and healthy controls using real-time PCR (qPCR) showed that the average expression of miRNA-29a-3p was 21.32 in latent TB and 8.69 in active TB (Fig.1). Statistical analysis using ANOVA test showed that miRNA-29a-3p was significantly increased in latent TB compared to active TB and healthy controls ($p = 0.000$) (Table 2).

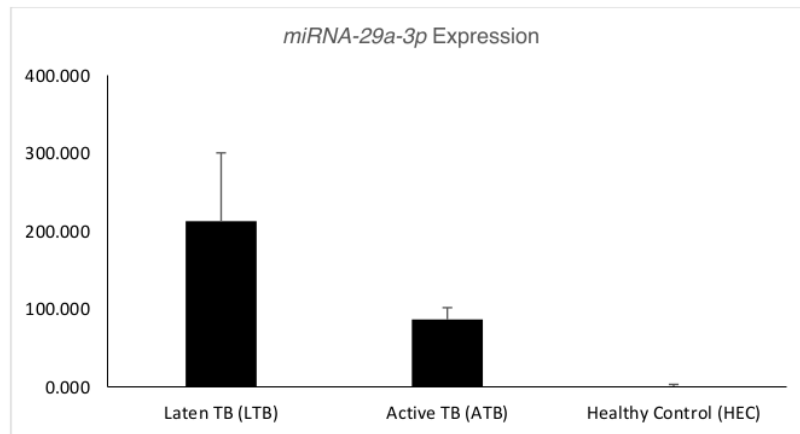


Fig.1 Expression of miRNA-29a-3p between Active TB, Latent TB and Healthy Control groups. Differences in miRNA-29a-3p expression between the three groups were analyzed using ANOVA assay (p = 0.000)

Table 2. MiRNA-29a Expression Examination Results in Active, Latent and Healthy TB Patients

Biological Groups Sample	N	Mean	SD	SE	CI 95%	P-Value
Active TB	50	8.69	15.18	2.14	4.37 13.00	0.000
Latent TB	33	21.32	87.62	15.25	-9.74 52.39	
Healthy Control	30	0.83	2.73	0.46	-0.18 1.85	

MiRNA-29a-3p Expression Correlation with *IFN-γ* in Active Pulmonary TB Patients and Latent TB

The ELISA method was used in this investigation to assess the levels of IFN- γ T cells in patients with active pulmonary TB, latent TB, and healthy controls. When the findings were further examined using the ANOVA test, it was discovered that the IFN- γ levels in the three groups varied significantly (p=0.000) (Table 3). The IFN- γ was present in active pulmonary TB at an average level of 73.92, latent TB was 33.75 and healthy controls was 0.42, indicating that IFN- γ levels in active pulmonary TB were higher than latent and healthy TB.

Table 3. *IFN- γ* Examination Results in Active TB Patients, Latent TB and Healthy

Biological Groups Sample	N	Mean	SD	SE	CI 95%		P-Value
Active TB	50	73.92	48.77	6.89	60.05	87.78	0.000
Latent TB	33	33.75	39.16	6.18	19.86	47.64	
Healthy Control	30	0.42	0.21	0.03	0.34	0.50	

The receiver operating characteristic (ROC) Curve Analysis

Based on the ROC curve (Fig.2) which uses a cut-off value of 0.01, It demonstrates the potential of miRNA-29a-3p as a sensitive biomarker of active pulmonary tuberculosis infection, with a sensitivity of 86% and specificity of 73% and an Area Under the Curve (AUC) of 0.76 (95% Confidence Interval 0.668 - 0.858). miRNA-29a-3p had a significant correlation with active pulmonary tuberculosis ($p = <0.0001$) (Table 4).

MiRNA-29a-3p is a sensitive possible biomarker of latent tuberculosis infection, according to the ROC curve (Fig. 2), which utilizes a cut-off value of 0.01. It has an AUC of 0.808 and has a sensitivity of 84.8% and a specificity of 70% (95% Confidence Intervals 0.698 - 0.919). miRNA-29a-3p had a significant correlation with latent tuberculosis ($p = <0.0001$) (Table 4).

