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Original Article

Evaluating the effectiveness of anti-tuberculosis treatment by detecting *Mycobacterium tuberculosis* 85B messenger RNA expression in sputum

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ABSTRACT

Background: The antigen 85 complex (85B) is secreted in large quantities from growing mycobacteria and the presence of bacterial mRNA is an indicator of cell viability. The quantitative detection of 85B mRNA expression levels can be used to assess the success of anti-tuberculosis treatment outcomes to detect viable mycobacteria cells. Therefore, we evaluated the levels of 85B mRNA of *Mycobacterium tuberculosis* strains in patients with pulmonary tuberculosis.

Methods: Thirty patients with primary tuberculosis were included in this study. The sputum specimens of patients were collected on days 0, 15, and 30 days and were cultured and evaluated by 85B mRNA-based RT-qPCR.

Results: Overall, 23 of the studied tuberculosis strains were susceptible to the primary anti-tuberculosis antibiotics used in this study, 7 were resistant. By the 30th day of treatment, 85B mRNA was detected in only one of the susceptible strains, but in all 7 of the resistant strains, though the relative gene expression varied between the strains. This difference between the susceptible and resistant strains at day 30 was statistically significant ($p < 0.05$).

Conclusion: 85B mRNA expression levels could be used to follow up on primary tuberculosis cases. 85B mRNA seems to be a good diagnostic marker for monitoring anti-tuberculosis treatment outcomes.

Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is an obligate human pathogen that causes tuberculosis (TB), which is still a mortal public health problem [1]. In the WHO's global TB report (2019), approximately 10.0 million (range: 9.0–11.1 million) people had TB in 2018 and there were an estimated 1.2 million TB deaths among HIV-negative people [2]. Current laboratory methods for the diagnosis of TB and follow-up of anti-TB treatment take a long time due to the slow growth rate of *M. tuberculosis* [3]. The success of an anti-TB treatment is usually correlated with the conversion of a sputum culture from positive to negative [4]. Usually, the first-line anti-TB regimen includes five antimicrobial agents (isoniazid, rifampin,

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ethambutol, streptomycin and pyrazinamide), and the treatment choice may be changed after obtaining the results of antimicrobial susceptibility tests [3]. Nucleic acid amplification tests (NAATs) of mycobacterial DNA/rRNA have proven to be fast and reliable diagnostic tests for TB diagnosis and follow-up on the success of anti-TB therapy [5]. Many NAAT methods, including those based on IS6110, 65 kDa heat shock protein, and 16S rRNA detection, are used either in TB detection or follow-up after treatment [6–10]. Unfortunately, these methods cannot differentiate between viable and non-viable *M. tuberculosis* [11]. The antigen 85 complex of *M. tuberculosis* and *Mycobacterium bovis* BCG generates a humoral response in TB patients that is specific for growing mycobacteria [12]. The antigen 85 complex includes three 30–32-kDa proteins that are located in the extracellular space and secreted during the growth of *M. tuberculosis* by an energy-dependent process [12]. The half-life of bacterial mRNAs are very short, compared to rRNA or genomic DNA, and therefore mycobacterial mRNA may be a better target for the detection of mycobacterial viability [13]. Also, the characteristics of mRNA-based assays suggest that these assays may distinguish between viable and non-viable organisms. These assays may be useful for monitoring the efficacy of anti-TB therapy [14].

We aimed to detect *M. tuberculosis* 85B mRNA from sputum specimens and evaluate the performance of using mRNA to monitor the response to anti-TB therapy using reverse-transcription quantitative PCR (RT-qPCR) in sputum specimens from TB patients receiving first-line anti-TB treatment.

Materials and methods

Study area and groups

This prospective study took place between October 2017 and October 2018 and involved the following participants:

1. Medical Microbiology Department, Medical Faculty of Beykent University.
2. Chest Diseases Clinics of Istanbul Sureyyapasa Chest Diseases and Thoracic Surgery training and research hospital.
3. Chest Diseases Clinics of Cerrahpasa Medical Faculty, Istanbul University-Cerrahpasa.

