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ORIGINAL ARTICLE

Evaluation of miR-let-7f, miR-125a, and miR-125b expression levels in sputum and serum samples of Iranians and Afghans with pulmonary tuberculosis

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ABSTRACT

Background and Objectives: The role of microRNAs (miRNAs) in tuberculosis infection is well established. As microRNAs are able to change expression profiles according to different conditions, they can be useful biomarkers. Iranians and Afghans with tuberculosis were studied for three immune-related miRNAs (miR-let-7f, miR-125a, and miR-125b).

Materials and Methods: A total of 60 Iranian and Afghan patients with active pulmonary TB were enrolled in the Pulmonary Department of the Pasteur Institute of Iran. Serum and sputum samples were collected simultaneously from all participants. A Real-time PCR was conducted to detect differentially expressed miRNAs.

Results: Iranian ($P<0.0001$) and Afghan ($P<0.0001$) serum samples and Afghan ($P<0.0001$) sputum samples overexpressed miR-125a, whereas Iranian sputum samples showed downregulation ($P=0.0039$). In both Iranian ($P<0.0001$; $P=0.0007$) and Afghan ($P<0.0001$; $P<0.0001$) serum and sputum samples, miR-125b was overexpressed. Furthermore, miR-let-7f downregulation was observed in serum and sputum samples ($P<0.0001$), whereas Iranian sputum samples had no statistically significant differences ($P=0.348$).

Conclusion: Overexpression of miR-125a and miR-125b has been detected in Iranian and Afghan samples. In both races, miR-let-7f downregulation has been confirmed. Identification of miRNA profiles under different conditions opens the door to evaluating potential new biomarkers for diagnosis, disease monitoring, and therapeutic markers in TB infection.

Keywords: *Mycobacterium tuberculosis*; MicroRNA; Sputum; Serum

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INTRODUCTION

Tuberculosis (TB) infection remains a relevant global health issue affecting millions of people. In the history of medicine, TB has been one of the greatest epidemiological challenges. In the latest World Health Organization (WHO) global TB report, 10.6 million (125-143 cases per 100 000 population) new TB cases were estimated, as well as 1.6 million deaths related to TB (1). About 15% of all newly infected TB cases were unable to receive adequate treatment. An active TB case infects between ten and fifteen people each year (2). Moreover, an epidemiological report predicted that *Mycobacterium tuberculosis* (MTB) would infect almost 225 million people between 1998 and 2030 without effective control and treatment (3). As a whole, TB treatment, diagnosis, and control strategies may not be as effective as they should be, and some cases may go undiagnosed and untreated. As a result, rapid detection and efficient treatment are essential for TB infection control (4). TB cases in 2021 were mostly located in the developing WHO regions of Africa, Southeast Asia, and Southwest Asia (1). It is necessary to acquire comprehensive information about MTB, especially in countries like Iran where Afghan immigrants are prevalent, so that appropriate control strategies can be adopted and "END TB" goals can be achieved (5). The control of TB infection remains largely suboptimal despite intensive research, and the mechanisms of its progression are unclear. Hence, it is imperative to understand new ways to improve TB control. Biomarkers that are distinctive and specific may be crucial for rapid and precise detection and treatment in this context. A variety of studies have examined different aspects of epigenetic mechanisms and TB infection and shown that specific epigenetic mechanisms at every stage of TB infection may serve as potential biomarkers (6, 7). Epigenetic biomarkers are currently included in small numbers of clinical investigations.

Non-coding RNAs, including miRNAs, are often thought of as epigenetic mechanisms, as they regulate biological processes (8). The first report of these conserved miRNAs was published in 2001 (9). It is known that many regulatory RNAs bind specifically to different target mRNAs encoded by the host genome and are therefore capable of acting as endogenous gene silencers post-transcriptionally (10). MTB modification of the host epigenome and interference with miRNA transcription are possible effects of MTB (8). A number of strategies are employed by this pathogenic organism in order to escape host immune responses by inducing miRNA expression. During TB infection, miR-125a, miR-125b, and miR-let-7f play an important role in altering immune response through the MTB-macrophage interaction. According to research, miR-125a inhibits autophagy and antimicrobial effects of MTB by regulating innate host defense (11). Apoptosis of macrophages is also considered to be a major host defense mechanism against MTB, and overexpression of miR-125b suppresses its apoptosis and inflammation (12). Furthermore, miR-let-7f is inhibited by MTB secreting effector early secreted antigenic target of 6 kDa (ESAT-6) in a manner dependent on ESAT-6. MTB survival is suppressed by miR-let-7f overexpression and inflammatory cytokines are produced as a result. Thus, miR-125a, miR-125b, and miR-let-7f play key roles in regulating the host response to MTB infection. Inhibition and overexpression of these miRNAs can increase or decrease inflammation and immune responses (13).

