

Original Article

Possible relation between expression of circulating microRNA and plasma cytokine levels in cases of pulmonary tuberculosis

Mahmut Ulger¹, Mehmet Sami Serin¹, Seda Tezcan Ulger², Gonul Aslan², Ahmet Ilvan³, Eyup Naci Tiftik⁴, Gurol Emekdas²

¹ Mersin University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Mersin, Turkey

² Mersin University, Faculty of Medicine, Department of Medical Microbiology, Mersin, Turkey

³ Istanbul Aydin University, Faculty of Medicine, Department of Chest Diseases, Istanbul, Turkey

⁴ Mersin University, Faculty of Medicine, Department of Hematology, Mersin, Turkey

Abstract

Introduction: Tuberculosis (TB) is a life-threatening infection and early diagnosis is critical for treatment and prevention of transmission. There is evidence of correlation between miRNA expression and cytokine regulation during TB infection. The aim of this study was to determine the relationship between expression levels of miRNAs in plasma and cytokine levels as a potential biomarker for genetic predisposition and/or early diagnosis of TB infection.

Methodology: The expression levels of 86 miRNAs were examined in plasma samples of 44 TB patients and 44 healthy controls by qRT-PCR using BioMark™ 96.96 Dynamic Array (Fluidigm Corporation, South San Francisco, CA, USA) system. The levels of plasma TNF- α , IFN- γ , IL-1 β , IL-4, IL-6, IL-8, IL-10, and IL-12/P40 were examined with ELISA.

Results: We identified dysregulation of 18 miRNAs which included upregulation of miR-1, miR-7-5p, miR-9-5p, miR-10a-5p, miR-10b-5p, miR-100-5p, miR-106b-5p, miR-128-3p, miR-133a-3p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-205-5p, miR-210-3p, and miR-296-5p, and downregulation of miR-15b-5p, miR-16-5p, and miR-25-3p in plasma samples of patients with pulmonary TB ($p < 0.05$). A significant correlation between the expression levels of miR-1, miR-7-5p, miR-9-5p, miR-10a-5p, miR-10b-5p, miR-15b-5p, miR-100-5p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-210-3p and cytokine levels of TNF- α , IFN- γ , IL-1 β , IL-8 and IL-10 was identified ($p < 0.05$).

Conclusions: We demonstrated that altered expression levels of plasma miRNAs consistent with immunological response have the potential to serve as non-invasive biomarkers for early diagnosis of pulmonary TB. Additional investigations with larger sample sizes will be required to confirm our findings and to determine if miRNAs can be possible targets for TB management strategies.

Key words: Tuberculosis; miRNA; Cytokine; Biomarker; qRT-PCR.

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Introduction

Tuberculosis (TB), caused by the bacillus *Mycobacterium tuberculosis*, is a communicable respiratory disease [1]. TB is one of the top 10 causes of death worldwide, and the leading cause of death from a single infectious agent. Globally, an estimated 8.9-11.0 million people contracted TB and 1.4 million died in 2019 [2]. Approximately 5 to 10% of *M. tuberculosis*-infected people tend to develop active TB during their lifetime, the remaining ~90-95% infected people remain asymptomatic [3,4]. Diabetes or co-infection with Human Immunodeficiency Virus (HIV) are risk factors in the development of the disease. In some patients, the role of host genetic factors in the regulation of disease development and progression, may contribute to the development of active TB [5].

The conventional methods for diagnosis of *M. tuberculosis* are primarily based on the detection of acid-fast *Bacilli* in smears by microscopy and then bacterial culture with selective medium. Depending on the type of specimen, the sensitivity of the acid-fast staining of smears is low. Bacterial culture is regarded as the gold standard method of diagnosis. However, the slow-growing nature of *M. tuberculosis* makes it time consuming and is the main disadvantage of the culture method of diagnosis [6,7]. Current methods such as microscopy, culture, or molecular testing, are still insufficient for diagnosing TB infection, so the development of new, sensitive, and effective biomarkers will be particularly beneficial for TB management [8,9].