Fifty-five sputum samples were obtained with highly suspicious clinical/radiological and histopathological evidence of pulmonary TB from patients who were referred to the outpatient clinics of Istanbul Sureyyapasa Chest Diseases and Thoracic Surgery training and research hospital. A total of 30 patients were included after TB was confirmed in the sputum samples by both positive Lowenstein–Jensen (LJ) tests and the BACTEC MGIT 960 method (BD Diagnostics, Sparks, MD) on day 0. The sputum samples of TB-positive patients were taken on days 0, 15, and 30. The sex distribution (M/F) and mean age of the patients, respectively, were 16/14 and 38.63 ± 16.33 years (range 21–76 years). All participants signed a written informed consent form that was approved by the Clinical Research Ethics Board of Istanbul University, Cerrahpasa Faculty of Medicine (No: 83045809/604.01, Date: 08.10.2015). The same Institutional Ethics Board also approved this study. Total RNA isolation and 85B mRNA studies were performed in the Microbiology laboratory of Beykent University.

Processing of specimens

The analysis was performed at the Microbiology Laboratory of Beykent University. Sputum samples were processed for microscopy (smear examination) and cultured in the BACTEC MGIT 960 and LJ systems. Samples were decontaminated with *N*-acetyl-L-cysteine and 2% sodium hydroxide (NALC–NaOH) and 500-μL aliquots of decontaminated sample were stored at −70 °C for RT-qPCR. The mycobacterial isolates obtained in culture were subjected to limited biochemical testing for species characterization using the BACTEC NAP TB Differentiation Test Kit (Becton Dickinson, Sparks, MD, USA).

Mycobacteria growth Indicator tube drug susceptibility testing

Mycobacteria growth indicator tube drug susceptibility testing (MGIT-DST) was performed using the BACTEC MGIT 960 SIRE kit according to the manufacturer's instructions [15]. In the test, 800 μL of OADC supplement and 100 μL of drugs were added to each MGIT tube. A few colonies on the LJ medium were suspended in PBS and vortexed to prepare a suspension. A 1:5 dilution of this suspension was prepared using PBS and 500-μL samples were inoculated into each drug-containing tube and a growth control tube. The final drug concentrations were 1.0 μg/mL for streptomycin, 0.1 μg/mL for isoniazid, 1.0 μg/mL for rifampin, and 5.0 μg/mL for ethambutol. Drug susceptibility testing of pyrazinamide was performed in a different set of samples by monitoring the growth of *M. tuberculosis*, in liquid medium at pH 5.9 containing 0.1 μg/mL of pyrazinamide, by the BACTEC MGIT 960 system. A growth control tube with growth supplement but without drug was also included and the relative growth ratios between the drug-containing tube and drug-free growth control tube were determined by the system's software algorithm. The susceptibility results were reported by the MGIT 960 system.

Molecular methods

85B mRNA detection with RT-qPCR

85B mRNA from *M. tuberculosis* was extracted from decontaminated samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, including a previously-described modification [16]. The RNA concentration and purity ratios (OD260/280, OD260/230) were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Purified RNA was stored at −70 °C until analysis. The primers and probes used were specific to the 85B sequence and amplify a 130-bp region (GenBank Accession number: X62398).