Together, miRNAs can play a crucial role in TB infection. Epigenetic analysis appears to offer innovative and more effective ways to control TB infection and develop targeted therapeutics. The current study used serum and sputum samples of Iranian and Afghani patients with active tuberculosis and healthy controls to examine differentially expressed miRNAs, miR-125a, miR-125b, and miR-let-7f, so that the levels of expressed miRNAs could be compared between Iranian patients and Afghani migrants.

MATERIALS AND METHODS

Human subjects. In this study, 60 Iranian and Afghan patients with active pulmonary tuberculosis from the Pasteur Institute of Iran were enrolled. The study included participants who had typical symptoms of pulmonary TB, such as fibrocavitary lung infiltrates on chest radiographs. As well as positive sputum culture and Ziehl-Neelsen stain for acid-fast bacteria. A Lowenstein-Jensen (LJ) culture medium was used after N-acetyl-cysteine (NAC) treatment for sputum culture. The culture tubes were incubated at 37°C for the first week and observed weekly thereafter for 8 weeks. For the identification of MTB in LJ culture medium, biochemical tests, including niacin production and nitrate tests, were performed. Inclusion of patients with another coexisting disease and

consumption of any drugs was not permitted. A total of sixty racially, age-, and sex-matched subjects were recruited as controls (Table 1). TB infections, both active and latent, were not present in the healthy control subjects. Aside from that, none of them had any clinical signs of infection. In all Iranian and Afghan participants, sputum and serum samples were collected simultaneously.

The study was approved by the Pasteur Institute of Iran ethics committee (IR.PII.REC.1398.046) and followed the Helsinki Declaration. The study was conducted with informed consent from all subjects.

Sample preparation and RNA isolation. In both the Iranian and Afghan study groups, sputum and venous blood were collected. For the collection of cell-free serum, blood was drawn into a sterile polyolefin resin tube without anticoagulants. Following 20 minutes of standing at room temperature, samples were centrifuged at 3,000 rpm for 10 minutes. In order to conduct the analysis, the supernatant serum was quickly collected, aliquoted, and stored at -70°C. Furthermore, the sputum samples were homogenized, as previously described (14). In order to conduct the analysis, the supernatant was collected, aliquoted, and immediately stored at -70°C.

The miRNAs preserved in sputum and serum samples were extracted using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Valencia, CA, USA). Each sputum and serum sample was tested in gel electrophoresis and the concentration and quality of RNA were determined using a Nanodrop spectrophotometer (ND-1000; Nanodrop Technologies). Equal amounts of RNA from each patient and control sample was divided into four groups (designated Iranian active TB group, Iranian control group, Afghan active TB group, and Afghan control group). Isolated miRNAs were used for cDNA synthesis by Quanti-Tect® Reverse Transcription kit.

Real-time RT-PCR. To detect differentially expressed miRNAs, miR-125a, miR-125b, and miR-let-7f, real-time quantitative reverse transcription PCR (Real-Time qRT-PCR) was performed using a standard TaqMan® PCR kit protocol on LightCycler® 96 System (15). Real-Time qRT-PCR was carried out on 60 ng of total RNA in a final volume of 20 µL RT-PCR reactions and incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. The 20 µl volumes of reaction included 1 µl product, 10 µl YTA SYBER Master Mix (YT2551), 1.4 µl forward and reverse primers (10 µM). Table 2 displays the characteristics of Stem-loop RT primers used in this experiment. All primers were designed by miRNA Primer Design Tool software (http://genomics.dote.hu:8080/mirnadesigntool/). Each sample was normalized based on its amount of U6 RNA and analysis was done through calculation of delta CT values. Finally, using the melting curve, the specific and exclusive peak of Real-Time qRT-PCR products were evaluated for each sample, and the specificity of Real-Time qRT-PCR amplification was confirmed. The average of the control delta CT values in the fold changes calculation has been used for $2^{-\Delta\Delta Ct}$ method. A triplicate analysis of each RT reaction was run, including no-template controls.