MicroRNAs (miRNA) are generally 18-25 nucleotide (nt) in length and highly conserved non-coding RNA fragments that regulate gene expression at the post-transcriptional level by interacting with 3' untranslated regions (3'-UTRs) of target gene mRNAs [5,6,9]. miRNAs take part in many important metabolic processes, like proliferation, apoptosis, or cell death [10].

miRNAs are found in various organisms and regulate both adaptive and innate immune responses to pathogens by regulating T cell development, differentiation of B cells, antibody production and disease progression [4]. Moreover, miRNAs are more stable than mRNAs [1] and according to recent studies circulating serum miRNAs may be considered as biomarker candidates for the early diagnosis of diseases such as cancer and some infectious diseases [5,10].

M. tuberculosis infection control mechanisms initiate with pathogen recognition and uptake by lung macrophages. This induces cytokine and chemokine production, expression of immune receptors, and initiation of host defense mechanisms [9]. Recent evidence suggests that *M. tuberculosis* manipulates immune reactions by dysregulation of the host miRNAs involved in macrophage polarization [11] or inhibition of the production of important cytokines, such as interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) [4]. Based on this evidence, miRNAs play important role in the pathogenesis of *M. tuberculosis* infection by regulation of immune responses related to the switch between latent and active infection [9,11].

The aim of the study was to determine the possible relation between the expression levels of circulating miRNAs in plasma and cytokine levels of TNF- α , IFN- γ , IL-1 β , IL-4, IL-6, IL-8, IL-10, and IL-12/P40 and to perform the preliminary research to create a TB miRNA panel to be used as a potential biomarker to determine genetic predisposition and/or the early diagnosis of TB.

Methodology

Patients and healthy controls

A total of 44 microbiological culture-proven TB patients applied to the Chest Disease Clinic, Faculty of Medicine, Mersin University, Turkey and 44 similar blood samples of healthy controls from the blood banking unit of Mersin University hospital, between May 2014 and May 2015, were included in this study. The TB patients included 40 (90.9%) male, 4 (9.1%) female patients with a mean age of 32.11 ± 8.77 (mean \pm SD; range: 25-75). All control blood samples were obtained from healthy blood donors and were negative

for anti-HBsAg, anti-HCV, anti-HIV 1/2, and anti-syphilis when tested by ELISA. Among the healthy blood donors 42 (95.4%) were male and two (4.6%) were female and the mean age was 36.09 ± 7.09 (mean \pm SD; range: 21-54). All these participants \geq 18 years of age.

The study was reviewed and approved by the local ethics commission of Mersin University Clinical Research Ethics Committee (The Ethic Commission Approval Report No. #2013/368 and date 14.11.2013). All patients and controls signed the consent forms and were informed regarding the purpose of the proposed study. Our study was also carried out in accordance with the Helsinki Declaration (The Ethic Commission Approval Report No. 9/13 and date 11/21/2008).

Sample preparation

Peripheral venous blood samples from each subject were collected and drawn into ethylene diamine tetra acetic acid (EDTA) containing tubes and centrifuged at 4000x g for 15 min for plasma separation. Plasma samples were aliquoted into a nuclease free micro centrifuge tube. 250 μ L of plasma sample was transferred to a new micro centrifuge tube and stored at -80 $^{\circ}$ C until nucleic acid isolation.

Isolation of total RNA including miRNAs were performed by using High Pure miRNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions and then stored at -80 $^{\circ}$ C until the experiment.