Table 4. Potential of miRNA-29a-3p as Biomarker of Active TB and Latent TB

Target	Biological Group Sample	P value	Sensitivity	Specificity
<i>miRNA-29a-3p</i>	Active TB	<0,0001	86 %	73 %
	Latent TB	<0,0001	84.8 %	70%

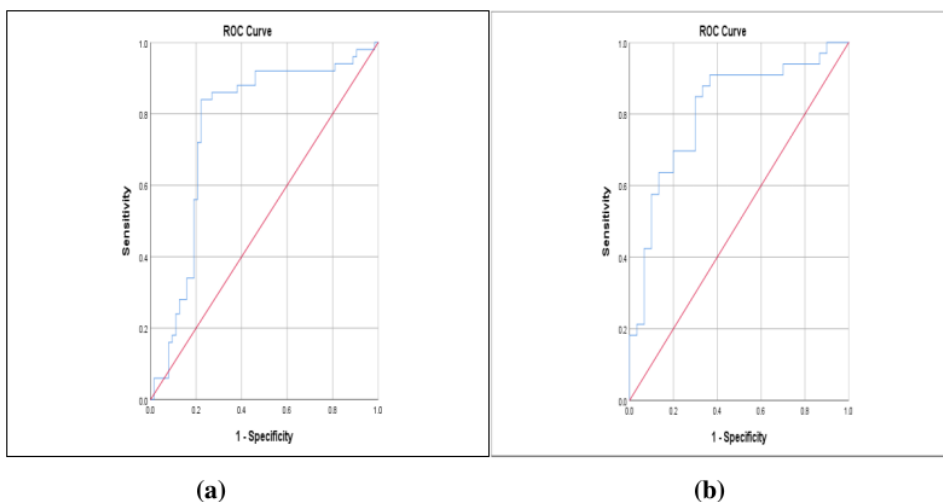


Fig.2. miRNA-29a-3p as a potential biomarker for active TB and latent TB
a) Potential of miRNA-29a as a biomarker of Active TB infection (p=. <0.0001);
b) Potential of miRNA-29a as a biomarker of latent TB infection (p=. <0.0001)

Discussion

Misdiagnoses that might raise morbidity and mortality rates are a persistent concern in the diagnosis of TB. In nations where the disease is endemic, national tuberculosis programs still rely on direct smear microscopy, solid cultures, radiography, skin testing, and the Tuberculin Skin test (TST) [9]. Sputum production for a TB confirmatory test is not permitted in infants under the age of six. TST is not specific for the identification of Mycobacterium tuberculosis. The gold standard for diagnosing tuberculosis, Mycobacterium tuberculosis culture, takes a long period and is not ideal for extrapulmonary TB. Due to the difficulty of sputum sampling, the identification of smear-negative and extra-pulmonary tuberculosis continues to provide a significant clinical challenge in pediatric TB. The newly developed FERON-TB test can aid in cases of TB but has a limitation that prevents it from making the distinction between latent and active TB and cannot be used to manage TB patients who are on anti-tuberculosis medications. Therefore, a more effective diagnostic technique is required, one that uses alternative specimens including blood, feces, and urine that may be obtained from people of all ages. MicroRNA is one of the biological indicators being examined for TB (miRNA). A novel bio-diagnostic called miRNA has been developed, and it has been widely linked to conditions like diabetes, heart disease, cancer, psoriasis, pregnancy, and infectious disorders. Mycobacterium TB is one of many microbes that can alter epigenetic patterns when

it infects a host. The epigenetic process is a change in the expression of an inherited gene but there is no change in the DNA sequence of the related gene. Epigenetic processes include DNA methylation, histone modification and microRNA (miRNA) [1,15].

The function of miRNA, a type of single-stranded RNA molecules with a length of 18–25 nucleotides, is to limit the function of its target genes at the post-transcriptional stage of gene expression. miRNA is a ribonucleic acid that does not code for a protein with a final transcript that interacts with the target gene encoding the messenger RNA (mRNA). To regulate the translation of mRNA, miRNA works in conjunction with other regulatory components including transcription factors. The majority of miRNAs are stored in areas of the genome that were previously thought to be non-coding regions. An estimated 2% to 5% of human genes are miRNA genes, which are dispersed across the genome. In many cases, polycistronic transcripts encode miRNAs. It is estimated that more than one-third of human genes are regulated by miRNAs because one miRNA might have several target mRNAs [16,17].

Based on the results of this study, 33 household contacts with an ELISA-based Interferon-Gamma Release Assay (IGRA) with positive results in the latent TB group and 30 people with negative Interferon-Gamma Release Assay (IGRA) results were designated as healthy groups.