Statistical analysis. If applicable, values were presented as mean + standard deviation (SD), number or median and range. Student t-test was used when

Table 1. Characteristics of participants

	Iranian TB Positive (n = 30)	Iranian Healthy Control (n = 30)	Afghan TB Positive (n = 30)	Afghan Healthy Control (n = 30)
Gender				
Female	15	16	11	9
Male	15	14	19	21
Age				
Median	31.5	25.5	30	36.5
Min.	20	20	22	20
Max.	50	50	50	50
BMI (Mean ± SD)*	21.2 ± 0.34	18.2 ± 0.31	21.2 ± 0.23	19.0 ± 0.29

TB, tuberculosis; BMI, Body Mass Index; SD, standard deviation; * P<0.000001

Table 2. Stem–loop RT primer characteristics of evaluated miRNAs

Gene Name	Stem-loop primers* UGAGGUAGUAGAUUGUAUAGUU
miR-Let7f	GTTGGCTCTGgtgcagggtccgaggtATTCGCACCAGAGCCAACAACTAT UCCCUGAGACCCUUUAACCUGUGA
miR-125a	GTTGGCTCTGgtgcagggtccgaggtATTCGCACCAGAGCCAACTCACAG UCCCUGAGACCCUAACUUGUGA
miR-125b	GTTGGCTCTGgtgcagggtccgaggtATTCGCACCAGAGCCAACTCACAA

*Bold sequence: cDNA primer binding site to microRNA
Italic-bold sequence: Stem formation site
Lowercase sequence: reverse primer binding site

comparing samples from two groups when they were normally distributed to compare numerical variables. In the absence of normally distributed data, Mann–Whitney U and Kruskal–Wallis tests were applied to compare miRNA levels. Statistics were considered significant when P values were less than 0.05. The analysis was carried out using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0.2 (GraphPad, La Jolla, CA, USA).

RESULTS

In this study, all participants were Iranian and Afghan immigrants referred to the Pulmonary Department of the Pasteur Institute of Iran. All patients were infected with sensitive strain of MTB. Mean age and sex of Iranian and Afghan active TB groups and related healthy controls was not significantly different (Table 1; P>0.05). Baseline body mass index (BMI) in the Iranian and Afghan active TB groups was significantly lower than of each related control cases (Table 1; P<0.05). In addition, all participants were HIV negative and the Iranian and Afghan active TB groups showed clinical signs and symptoms of active pulmonary TB. These recorded symptoms comprised 98.3% cough, 78.3% fever, 83.3% weight loss, 69.8% night sweats, and 81.6% hemoptysis. Healthy controls involved in the study were free of active TB infection, latent TB infection, and any clinical symptoms of any infectious disease.

Several miRNA genes (miR-125a, miR-125b, and miR-let-7f) were selected for analysis in the current study, including miR-125a, miR-125b, and miR-let-7f, because they have important functions in modulating immune responses. During TB infection, all three of these miRNAs alter immune responses through the MTB-macrophage interplay. In Fig. 1, the relative expression levels of the miRNA genes in serum and sputum samples of Iranian and Afghan immigrants have been shown. Overexpression of miR-125a gene has been detected in both serum samples of Iranian (P<0.0001) and Afghan (P<0.0001) and sputum samples of Afghan (P<0.0001) subjects, while Iranian sputum samples showed its downregulation (P=0.0039). The overexpression of miR-125b found in all serum and sputum samples of Iranian (P<0.0001; P=0.0007) and Afghan (P<0.0001; P<0.0001) patients. Both serum and sputum samples from two races showed downregulation of miR-let-7f (P=0.0001), while Iranian sputum samples showed no statistically significant difference (P=0.348).

Using Kruskal-Wallis tests, the miRNA genes (miR-125a, miR-125b, and miR-let-7f) in Iranian and Afghan participants are compared in Fig. 2. All sputum and serum samples of two races showed statistically significant differences in miR-125a, miR-125b, and miR-let-7f. A significant increase in miR-125a expression was observed in Afghani sputum and serum samples (P<0.0001) compared to Iranian samples. In both serum (P<0.0001) and sputum (P=0.0009) samples of Iranian and Afghan patients, miR-125b expression levels were nearly the same. Additionally, miR-let-7f expression was predominantly decreased in serum samples from Iranian and Afghani subjects compared with sputum samples.