Reverse transcription reaction

Isolated RNA samples were reverse transcribed into complementary DNA (cDNA) in 7 μ L final reaction volumes using miScript II Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). All reactions were performed as specified in the manufacturer's protocol: 3.5 μ L total RNA were added to 3.5 μ L of the reverse transcription (RT) reaction mix (10X miScript Nucleic Mix, miScript Reverse Transcriptase Mix, 5X miScript HiSpec Buffer and Nuclease free water). The reverse transcription reaction was carried out using the PikoReal™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with the following conditions: 37 $^{\circ}$ C for 60 min, 95 $^{\circ}$ C for 5 min and 4 $^{\circ}$ C until further processing or storage. cDNA samples were stored at -80 $^{\circ}$ C until PCR analysis.

Pre-amplification

After reverse transcription, a pre-amplification was performed using the miScript PreAmp PCR Kit (Qiagen GmbH, Hilden, Germany) according to the

manufacturer's specifications. Briefly cDNA samples were diluted with Low EDTA [0.1 mM] TE Buffer. Pre-amplification reaction was done at 10 µL final reaction volume, 2 µL of cDNA samples were added to 8 µL of the PreAmp mix (5X miScript PreAmp Buffer, HotStartTaq DNA Polymerase, Primer Pool [Quanta BioSciences, Beverly, MA, USA], miScript PreAmp Universal Primer and nuclease free water). miScript PreAmp PCR amplification condition was carried out as follows: one cycle at 95 °C for 15 min followed by 12 cycles at 95 °C for 30 s, 60 °C for 3 min and then rest period at 4 °C in a PikoReal™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA).

Following the pre-amplification, the reaction was treated with Exonuclease I (New England Biolabs, Ipswich, MA, USA) to remove any unincorporated pre-amplification primer. Specifically, 10 U of ExoI was added to each sample and the samples were incubated at 37 °C for 30 min followed by heat inactivation of ExoI at 95 °C for 15 min.

Determination of miRNA expression with real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) reactions were performed using the high-throughput BioMark Real-Time PCR system (Fluidigm Corporation, South San Francisco, CA, USA) as described previously [12], with minor modifications.

The list of 84 miRNAs that were investigated for expression levels are as follows: hsa-miR-1, hsa-let-7a-5p, hsa-let-7c, hsa-miR-7-5p, hsa-miR-9-5p, hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-15a-5p, hsa-miR-15b-5p, hsa-miR-16-5p, hsa-miR-17-3p, hsa-miR-17-5p, hsa-miR-18a-5p, hsa-miR-19a-3p, hsa-miR-19b-3p, hsa-miR-20a-5p, hsa-miR-21-5p, hsa-miR-22-3p, hsa-miR-23a-3p, hsa-miR-24-3p, hsa-miR-25-3p, hsa-miR-26a-5p, hsa-miR-26b-5p, hsa-miR-27a-3p, hsa-miR-29a-3p, hsa-miR-30d-5p, hsa-miR-30e-5p, hsa-miR-31-5p, hsa-miR-34a-5p, hsa-miR-92a-3p, hsa-miR-93-5p, hsa-miR-96-5p, hsa-miR-100-5p, hsa-miR-103a-3p, hsa-miR-106b-5p, hsa-miR-107, hsa-miR-122-5p, hsa-miR-124-3p, hsa-miR-125b-5p, hsa-miR-126-3p, hsa-miR-128, hsa-miR-130b-3p, hsa-miR-133a, hsa-miR-133b, hsa-miR-134, hsa-miR-141-3p, hsa-miR-143-3p, hsa-miR-145-5p, hsa-miR-146a-5p, hsa-miR-148a-3p, hsa-miR-150-5p, hsa-miR-155-5p, hsa-miR-184, hsa-miR-191-5p, hsa-miR-192-5p, hsa-miR-193a-5p, hsa-miR-195-5p, hsa-miR-196a-5p, hsa-miR-200a-3p, hsa-miR-200b-3p, hsa-miR-200c-3p, hsa-miR-203a, hsa-miR-204-5p, hsa-miR-205-5p, hsa-miR-206, hsa-miR-208a, hsa-miR-210, hsa-miR-211-

5p, hsa-miR-214-3p, hsa-miR-215, hsa-miR-221-3p, hsa-miR-222-3p, hsa-miR-223-3p, hsa-miR-224-5p, hsa-miR-296-5p, hsa-miR-372, hsa-miR-373-3p, hsa-miR-374a-5p, hsa-miR-375, hsa-miR-376c-3p, hsa-miR-423-5p, hsa-miR-499a-5p, hsa-miR-574-3p, hsa-miR-885-5p.