According to the sample characteristic data, there were three groups of patients with active pulmonary TB: positive IGRA household contacts, and negative IGRA household contacts ($p = 0.000$). This result is similar with WHO data showing that more males than women are diagnosed with smear-positive TB [18,19].

Inactive and active tuberculosis, as well as healthy controls, have different gene expression profiles in macrophages and NK cells, according to several investigations. MiRNAs control changes in cellular make-up and related gene expression in tuberculosis patients. It has been discovered that a number of miRNAs control T cell differentiation and operation. Additionally, miRNAs have been demonstrated to have a significant role in controlling the activity of natural killer (NK) cells, macrophages, innate immune cells, and DCs [20].

The primary activities of dendritic cells, macrophages, and NK cells (NKC) are significantly regulated by miRNAs. Several studies have demonstrated alterations in the gene expression on macrophages and NK cells in cases of active and latent tuberculosis as well as in healthy people. miRNA regulates gene expression changes, miRNA regulates T cell differentiation and function [21].

MiRNA-29a-3p is one of the miRNAs that has been linked to *M. tuberculosis* infection. As a result of Mycobacterium infection in host cells, miRNA-29a-3p is overexpressed. miRNA-29a-3p suppresses the immune response against *M. tuberculosis* by decreasing IFN-

γ. Along with targeting the 3' UTR of IFN-mRNA, miRNA-29a-3p promotes IFN-mRNA and joins it with the protein Argonaute 2 (Ago2) to create an RNA silencing complex, which then reduces post-transcriptional IFN-expression.

In addition, several studies have shown that *miRNA-29a-3p* also targets the myeloid cell leukemia-1 (Mcl-1), anti-apoptotic proteins Bcl-2, GTP binding protein Cdc42 and p85 kinase, thus demonstrating the role of *miRNA-29a-3p* in regulating the apoptotic pathway. Involved in an anti-tuberculosis response, overexpressed miRNA-29a-3p in tuberculosis infection prevents macrophage phagocytosis by inhibiting IFN-γ and increasing cell apoptosis [10].

Using real-time PCR, miRNA-29a-3p was studied in this study's active TB, latent TB, and healthy controls (qPCR). The results showed that the mean expression of miRNA-29a-3p was 21.32 in latent TB and 8.69 in active TB and 0.83 healthy individuals (Fig.1). When compared to active TB and healthy individuals, miRNA-29a-3p levels in latent TB were considerably higher ($p = 0.000$), according to statistical analysis using the ANOVA test. This result is consistent with the findings of Ndzi, E. N. et al study [9], which demonstrated that hsa-miR-155-5p, hsa-miR-361-5p, and hsa-miR-29a-3p were significantly upregulated in active TB patients compared to healthy controls, and that hsa-mir-29a-3p and hsa-mir-361-5p were also significantly increased (all $p < 0.05$). miR-29a-3p showed good performance (81.37%) in differentiating between active TB patients with healthy individuals and a good diagnostic role (84.35%) in differentiating active TB from latent TB. miR-29a-3p is a valuable marker of infection, particularly for establishing the diagnosis of tuberculosis (TB), because it can distinguish between active TB, latent TB, and healthy controls in the blood. Additionally, blood (plasma) testing for miR-29a-3p is preferable to sputum testing since blood is simpler to collect and can be used for pediatric and extrapulmonary TB diagnosis [9,11].

Additionally, the findings of this study are consistent with those of Fu et al. (2011) [10], who discovered that active TB patients had 59 miRNAs that were elevated and healthy controls had 33 miRNAs that were downregulated. One of them that is upregulated is miRNA-29a, besides miRNA-29a can increase susceptibility to TB by inhibiting INF-γ expression by T cells.

Similarly, according to the findings of Sharbati et al [22]; Wu et al, [23]; Draz et al, [24]; Zhou et al, [3]; Lu et al, [4], blood miRNA-29a-3p was considerably overexpressed in patients with active and latent TB as compared to healthy controls. Thus, miRNA-29a-3p has potential as a candidate biomarker for the diagnosis of pulmonary tuberculosis.

