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Correlation of Sputum mir-144 Copy Levels with Treatment Response among Pulmonary Tuberculosis Patients

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ABSTRACT

Background: *MicroRNAs are approximately 22 nucleotides (nt) in length, small non-coding RNAs (ncRNAs), playing a vital role during post-transcriptional regulation of mycobacterial infection. Few sputum microRNAs (sputum miR) were evaluated in literature for their role as potential biomarkers in diagnosing Pulmonary Tuberculosis (PTB), but the correlation with a full course of Tuberculosis (TB) treatment is yet to be ascertained.* ***Methods:*** *Sputum samples of forty-six PTB patients were collected for Acid Fast Bacilli (AFB) staining and sputum microRNA-144 (sputum miR-144) copy analysis, before, during, and at the end of treatment as per Indian National guidelines. Twelve patients of Asthma or Chronic Obstructive Pulmonary Disease (COPD), not in infective exacerbation, were recruited as controls. Sputum miR-144 copy levels were measured by quantitative Real-Time PCR (qRT-PCR) method. p****Results:*** *The difference between the baseline sputum miR-144 copy levels of patients and that of controls was found to be significantly higher (p <0.001). Significant up-regulation of sputum miR-144 copy levels at the baseline, as compared to controls, and significant downregulation during and after the full course of treatment was noted (all p <0.001).* ***Conclusion:*** *The potential role of sputum miR-144 as a simple and non-invasive surrogate biomarker in the diagnosis and treatment of PTB gains importances.*

Keywords: Biomarkers, *Mycobacterium tuberculosis*, Real-time polymerase chain reaction, MicroRNAs, Antitubercular agents

INTRODUCTION

Globally, Tuberculosis (TB) is the leading cause of mortality from a single infectious agent [1]. The incidence was found to be 10 million affected people in 2018, as per the recent Global TB report 2019 [1]. India is one of the 14 countries mentioned in the three high burden countries given by the World Health Organisation (WHO) for the period 2016-2020 namely TB, Multi-Drug Resistant TB (MDR-TB), and Human Immunodeficiency Virus/Tuberculosis (HIV/TB) co-infection [1]. With the emergence of molecular biology in the field of TB diagnostics, newer technologies are explored to provide a faster and accurate diagnosis of TB.

Up to 60% of the human genome is estimated to be under the regulation of miRNAs [2]. Discovered more than 25 years ago, these small non-coding miRNAs are critical in the embryogenesis of the lung [3]. miRNAs have been linked to the pathogenesis of various respiratory diseases like lung cancer, asthma, Chronic Obstructive Pulmonary Disease (COPD), pneumonia, pulmonary tuberculosis, and Pulmonary Hypertension (PH) [3-6]. If the triggers and the mechanisms behind their dysregulation are known, targeted therapies are possible. Few studies have probed the role of miRNAs in the pathogenesis of tuberculosis [7-8]. To date, multiple serum miRNAs have been studied for their

role in molecular pathogenesis, diagnosis of tuberculosis, and their potential to discriminate latent TB from active tuberculosis, using different sequencing mechanisms [9-14].

While many studies have evaluated the role of circulating serum miRNAs in TB, their significance in the sputum sample has been analyzed only in a few studies [15-16]. Sputum culture evaluation is considered the gold standard in the diagnosis of Pulmonary Tuberculosis (PTB). But owing to the slow doubling time of Mycobacterium tuberculosis, results take approximately 6 weeks. Sputum smear microscopy is used for day-to-day practice as it is easy to perform and cheap but lacks sensitivity [17]. The emergence of molecular tests has drastically reduced the diagnosis time, but they too have some drawbacks-minimum volume of sputum needed to run the test and expensive kits/cartridges. Hence our quest for better diagnostic tools for TB remains significant to date.

Mycobacterium tuberculosis (MTB) induces the expression of miR-144*/miR-144-5p in the monocytes and macrophages of human beings, which in turn causes inhibition of DNA Damage Regulated Autophagy Modulator 2 (DRAM2). Because of DRAM2 inhibition, there is decreased autophagy induction leading to increased miR-144* levels in Peripheral Blood Mononuclear Cells (PBMC) of TB patients [18]. The validation of circulating serum miRNAs with sputum miRNAs gains importance since it is a non-invasive test and all our current diagnostic tools are sputum-based assays. Recently, Yan Lv et al., have observed a significant correlation of sputum and serum miRNA 144 levels with initial treatment response i.e., after 1 month of antitubercular therapy [16]. As the treatment duration of PTB varies between 6-8 months, correlation of miR-144 copy levels with sputum smear microscopy throughout the course is needed to ascertain that the initial down-regulation after ATT noted by previous studies was not by chance. Hence, we conceptualized a study to correlate the sputum miR-144 copies with treatment response during the full course of anti-TB chemotherapy. The initial results of our study have been previously reported in the form of an abstract [19].