Previously lyophilized miRNA primers were prepared by diluting according to the manufacturer's instructions and 6 µL was dispensed to each assay well. Pre-amplified cDNA samples were diluted 1:5 as stated above. Specifically, a 6 µL sample mixture was prepared for each sample containing 3 µL TaqMan Universal PCR Master Mix, No Amp Erase UNG, (Applied Biosystems, Foster City, CA, USA), 0.3 µL 20X DNA Binding Dye Sample Loading Reagent (Fluidigm Corporation, South San Francisco, CA, USA), and 0.7 µL nuclease free water and pipetted into a 96 well plate at final volume of 4 µL; and 2 µL of 1:5 diluted pre-amplified cDNA pipetted into each well and mixed. This mixture was pipetted into sample inlets of the 96.96 Dynamic Arrays (Fluidigm Corporation, South San Francisco, CA, USA). The Bio Mark IFC controller HX (Fluidigm Corporation, San Francisco, CA, USA) was used to distribute the assay mix and sample mix from the loading inlets into the 96.96 Dynamic array reaction chambers for qRT-PCR by Fluidigm's Integrated Fluidic Circuit Technology. After loading, the chip was placed in the BioMark Instrument for the real-time PCR step; for UNG inactivation and hot start protocol, samples were firstly held for 2 min at 50 °C, then 70 °C for 30 min, 25 °C for 10 min, followed by 23 cycles with 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. Melting curve analysis was performed to evaluate the specificity of the PCR products at range 60-95 °C.

Determination of cytokine levels

Plasma TNF-α (DIASource, Louvain-la-Neuve, Belgium, KAP 1751, detection limit: 0.7 pg/mL), IFN-γ (DIASource, KAP 1231, detection limit: 0.03 IU/mL), IL-1β (DIASource, KAP 1211, detection limit: 0.35 pg/mL), IL-4 (RayBiotech, Peachtree Corners, GA, USA, ELH-IL4, detection limit: 5 pg/mL), IL-6 (DIASource, KAP 1261, detection limit: 2 pg/mL), IL-8 (DIASource, KAP 1301, detection limit: 1.1 pg/mL), IL-10 (DIASource, KAP 1321, detection limit: 1.6 pg/mL) and IL-12/P40 (CUSABIO, Houston, TX, USA, CSB-E04598h, detection limit: 7.8 pg/mL-500 pg/mL) levels were examined using commercially available ELISA kits. Tests and evaluation procedures were carried out according to the manufacturer's recommendations. The reactions were visualized by

adding the substrate solution (3,3',5,5'-tetramethylbenzidine [TMB]), and absorbance at 450 nm was measured with an ELISA plate reader (Biotek ELX800, Winooski, VT, USA).

Statistical analysis

All statistical analyses for the determination of miRNA expression profile were performed using the Biogazelle’s qbase PLUS 2.0 (Ghent, Belgium) software which uses global means normalization method to troubleshoot the housekeeping gene problem in circulation. RNU48 was used as endogenous control. This qRT-PCR profiling platform consists of 84 miRNAs and was analyzed by using global mean normalization. Student’s t-test or Mann-Whitney U test were performed to compare the differences in miRNA levels between TB patients and healthy controls and the one-way ANOVA test was used for three or more groups. *p* < 0.05 was considered statistically significant.