The primers and probes were specific to 85B sequence and multiplied a specific 130 bp region of 85B mRNA (GenBank Access numberı: X62398); 85B mRNA forward (5-TCAGGGGATGGGGCCTAG-3), 85B mRNA reverse (5-GCTTGGGGATCTGCTGCGTA-3) and 85B mRNA probe (5-FAM-TCGAGTGACCCGGCATGGGAGCGT-Tamra-3) [16,17]. Mycobacterial 16S reference gene was used for normalization of 85B mRNA gene expression levels. For the 16S reference gene; RT primer (5-CCCAGTAATTCC-3), 16S Reverse primer (5-CGCTCGCACCCTACGTATTAC-3), 16S Forward primer (5-TTCTCTCGGATTGACGGTAGGT-3) and 16S Probe (5-6FAM-AGCACCGGCCAACTACGTGCCAG-TAMRA-3) sequences were used [18]. RT-qPCR was performed using a LightCycler 480 II Real-Time PCR System (Roche Diagnostics GmBH) using the 2X OneStep qRT-PCR Mastermix Kit (PrimerDesign Ltd., Southampton, UK) according to the manufacturer's instructions. The qPCR reaction was performed in a total volume of 20 μL consisting of 5 μL of total RNA (40 ng/μL), 10 μL of 2X qPCR Mastermix Kit (PrimerDesign Ltd. Southampton, UK), 1 μL of primer/probe mix (FAM reporter), and 4 μL of nuclease free water. PCR conditions were as follows: reverse transcription at 55 °C for 10 min and enzyme activation at 95 °C for 2 min followed by 50 cycles of denaturation for 10 s at 95 °C and annealing for 60 s at 60 °C. LightCycler 480 software (version 1.5) automatically analyzed the results. 85B mRNA and 16S RNA

Table 1
The distrubution of MGIT 960 and 85B mRNA/16S rRNA results of 30 *M. tuberculosis* positive patients with pulmonary tuberculosis before (day 0) and after the start of the tuberculostatic drugs.

	MGIT n(%)		85B mRNA/16S rRNA n (%)	
	Positive	Negative	Positive	Negative
Day 0	30 (100)	0 (0)	30 (100)	0 (0)
Day 15	17 (56.6)	13 (43.3)	17 (56.6)	13 (43.3)
Day 30	7 (23.3)	23 (76.6)	8 (26.67)	22 (73.3)

results were analysed according to the deltaCt method by relative quantation modüle of software.

Statistical analyses

The Pearson correlation coefficient was used to assess the MGIT 960 and 85B mRNA comparison for day-30 samples. Student's t-test was used to compare the mean ages and sex between groups. The Mann–Whitney U test was used to compare the 85B mRNA expression between the resistant and susceptible groups. All statistical analyses were performed using the SPSS version 20 (IBM Corporation, Somers, NY, USA) and MedCalc version 13 (MedCalc, Mariakerke, Belgium) statistical software packages.

Results

The sex distribution (M/F) and mean age of the patients, respectively, were 16/14 and 38.63 ± 16.33 years (range 21–76 years). The distribution of the MGIT 960 and 85B mRNA results of the 30 *M. tuberculosis*-positive patients with pulmonary TB before (day 0) and after the start of the anti-TB drugs is shown in Table 1. There were 100% correlations between the MGIT 960 and 85B mRNA levels for samples from days 0 and 15, but the Pearson correlation coefficient was 0.914853 for the day-30 samples.

Table 2 shows the drug susceptibility testing results of patients 1, 2, 4, 6, 13, and 16, who had resistant and multidrug-resistant TB (MDR-TB) on days 0 and 30 and one susceptible strain on day 0. While five of the strains were MDR, the other two strains were isoniazid- and streptomycin-resistant. The highest resistance ratio was observed for isoniazid (23.3%), followed by streptomycin (20%). Only one susceptible strain indicated positivity (480 level) on day 30, whereas the other 22 *M. tuberculosis* strains were negative for 85B mRNA on day 30. We suggested it as false positive.

Table 3 shows the 85B mRNA expression levels and culture results of antibiotic-susceptible and -resistant *M. tuberculosis* strains on days 0, 15, and 30. The 85B mRNA levels of seven MDR *M. tuberculosis* strains on days 0, 15, and 30 are shown in Fig. 1. There are seven strains with MDR TB. In the comparison of the 85B mRNA levels between the resistant and susceptible *M. tuberculosis* strains on days 0 and 30, the resistant strains had significantly different 85B mRNA levels than the susceptible strains ($p < 0.05$ [p:0.026]) (Fig. 2).