MATERIAL AND METHODS

Study Design

This is an analytical (observational) study to explore the correlation between sputum miR-144 levels with response to PTB treatment.

Subjects

Adult (>18 years) patients attending Pulmonary Medicine Outpatient Department or admitted under Pulmonary Medicine Department with cough for 2 weeks or more, along with fever, loss of appetite and weight, malaise, and night sweats, and diagnosed to have sputum smear-positive/smear-negative Pulmonary tuberculosis, as per Revised National Tuberculosis Control Program (RNTCP)-Technical and Operational Guidelines (TOG) for TB control in India 2016, were enrolled in the study [20]. Both new cases and previously treated patients were enrolled, after written informed consent. Patients with the following conditions were excluded from the study: Pregnant women, patients with Drug Resistant-TB (DR-TB), Extrapulmonary Tuberculosis (EPTB), HIV/TB co-infection, or any immunosuppressive conditions, taking immunosuppressive therapy, malignancy (of any organ), psychiatric illness, hemolytic anemia, thalassemia, ischaemic heart disease, type 2 diabetes mellitus, grave's disease and those who were not willing to participate. Patients, newly diagnosed with any of the above-mentioned conditions during the study period, were also excluded. The study was approved by the Institutional Human Ethics Committee (Faculty/2016/03/27) and was conducted in compliance with the Declaration of Helsinki [21].

Initially, 12 controls (Stable Asthmatics or COPD) were enrolled in the study for analyzing the difference in baseline miR-144 copies among PTB patients and controls. The enrollment of the patients was continued after determining an initial difference between the two patient cohorts. Thus, a total of 46 patients and 12 controls were enrolled for this study.

Data Collection and Follow up

Sputum AFB samples were collected as per RNTCP protocol (2 samples-one 'spot' and one early morning sample) along with one sputum sample for miR-144. All sputum samples were positive for AFB at the baseline, except for one. Computed-Tomography (CT) of Thorax followed by Bronchoalveolar Lavage (BAL) was done for the smear-negative patient and BAL was found to be positive for AFB. Patients were started on Category I (6 months duration) or II treatment (8 months duration) as per RNTCP. One patient did not follow up after the first month. Two patients remained

sputum AFB positive at the end of the Intensive Phase (IP) but their sputum Cartridge Based-Nucleic Acid Amplification Test (CB-NAAT) sample did not detect resistance to Rifampicin. All patients showed clinical improvement at the end of treatment and were sputum AFB negative. The end of treatment sputum sample for miR-144 analysis (third sample) was collected for all these patients.

RNA Isolation

miRNA was isolated using the HELINI™ PureFast Total RNA (miRNA) Mini spin Prep Kit (Helini, Biomolecules, Pvt India Ltd, India; Cat. No. 2008-25/50/100 preparations) according to the manufacturer's protocol.

Quantitative Real-Time PCR

Helini microRNA Real-Time PCR kit (Helini, Biomolecules, Pvt India Ltd, India), which includes cDNA mix, miR-144-3p cDNA primer mix, internal control-cDNA primer mix, probe PCR Master mix, and miR-144-30 probe primer mix was used. Sputum miRNAs were quantified using CFX96TM Real-Time Systems (BIORAD, California, USA). All the steps were followed according to the manufacturer's instructions. Each reaction contained 2.5 microliters (µl) of sputum miRNA-cDNA in a total of 25 µl reaction. The qRT-PCR reaction was performed using the same kit with the following conditions: Taq enzyme activation at 95°C for 15 min., denaturation at 95°C for 20 sec., annealing/data collection at 56°C for 20 sec., and extension at 72°C for 20 sec.. Standard curves for absolute quantification was generated using commercially available synthetic miRNA oligonucleotides provided in the kit.

Results are reported as miRNA copy number per nanoliter of sputum, calculated based on the known copy number of the standards provided in the kit.

Analysis

Data were analyzed using IBM SPSS ver. 16.0 (IBM Co., Armonk, NY, USA). Quantitative data were recorded as mean ± standard deviation. Comparisons at baseline, end of IP, and end of anti-TB treatment were conducted by paired t-test. The other group comparisons were conducted by the use of the independent-samples t-test in one-way repeated measures of ANOVA (Post-hoc test: Bonferroni and Hochberg). Results were considered to be statistically significant when the p-value was <0.05.