The standard curves were created with the target standard cytokine concentration by plotting the mean absorbance (y axis) against the cytokine concentration (x axis) for each ELISA plate. Concentrations were determined with linear or non-linear regression analysis by the projection on the curve from absorbance values obtained in ELISA studies. Student’s t-test or Mann-Whitney U test was used to compare for the mean in independent samples. The χ^2 test or Fisher’s exact test was used for non-parametric analysis of categorical variables. Student’s t-test was also used to show possible relation between miRNAs displayed significant expression differences among groups and cytokines determined by significantly lower expression. ANOVA test was performed for comparing more than

two groups. Statistical significance was defined by a *p* value of less than 0.05. All statistical analyses were carried out using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA) software.

Results

miRNA expression levels

qRT-PCR results showed that among expression levels of the 84 miRNAs, 18 were obviously altered in the TB patients compared to the healthy control group (*p* < 0.05). Of the 18 miRNAs that had altered expression levels, 15 miRNAs were found to have statistically significant upregulated levels and the remaining 3 miRNAs were downregulated (*p* < 0.05). The upregulated miRNAs were miR-9-5p, miR-10a-5p, miR-10b-5p, miR-100-5p, miR-106b-5p, miR-128-3p, miR-133a-3p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-205-5p, miR-210-3p, and miR-296-5p. The downregulated miRNAs were miR-15b-5p, miR-16-5p, and miR-25-3p (Table 1 and Figure 1).

Cytokine ELISA results

Significant difference (*p* < 0.05) was observed in the levels of TNF- α , IFN- γ , IL-1 β , IL-8, and IL-10 between TB patients and the healthy control group based to the results of plasma cytokine ELISA (Table 2). However, there was no statistically significant difference in IL-4, IL-6 and IL-12/P40 levels between the TB patients and the healthy control group (*p* > 0.05).

Comparisons of miRNA expression levels with ELISA results

The expression levels of 18 miRNAs, which were statistically significant in the study population, were compared with statistically significant cytokine ELISA (TNF- α , IFN- γ , IL-1 β , IL-8 and IL-10) results. It was

Table 1. miRNA levels in TB patients vs control group.

miRNA	Fold changes	<i>p</i>
miR-1	2.9958 ↑	0.022557
miR-7-5p	2.5457 ↑	0.023824
miR-9-5p	3.8837 ↑	0.002693
miR-10a-5p	4.6541 ↑	0.003586
miR-10b-5p	3.169 ↑	0.008363
miR-15b-5p	-3.1434 ↓	0.011069
miR-16-5p	-2.3654 ↓	0.001673
miR-25-3p	-2.3751 ↓	0.001509
miR-100-5p	2.7933 ↑	0.023878
miR-106b-5p	2.8035 ↑	0.035662
miR-128-3p	2.0569 ↑	0.038605
miR-133a-3p	3.7196 ↑	0.031279
miR-143-3p	3.2121 ↑	0.014401
miR-193a-5p	3.8431 ↑	0.01085
miR-200b-3p	4.2989 ↑	0.00744
miR-205-5p	2.531 ↑	0.023051
miR-210-3p	5.1161 ↑	0.015976
miR-296-5p	3.4983 ↑	0.019468

Figure 1. miRNA levels in TB patients vs control group.

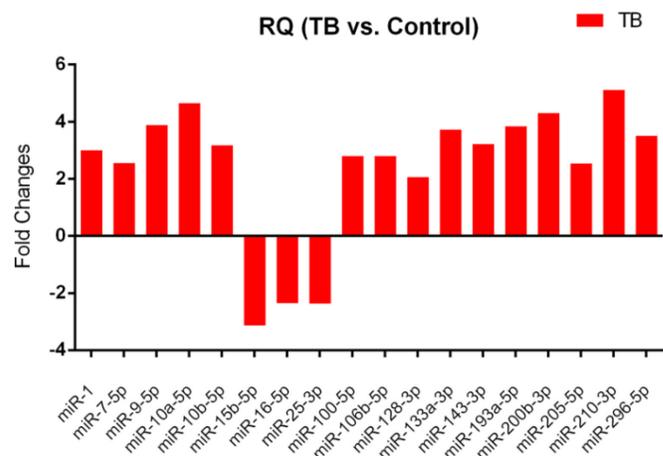


Table 2. Plasma cytokine ELISA results of TB patients and control group.