DISCUSSION

Epigenetic mechanisms such as miRNAs regulate gene expression at the post- transcriptional level and

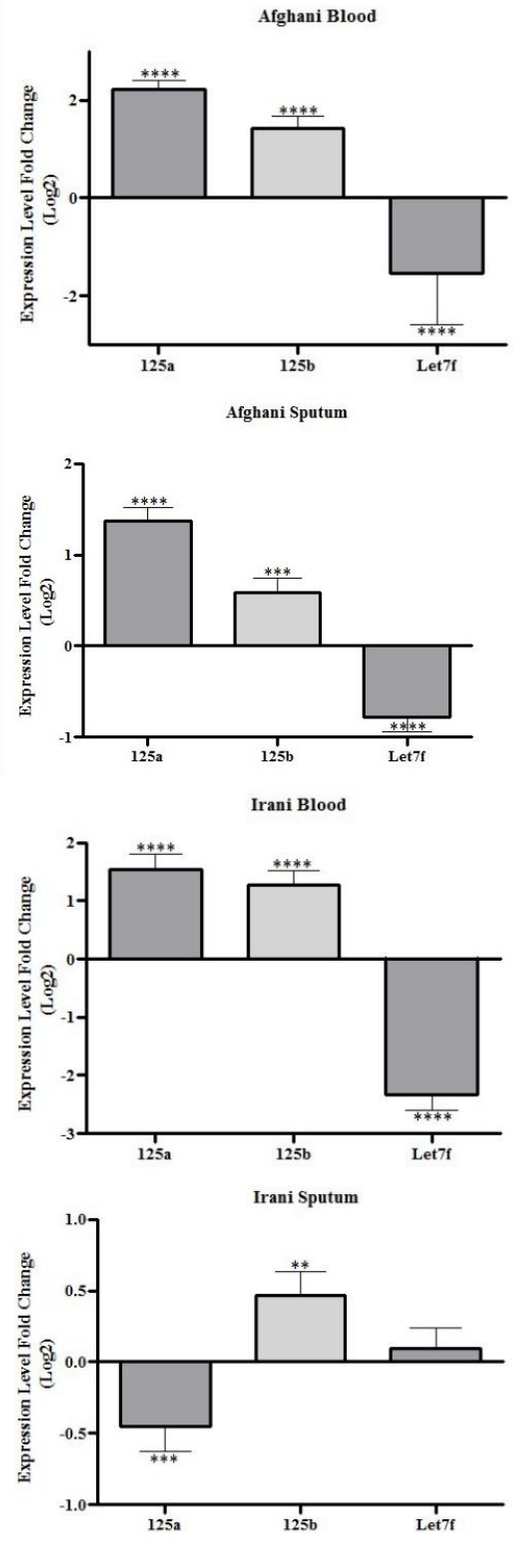

Fig. 1. Expression level of miRNA genes (miR-125a, miR-125b, and miR-let-7f) compared to control in serum and sputum samples of Iranian and Afghan immigrants; Mann–Whitney U test. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

play a key role in immune regulation. Several miRNAs are found in the human genome, which target specific mRNAs and regulate cellular processes, including anti-inflammatory and pro-inflammatory signals (16). It has been shown that altering the epigenome in TB infection can change the host transcriptional profile (6), though the epigenetic mechanisms are not yet fully understood (17). There has been extensive research on the crosstalk between miRNA expression and TB infection in recent years (6). In addition, there are some evidences that confirm population differences in microRNA expression (18). Distinct genetic variation and heterogeneity of the Iranian and Afghan population has been detected (19, 20). Identifying the relationship between miRNA expressions in TB patients of Iranian and Afghan races may provide important information regarding TB pathogenesis, host susceptibility, and more effective control. To assess the differential expression of miRNAs, miR-125a, miR-125b, and miR-let-7f, in Iranian and Afghani patients with active tuberculosis and healthy controls, this study used serum and sputum samples. This was done in order to compare miRNA expression levels between Iranian patients and Afghan migrants. In the course of TB infection, these three miRNAs interact with macrophages to alter immune responses and outcomes. It was found that the miR-125a gene was significantly overexpressed in the serum samples of Iranians (P<0.0001) and Afghans (P<0.0001), and in the sputum samples of Afghans (P<0.0001), while the miR-125a gene is significantly downregulated (P=0.0039) in Iranians sputum. By inhibiting autophagy and antimicrobial effects against MTB, miR-125a regulates the innate host defense (11). Overexpression of miR-27a, miR-33, miR-144, and miR-125a in TB patients, as well as their effects on autophagosome formation, has been shown (21, 22). It was reported that treated TB patients had a significantly higher level of miR-125a in their serum than untreated TB patients (23). Further, miR-125a is reported to be significantly upregulated by MTB infection in infected cells (RAW264.7/THP-1 cells), dependent on toll like receptor 4 (TLR4) signaling (24). The expression of miR-125a in macrophages after TB infection is primarily mediated by TLR4 rather than TLR2. A large number of immune cells, including macrophages, express TLR4. As a result of a pathogenic infection, TLR4 activation is closely related to inflammation and autophagy. Accordingly, miR-125a overexpression may be partially