Discussion

To understand the killing efficacy of a TB treatment, at least two months are required to observe the conversion of a sputum culture from positive to negative, which has been shown to correlate with the relapse rate 2 years after the finalization of therapy [17]. In this study, we measured 85B mRNA levels in sputum collected from TB patients that received one month of therapy. We aimed to evaluate the usefulness of 85B mRNA levels as a surrogate marker of TB treatment effectiveness. Our main aim was to shorten the duration of tests that evaluate the efficiency of anti-TB treatment regimens.

In our previous study, RT-qPCR targeting of the 85B gene of *M. tuberculosis* seemed to be a more useful and rapid technique than DNA-based methods for detecting live *M. tuberculosis* bacilli from sputum specimens [19]. There is great need for TB surrogate biomarkers that can help us monitor anti-TB treatment outcomes and predict treatment success. There were no differences between the MGIT 960 system and 85B mRNA expression results for days 0

Table 2
Drug Susceptibility Testing (DST) results of 1, 2, 4, 6, 13 and 16 patients with resistant and multidrug-resistant tuberculosis (MDR-TB) strains and 12 patient with one susceptible strain on day 0 and 30.

No	Gender	Age	Antituberculosis antibiotics (day 0 and 30)				
			Isoniazid	Rifampin	Pyrazinamide	Ethambutol	Streptomyocin
1	F	50	R	R	S	S	R
2	F	22	R	R	S	S	R
4	M	16	R	R	S	S	S
5	F	23	R	S	S	S	R
6	M	57	R	R	R	R	R
12[a]	E	18	S	S	S	S	S
13	F	16	R	R	R	R	R
16	M	67	R	S	S	S	R

[a] No growth was observed on day 30.

Table 3
Quantification of the bacillary load (log) and culture results of eight patients (one susceptible and seven resistant strains) monitored during 30 days of specific treatment by 85B mRNA/16S rRNA relative fold change (log).

No	Gender	Age	Day 0		Day 15		Day 30	
			MGIT	85B mRNA/16S rRNA	MGIT	85B mRNA/16S rRNA	MGIT	85B mRNA/16S rRNA
1	K	50	Positive	5.12	Positive	4.13	Positive	3.51
2	K	22	Positive	5.10	Positive	4.10	Positive	3.61
4	E	16	Positive	5.06	Positive	4.15	Positive	3.98
5	K	23	Positive	4.99	Positive	4.05	Positive	3.56
6	E	57	Positive	4.94	Positive	3.99	Positive	3.45
12	E	18	Positive	5.05	Negative	2.83	Positive	2.55
13	K	16	Positive	4.95	Positive	4.18	Positive	4.04
16	E	67	Positive	4.90	Positive	3.95	Positive	3.47