Cytokines	TB Patients (n = 44)		Control (n = 44)		p values
	Positive	Negative	Positive	Negative	
TNF- α	43	1	0	44	< 0.0001
IFN- γ	43	1	0	44	< 0.0001
IL-1 β	43	1	0	44	< 0.0001
IL-8	42	2	4	40	0.0016
IL-10	42	2	4	40	0.0012

determined that statistically significant differences were found in the expression levels of 11 miRNAs: miR-1, miR-9-5p, miR-10a-5p, miR-10b-5p, miR-15b-5p, miR-100-5p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-210-3p, miR-296-5p ($p < 0.05$) (Table 3).

Discussion

miRNAs play an important role in regulating the post transcriptional level of gene expression, including stem-cell differentiation, embryogenesis, hematopoiesis, metabolism and secretions, and also have a significant association between the dysregulated miRNA and immune responses to various infectious diseases [10,13]. The growing evidence of altered expression levels of specific miRNAs in serum or plasma suggests that they are potential non-invasive biomarkers for the diagnosis of human infectious diseases or for detecting alterations in physiological conditions [8,13,14]. TB remains a devastating disease and has enormous impact on global health [15]. TB biomarkers would constitute extremely useful tools for different situations of TB including protection by vaccination, discrimination of latent and active disease

and prediction of treatment outcome and relapse risk [15,16]. Therefore, analysis of a set of *M. tuberculosis*-associated miRNAs in plasma will distinctly develop the diagnosis of TB infection.

Several studies have determined that a few miRNAs were differentially expressed during pulmonary TB infection. The expression levels of multiple miRNAs that were found to be significantly upregulated included miR-10b-5p, miR-16, miR-21, miR-29a, miR-31, miR-103a-3p, miR-107, miR-148a-3p, miR-155, miR-193a-5p, miR-200b-3p, miR-296-5p, miR-361-5p, miR-889, and miR-576-3p compared with those in healthy controls [16-22]. Expression levels of miR-1, miR-22-3p, miR-15b-5p, miR-143, miR-146a, miR-151a-3p, miR-320a, and miR-769-5p were significantly downregulated in patients versus healthy controls [20-24]. In this study we used the Biomark qRT-PCR assay to identify miRNAs related with TB infection and to determine which miRNAs have potential for detecting this infectious disease in individual plasma samples from TB patients vs controls. We identified numerous miRNAs to be stable in plasma. We identified the role of 18 of the 84 miRNAs commonly found in plasma. Differential expression levels of these miRNAs were

Table 3. The comparison of cytokine ELISA results and the expression levels of the 18 miRNAs. Expression levels of the 11 statistically significant miRNAs are shown in bold.

miRNA	p values				
	TNF- α	IFN- γ	IL-1 β	IL-8	IL-10
miR-1	0.0296	0.0296	0.0296	0.0294	0.0294
miR-7-5p	0.1171	0.1171	0.1171	0.1164	0.1164
miR-9-5p	0.0025	0.0025	0.0025	0.0024	0.0024
miR-10a-5p	0.0068	0.0068	0.0068	0.0067	0.0067
miR-10b-5p	0.0488	0.0488	0.0488	0.0485	0.0485
miR-15b-5p	0.0202	0.0202	0.0202	0.0258	0.0258
miR-16-5p	0.8294	0.8294	0.8294	0.8272	0.8272
miR-25-3p	0.6662	0.6662	0.6662	0.6620	0.6620
miR-100-5p	0.0296	0.0296	0.0296	0.0294	0.0294
miR-106b-5p	0.2402	0.2402	0.2402	0.2377	0.2377
miR-128-3p	0.0553	0.0553	0.0553	0.0551	0.0551
miR-133a-3p	0.1830	0.1830	0.1830	0.1797	0.1797
miR-143-3p	0.0148	0.0148	0.0148	0.0146	0.0146
miR-193a-5p	0.0207	0.0207	0.0207	0.0107	0.0107
miR-200b-3p	0.0207	0.0207	0.0207	0.0204	0.0204
miR-205-5p	0.0577	0.0577	0.0577	0.0574	0.0574
miR-210-3p	0.0202	0.0202	0.0202	0.0197	0.0197
miR-296-5p	0.0055	0.0055	0.0055	0.0054	0.0054