MiRNA-29a-3p has the potential to act as a biomarker of active pulmonary tuberculosis infection, according to ROC curve analysis, which used a cut-off value of 0.01 and revealed that it has a sensitivity of 86%, a specificity of 73%, and an AUC of 0.763 (95% Confidence Interval 0.668 - 0.858). miRNA-29a-3p had a significant correlation with active pulmonary tuberculosis ($p < 0.0001$) (Fig.2). The AUC for MiRNA-29a-3p latent tuberculosis is 0.808 and it has a sensitivity of 84.8 percent and a specificity of 70 percent (95 percent Confidence Interval 0.698 - 0.919). miRNA-29a-3p had a significant correlation with latent tuberculosis ($p = < 0.0001$) (Fig.2).

IFN- γ levels in the groups with active pulmonary TB, latent TB, and healthy controls were assessed in this investigation. According to the ANOVA test, there were significant differences among the three groups ($p = 0.000$) (Table 3). However, there was no association between IFN- γ levels in the three groups and miRNA-29a-3p expression, where in active pulmonary TB ($R = 0.005$; $p = 0.62$), latent TB ($R = 0.010$; $p = 0.38$) and healthy control ($R = 0.060$; $p = 0.19$). This result is similar to a study by Afum-Adjei Awuah A, et al. [12] that looked at the dynamics of IFN- γ and miR-29a expression of CD4+ T cells from patients with active tuberculosis (TB) ($n = 32$) and household contacts infected with *M. tuberculosis* ($n = 19$) in Ghana. There was no interdependence between miR-29a and IFN- γ , as they discovered no statistically significant correlation for either PPD-induced TB patients ($R = 0.20$; $P = 0.157$); latent TB patients ($R = 0.37$; $P = 0.118$); or SEB-induced TB patients ($R = 0.20$; $P = 0.167$); or latent TB ($R = 0.22$; $P = 0.367$).

These findings are in accordance with a prior study by Kleinstauber et al. [13], that found no association between miR-29a-3p expression and IFN- γ levels in young patients with pulmonary TB. Additionally, this study was carried out in a nation with a high TB incidence. Although our findings do not rule out a role for miR-29a-3p in the regulation of IFN- γ , it is doubtful that miR-29a-3p has a significant inhibitory effect on CD4+ T cells during active pulmonary TB and latent TB infection. This could be due to other factors that contribute to the regulation of IFN- γ besides miR-29a-3p.

Although it is not the sole effector mechanism that contributes to the CD4 T cell-mediated immune system, IFN- γ production by CD4 T cells has a significant impact on *M. tuberculosis* immunity. The IFN- γ production was positively associated with miR-29a-3p expression during *M. tuberculosis* infection. When compared to uninfected controls, CD4+ or CD8+ T cells from *M. bovis* BCG-infected mice showed decreased miR-29a-3p levels and increased IFN- γ mRNA levels. Studies showed that miR-29a-3p directly targets IFN- γ mRNA, which in turn reduces

immune responses against intracellular infections. However, the evidence on the connection between IFN- γ and miR-29a-3p in humans are in stark contrast. In CD4+ T cells from children with active TB compared to latent TB, lower expression of miR-142-3p, miR-29a-3p, miR-26a-5p, and miR-21-5p was observed. In contrast, there was no association between IFN- γ expression and miR-29a-3p in children with TB compared to healthy controls in T cells expressing M. tuberculosis-specific IFN- γ . Additionally, miR-29a-3p downregulation in T cells by antagomir had no impact on I IFN- γ expression upon in vitro activation [25].

As for the limitations of this study, firstly, the number of latent TB patients included in this study was small in assessing the potential of miRNA-29a-3p as a biomarker, so further research with a larger sample size is needed. However, our results are consistent with previous studies regarding the use of miRNA-29a-3p as a biomarker for the diagnosis of active TB and latent TB. Second, there was no effect of miRNA-29a-3p expression on IFN- γ expression, so further research is needed to involve TB patients undergoing antituberculosis therapy to clarify the role of miRNA-29a-3p in inhibiting IFN- γ in CD4 T cells during TB infection.

Conclusion

There was an increase in miRNA-29a-3p expression in Active pulmonary TB and latent pulmonary TB. The miRNA-29a-3p expression was higher in latent TB compared to active TB and healthy controls. The miRNA-29a-3p is a potential biomarker to diagnose latent pulmonary TB and active pulmonary TB infection. There was no significant correlation between IFN- γ levels and miR-29a-3p expression.

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