RESULTS AND DISCUSSION

Data from 45 patients were considered for analysis. The demographic profile of the patients is given below (Table 1).

Table 1 Baseline patient characteristics

Parameter		n= 45
Gender	Male	36 (80%)
	Female	9 (20%)
Age, in years	Mean ± SD	47.02 ± 16.38
	Range	19-80
New case vs. Previously treated		40 (88.89%), 5 (11.11%)
Cavity in chest X-Ray	Yes	12 (26.67%), 33 (73.33%)
	No	
Thrice weekly vs. Daily fixed-dose Combination		39 (86.67%), 6 (13.33%)
Sputum smear grading, mean baseline miR-144 (copies/nl)	1+, 59.78	5 (11.11%)
	2+, 60.36	21 (46.67%)
	3+, 60.03	13 (28.89%)
	Scanty+/Negative, 59.66	6 (13.33%)

The patients were predominantly male (36, 80%) with a mean age of (47.02 ± 16.38) years. A significant number of them (40 (88.89%)) were new cases and 33 patients (73.33%) had cavities in chest X-ray at baseline. Concerning their

type of treatment, 39 patients (86.67%) took thrice-weekly treatment, and 6 (13.33%) were started on a daily Fixed-Dose Combination (FDC) therapy.

Figure 1 depicting the baseline miR-144 copies across the age distribution (19-80) years signifies no change in the values with increasing age. Across the gender, there was no difference in the miR-144 copy levels. These values at baseline were slightly higher among females than males (59.66 vs 61.91, male vs female), though the number of females in the study population is less. Interestingly, the correlation between sputum smear positivity grading at baseline (as per RNTCP) and the miR-144 copy levels were found to be insignificant. The miR-144 copy levels per nanoliter (nl) corresponding to the sputum smear grades for AFB 1+, 2+, 3+, scanty positive/negative were 59.78, 60.36, 60.03, and 59.66 respectively. No linear increase in miR-144 copy levels with increasing AFB smear grading was noted.

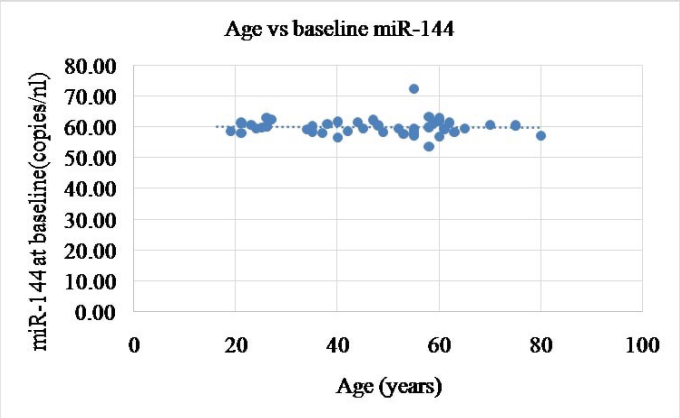

Figure 1 Scatter plot of individual sputum miR-144 copy levels at baseline against the patients' age

The distribution of individual miR-144 copy levels across the patients' age does not change with an increase in age and is clustered around 60 copies/nl.

The mean miR-144 copy level for the controls was (14.03 ± 4.21) copies/nl. The mean baseline value for patients was (60.11 ± 2.68) copies/nl. The difference between these two values was found to be statistically significant ($p < 0.001$) (Figure 2). While the mean copy level at the end of the intensive phase of treatment was (28.85 ± 3.68) copies/nl, the end of treatment value was found to be (18.64 ± 4.64) copies/nl. Interestingly, the overall difference between all the four values was also found to be statistically significant ($p < 0.001$). One-on-one comparisons between baseline and end of IP values, end of IP and end of treatment values, and end of treatment with baseline values were also found to be statistically significant ($p < 0.001$) (Figure 2). When the three values for all the patients across the treatment phases (from the baseline to the end of treatment) were expressed as a linear plot, a decreasing trend in sputum miR-144 copy levels was observed (Figure 3).