TNF- α : Tumor Necrosis Factor alpha; IFN- γ : Interferon gamma; IL: Interleukin; IL-1 β : Interleukin 1 beta.

established based on upregulation of miR-1, miR-7-5p, miR-9-5p, miR-10a-5p, miR-10b-5p, miR-100-5p, miR-106b-5p, miR-128-3p, miR-133a-3p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-205-5p, miR-210-3p, and miR-296-5p and downregulation of miR-15b-5p, miR-16-5p, and miR-25-3p in the human pulmonary TB patients compared to the healthy control group ($p < 0.05$).

The changes in miRNA expression profiles reflect universal responses to mycobacterial pathogens and suggest that miRNAs may be unique and potential biomarkers for TB disease. These findings reveal that miRNAs alone or in combination may discriminate between TB patients and healthy controls and have the potential to serve as novel biomarkers. A unique miRNA profile used to specifically detect or classify TB patients has not yet been defined. Our study is a significant contribution towards determining the appropriate miRNA pattern associated with TB.

Our observation that miR-10b-5p, miR-143, miR-193a-5p, miR-200b-3p, and miR-296-5p are significantly upregulated in the patients with pulmonary TB compared to the healthy control group is consistent with previous studies [3,17,22,25]. Our data on miRNAs associated with TB is likely to contribute to the development of therapeutics and non-invasive biomarkers for chronic disease caused by *M. tuberculosis*.

Results from the study of Zhou *et al* [23] suggested that downregulated miR-1 may be suitable to serve as a potential biomarker for the diagnosis of childhood TB. However quantitative data of the miRNAs expression levels from the current study is largely inconsistent to their study because our results indicated that miR-1 was upregulated in TB patients. Similarly, the expression level of miR-106b was found to be upregulated by 2.80-fold in the TB patients in our study. However, a previous study [26] found that a panel of miRNAs including miR-106b was significantly downregulated in the plasma from chronic obstructive pulmonary disease (COPD) patients when compared with normal smokers. These findings demonstrated that progressive reduction in the plasma miR-106b level might reflect persistent and systemic changes in COPD patients. Further studies are required to demonstrate the role of miR-1 and miR-106b in pathogenesis of pulmonary disease.

Recent evidence showed that miR-7 played an important role in the pathologies of lung-injury [27]. Similarly, Akbas *et al* [28] reported that increase in level of miR-7 associated with COPD and could be a potential biomarker. These data suggest the important role of miR-7 in the development of lung-related

diseases. Our study showed higher levels of miR-7-5p in plasma of TB patients compared to healthy controls. Our study also detected for the first time higher levels of miR-100-5p in plasma of TB patients compared with healthy controls. Likewise, miR-100-5p in mammals infected with influenza A virus was found to be upregulated as a result of lung disease, suggesting that it could be used as a target for specific miRNA inhibitors to control immune response or cell death [29]. This may be a response to lung injury caused by TB infection, but the mechanism needs to be clarified with further studies.