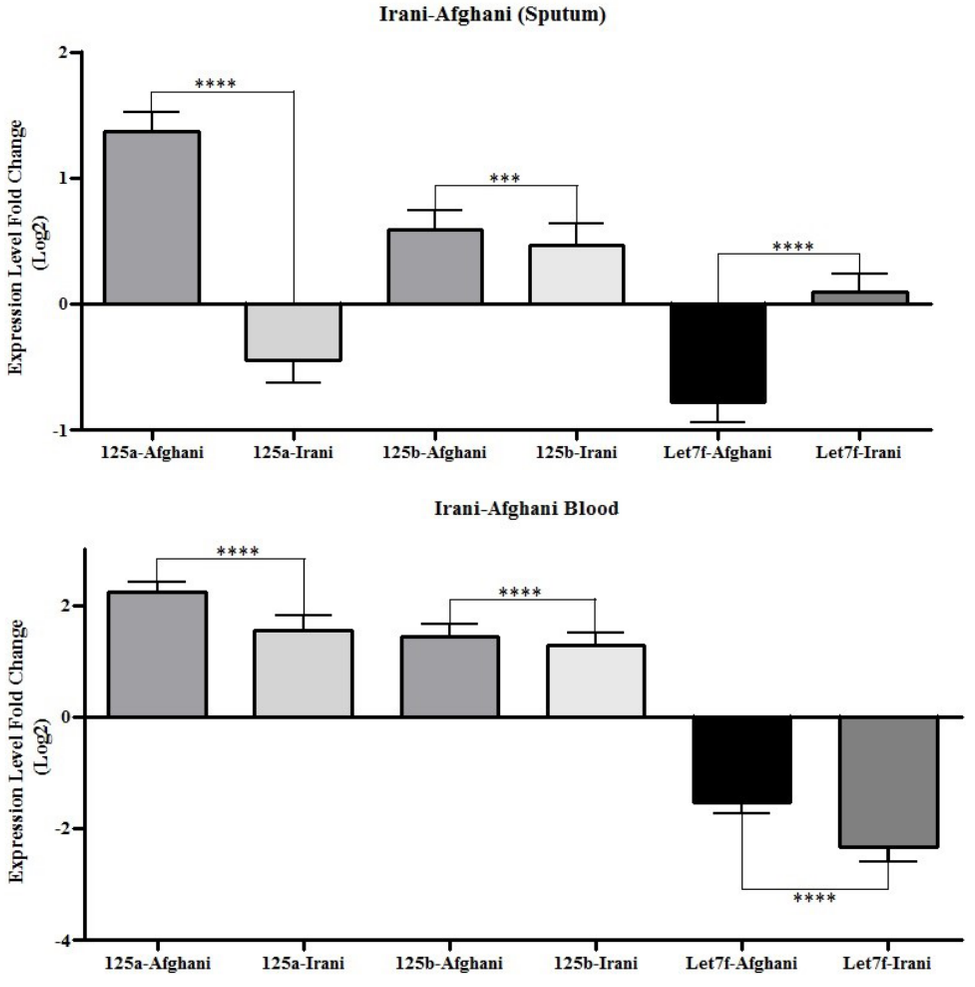

Fig. 2. Comparisons of miRNA levels (miR-125a, miR-125b, and miR-let-7f) between Iranian and Afghani serum and sputum samples; Kruskal–Wallis test. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

responsible for TB patients' TLR4 signaling (25).