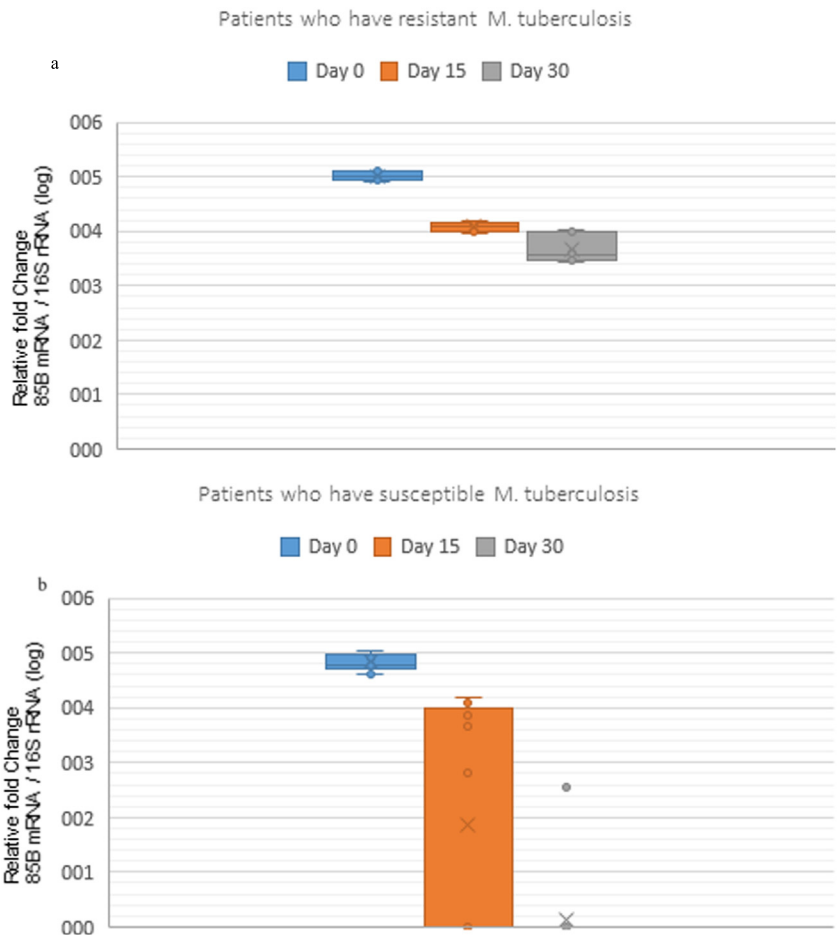

Fig. 1. (a) The distribution of 85B mRNA gene expression for antibiotic-resistant *M. tuberculosis* strains on days 0, 15, and 30. (b) The distribution of 85B mRNA gene expression for antibiotic-susceptible *M. tuberculosis* strains on days 0, 15, and 30.

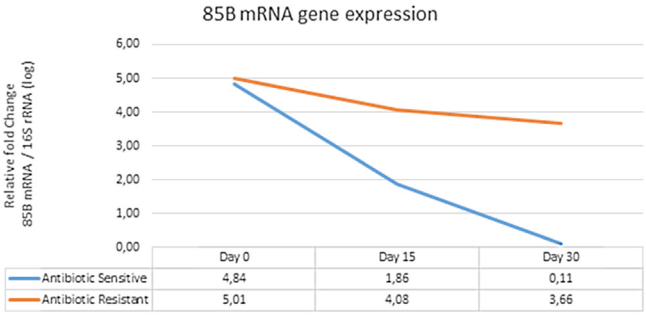

Fig. 2. Quantification of 85B mRNA gene expression (log) for antibiotic-susceptible and resistant *M. tuberculosis* strains on days 0, 15, and 30.

and 15, but there was only one more positive case for the 85B mRNA expression results than the MGIT 960 results. The positive case for 85B mRNA expression was also very weak (480), but the Pearson correlation coefficient decreased to 0.914853 for day-30 samples between the MGIT 960 and 85B mRNA results, whereas the correlation was 100% between these groups for the day-0 and day-15 samples. We suggested it as false-positive but decreased the correlation between RT-qPCR and culture. *M. tuberculosis* strains that were resistant to either isoniazid or rifampin showed no reductions in 85B mRNA expression in the presence of the anti-TB antibiotics. Only one strain, which was isolated from patient 12, showed very weak 85B mRNA expression but no growth in the MGIT 960 system.