Other miRNAs that were found to be upregulated in TB patients relative to controls were miR-9-5p, miR-10a-5p, miR-128-3p, miR133a, miR-205-5p, and miR-210. However, we did not find previous studies that explored their association with TB. There is some evidence that suggests that the expression levels of miR-9-5p, miR-10a-5p, miR-205-5p, and miR-210 were significantly upregulated in lung cancer tissues, and that their expression levels can be used as a poor prognostic and potential non-invasive biomarkers in patients [30-33]. Conversely, miR-133a serves as a tumor-suppressive and its downregulated levels suggest deterioration in lung cancer patients [34]. In addition, miR-128-3p has been reported to be downregulated in lung cancer tissues versus normal tissues [35]. Emerging evidence indicates that dysregulated miRNAs are associated with cancer tumorigenesis and progression. Further studies are required to establish their functions in active TB infection and contribution to the molecular mechanisms of lung injury.

The levels of miR-15b-5p, miR-16-5p, and miR-25-3p in plasma were significantly lower in the TB patients than that in the control group in the current study. However upregulated levels of miR-16 and miR-25 were identified as a signature for discriminating between healthy persons and pulmonary TB by Miotto *et al* [17]. It has also been stated that the expression of miR-16 may be associated with active TB [18,19]. In another study miR-15b-5p was found to be downregulated by 0.46-fold which is consistent with our study [22].

M. tuberculosis is an intracellular agent and interacts with secreted miRNAs from infected epithelial cells and immune cells associated with signaling pathways to enhance its survival inside the hosts [36]. Therefore, the eradication of *M. tuberculosis* requires prolonged antimicrobial treatment, which in turn can lead to development of drug resistance [37]. The expression levels of altered miRNAs potentially involved in the host immunological response during TB

infection are encouraging evidence and can create new opportunities for TB research, diagnosis and treatment [38,39]. Several miRNAs including let-7e, miR-21, miR-26a, miR-29a, miR-31, miR-142-3, miR-155, miR-210, and miR-223 are relevant to regulation of anti-TB immunity [38-41]. In this preliminary study, we intended to demonstrate the possible relationship between the altered levels of some cytokines and expressions of a group of circulating miRNAs. Our purpose was to determine if a TB miRNA panel can be used as a biomarker for detection of predisposition and/or early diagnosis of TB. At the end of the study, statistically significant differences were found between TNF- α , IFN- γ , IL-1 β , IL-8 and IL-10 cytokine levels in the TB patients compared to the healthy controls included in the study ($p < 0.05$). The results of the present study demonstrated the potential role of miR-1, miR-9-5p, miR-10a-5p, miR-10b-5p, miR-15b-5p, miR-100-5p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-210-3p, and miR-296-5p as promising biomarkers in the immune response against human TB, due to a correlation between a significant change in expression levels and cytokine levels ($p < 0.05$). The findings of our study enrich insights into miRNA expression levels and behavior in the immune response to TB, an intracellular bacterial infection.

Conclusions

In summary, the present study determined the involvement of miRNAs in the pathogenesis of TB. Our results demonstrated dysregulation of 18 miRNAs of which 15 were upregulated and three were downregulated in plasma of TB patients. The biological mechanism and therapeutic potential in TB infections of dysregulation in these miRNAs should be studied further. Our study represents a preliminary investigation of host response associated with miRNA expression status in patients with pulmonary TB infection. A combination of miR-1, miR-9-5p, miR-10a-5p, miR-10b-5p, miR-15b-5p, miR-100-5p, miR-143-3p, miR-193a-5p, miR-200b-3p, miR-210-3p, and miR-296-5 was identified as a potential non-invasive molecular marker consistent with immunological response for rapid diagnosis of TB infection. The data from the present study are likely to contribute to the development of therapeutics and biomarkers. Therefore, additional investigations in larger numbers of patients and healthy volunteers will be required to confirm our findings and to determine whether miRNAs are possible targets for TB management strategies.

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Corresponding author

Assoc. Prof. Dr. Mahmut ULGER, PhD
Mersin University, Faculty of Pharmacy, Department of
Pharmaceutical Microbiology,
Yenisehir 33169 /Mersin-Turkey
Tel: +90 (324) 341 2815–12152
Fax: +90 (324) 341 3022
Email: mahmutulg@yahoo.com.tr; mahmutulger@mersin.edu.tr

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