Overexpression of miR-125a in TB infection is highlighted in some studies from China, Peru, and India (11, 26, 27). In addition, miR-125b was over-expressed in all serum and sputum samples from Iranian (P<0.0001; P=0.0007) and Afghan (P<0.0001; P<0.0001) patients. Apoptosis of macrophages is an important host defense mechanism against TB, and miR-125b overexpression suppresses apoptosis and inflammation (12), which can improve response to treatments. A study revealed that expression levels of TNF-α, IL-6, IFN-γ, and nuclear factor kappa B (NF-κB) are negatively correlated with expression of miR-125b in peripheral blood mononuclear cells of patients with TB (28). Consequently, miR-125b appears to play an important role in the survival of MTB and the progression of the infection through its ability to regulate the expression of cytokines (29). Several Chines, Indian and American studies confirmed upregulation of miR-125b during TB infection (29-31). Furthermore, downregulation of miR-let-7f has been confirmed in all samples of serum as well as in samples of sputum from both races (P<0.0001), whereas no statistically significant differences have been observed in Iranian sputum samples (P=0.348). In a recent study, it was established that miR-let-7f inhibits NF-κB activity and its overexpression diminishes MTB survival and creates an inflammatory environment (13), which may increase response to treatment. As a result of MTB ESAT-6, miR-let-7f levels are reduced in macrophages infected with MTB. As a result of an effector secreted by ESAT-6, immune responses are modulated and bacteria can escape from phagosomes (13, 32). The transfection of macrophages with miR-let-7f family members decreases MTB survival in macrophages

(13). According to Kumar et al, miR-let-7f is a regulator of the NF-κB pathway, suggesting that it could contribute to the immune response to MTB (13). While many questions remain regarding the function of miR-let-7f, the findings indicate that expression of let-7 decreases as the disease progresses. As a result, reducing let-7 expression will be very beneficial for the bacterium. The reverse association between miR-let-7f expression and MTB survival is confirmed by some studies from China, Brazil, and Malaysia (33-35). As previous studies have shown, let-7 is also protective against TB infection in the present study (36, 37).

There have been numerous studies that examine the relationship between ethnicity and tuberculosis infection. In several studies, TB infections have been examined from the perspective of ethnic migration over time (38, 39). In the current study, miR-125a, miR-125b, and miR-let-7f levels of all sputum and serum samples differed statistically significantly between races. Expression level of miR-125a was increased in serum ($P<0.0001$) and sputum ($P<0.0001$) samples of Afghani subjects in comparison to Iranian subjects. The expression level of miR-125b was almost similarly increased in serum ($P<0.0001$) and sputum ($P=0.0009$) samples of Iranian and Afghani patients. In addition, miR-let-7f expression mostly decreased in serum samples of Iranian and Afghani subjects in comparison to their sputum samples. Several MTB strains have been associated with ethnicity and immigration of different racial groups in Taiwan, according to a review study by Dou et al. (40). Genetic differences or environmental factors during different situations may have caused the observed difference between the two evaluated races in this study. There are likely multiple reasons behind ethnic disparities, and the reasons are not fully understood (41). It is noteworthy that ethnicity has a strong association with induction of specific miRNA expression during TB infection (42).

In conclusion, it was shown that Iranian and Afghan serum and sputum samples overexpressed miR-125a gene, while Iranian sputum samples downregulated it. Also, overexpression of miR-125b was found in all serum and sputum samples collected from Iranian and Afghan patients. It was established that miR-let-7f was downregulated in all samples of serum and sputum of two races, whereas Iranian sputum samples did not show any statistically significant differences. A number of studies have demonstrated that host miRNA profiles can be manipulated to adjust immune genes involved in TB infection. An accurate identification of the miRNA profile under different conditions could lead to the identification of new biomarkers that could be used for the diagnosis, monitoring, or treatment of TB infections. Besides, utility of rapid miRNA testing based on printed electrochemical strip biosensors in biological fluids in a cheap and easy way can be fundamental to facilitate future development of miRNA profiling. In light of this, further studies are needed to understand further the regulatory role of miRNAs and mechanisms that are yet to be uncovered regarding their role in TB infection. Such studies will help to develop more effective TB treatments and ultimately reduce the global burden of this disease.

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