There are some related studies in the literature. Montenegro et al. [20] evaluated 52 patients with pulmonary TB by culture and RT-qPCR for a 30-day treatment. They detected 85B mRNA in the sputum samples of 52 patients with a confirmed diagnosis of pulmonary TB on day 0. Then, during treatment, they detected 85B mRNA in 13 patients on day 15 and in only three patients on day 30. Therefore, they concluded that *M. tuberculosis* mRNA in the sputum samples can be used as a prognostic marker and that 85B mRNA quantification is very promising as an early and reliable indicator for monitoring responses to treatment, drug resistance, re-infection, and relapse. In another study, Hellyer et al. [10] reported that exposing sensitive *M. tuberculosis* strains to isoniazid or rifampin for 24 h reduced the levels of 85B mRNA to <4 and <0.01%, respectively, but no reduction was observed in drug-free control cultures. In contrast, the levels of IS6110 DNA and 16S rRNA were not reduced in the same period. In a similar study, Li et al. [14] detected isocitrate lyase mRNA in the sputum of culture-positive TB patients who received a rifampin-based regimen for 1 month. At 2 months, isocitrate lyase mRNA levels in the sputum correlated more closely with liquid growth than solid culture medium.

Desjardin et al. [17] reported that levels of 85B mRNA declined after the initiation of anti-TB therapy and that 90% of patients converted negative after 2 months of treatment. They concluded that the rapid disappearance of *M. tuberculosis* mRNA from sputum suggests that it is a good indicator of microbial viability and a useful marker for rapidly assessing responses to chemotherapy. Meanwhile, Yasir et al. [21] studied 29 *M. tuberculosis*-positive specimens for culture and mRNA before initiating TB chemotherapy. They detected 27 (93.1%) and 28 (96.5%) of specimens as negative by

culture while, detecting 26 (89.6%) and 28 (96.5%) as negative by mRNA. They finally concluded that rapid decline in the mRNA levels correlated with rapid culture clearance after anti-TB therapy. Mdivani et al. [3] studied 65 patients with newly diagnosed TB who were treated with a standardized first-line anti-TB treatment. The sputum specimens were evaluated at week 2 and at months 1, 2, and 4 after therapy initiation. The overall agreement between the culture and mRNA RT-PCR results was 87.1%. They concluded that for monitoring the efficacy of treatment, the mRNA RT-PCR results were similar to those of culture at the follow-up time points.

In another study, Jou et al. [22] used an IS6110-targeted DNA PCR and all the *M. tuberculosis* samples, though 13 days of isoniazid treatment, continued to be positive. They demonstrated the superiority of an mRNA target in the detection of mycobacterial viability; on the other hand, their STN RT-PCR assay, which targeted the mRNA coding for the ubiquitous 85B antigen of mycobacteria, could not distinguish between those cultures that contained the antibiotic and those that did not. Subcultures on LJ agar confirmed the viability assessments of the STN RT-PCR. Our RT-qPCR-based 85B mRNA expression results are in line with the results of other studies.

In the study of Wallis and Johnson [23], a 72% decrease in the 85B mRNA levels was observed during treatment from days 0 to 4, and followed by a 99% decrease between days 7 and 14. In other words, bacterial CFU counts declined 85% within the first 4 days of treatment and by an additional 63% from days 4 to 14. Ninety percent of TB cases convert negative, as evaluated by 85B mRNA expression, after 2 months of therapy. Clearance of the 16S RNA followed an intermediate pattern. Our seven *M. tuberculosis* strains with MDR indicated positive results for 85B mRNA with mostly non-significant decreases. In such situations, the anti-TB treatment regimen should be shifted to second-line anti-TB antibiotics. This will supply an optimal treatment regimen for patients with MDR TB [23].

In conclusion, detecting mycobacterial viability via RT-qPCR of 85B rRNA to monitor the efficacy of anti-TB treatment seems to be a very promising way to follow-up TB patients under the anti-TB treatment. The advantage of this method is speed. It takes weeks to wait for the results of both solid and liquid mycobacterial cultures, whereas RT-qPCR takes a few hours. On the other hand, we had some limitations, such as limited financial resources, which only allowed us to study a 2-month period and prevented us from expanding the sample size beyond 30 patients. Research with larger samples is needed to indicate the advantage of 85B mRNA as a surrogate marker for the clinical and therapeutic monitoring of patients with pulmonary TB.

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Competing interests

None declared.

Ethical approval

Not required.

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