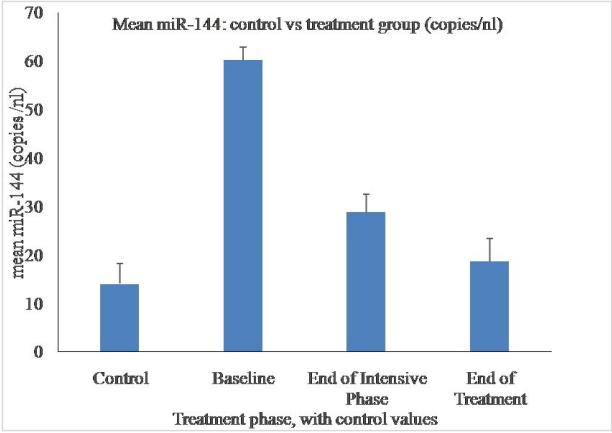

Figure 2 Comparison of miR-144 copies between controls and patients-at baseline, end of the intensive phase, and end of treatment

This bar diagram shows sputum miR-144 (copies/nl) among controls and patients. The copy levels are higher among patients, at baseline as compared to controls and there is a significant downregulation once treatment is initiated ($p < 0.001$). At the end of treatment, the sputum miR-144 copy levels are comparable to those of controls.

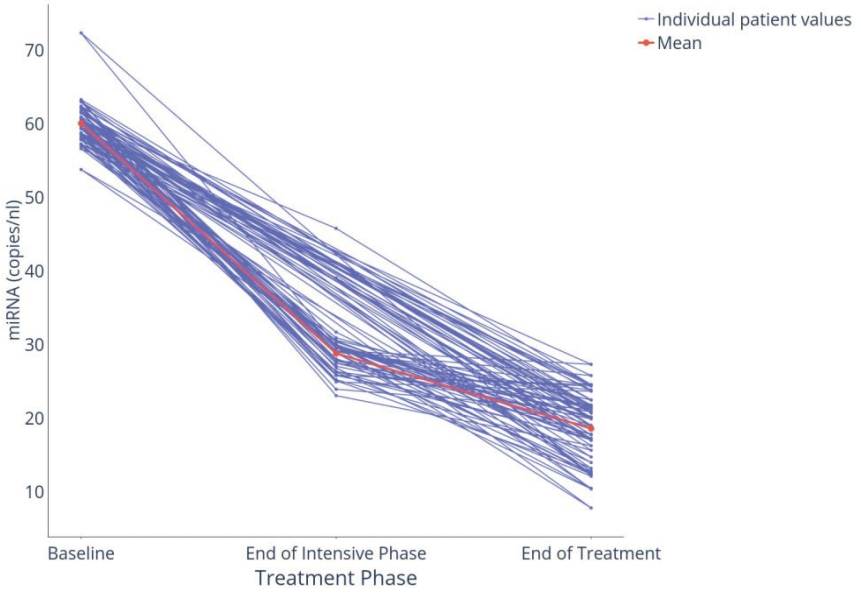

Figure 3 Linear plot showing individual sputum miR-144 copy levels throughout treatment

Individual sputum miR-144 copy levels are plotted throughout treatment. There is a significant downregulation among all the values with the mean value highlighted in red ($p < 0.001$).

While two of the patients remained sputum smear AFB positive (grades of positivity: both samples were 1+; baseline: 2+) at the end of IP, the rest had smear conversion by the end of 2 months of Intensive Phase. While one of them had been started on Category I Anti-tubercular therapy-Fixed-Dose Combination (CAT I ATT-FDC)-daily regimen, the other was on a thrice-weekly regimen. We analyzed their end of IP miR-144 copy levels with the baseline values. The percentage change in miR-144 copy levels from baseline to end of IP was calculated for the two patients (24.07%, 32.43%; Mean =28.25%). The overall percentage change for the 43 patients who had smear conversion at the end of IP was 52.99%. A two-tailed t-test for equality of means was performed and the difference between responders' and non-responders' miR-144 levels at the end of IP was found to be statistically significant ($p < 0.001$) (Table 2). Sputum CB-NAAT was done for them and was found to be MTB detected and Rifampicin sensitive. Hence the patients continued Continuation Phase (CP) as per drug-sensitive TB guidelines. miR-144 values of the non-responders showed down-regulation at the end of treatment correlated by their clinical improvement and sputum AFB negativity. No significant difference in the miR-144 values was observed between responders and non-responders at the end of treatment ($p=0.1960$).

Table 2 Comparison of sputum and miR-144 results between patients who had sputum smear conversion at the end of the Intensive phase and those with sputum smear-positive

Sputum AFB smear status-at the end of IP	Number of patients	Mean miR-144 copy levels at baseline	Mean miR-144 copy levels at the end of IP	Mean of change from the baseline at the end of IP %	Statistical analysis
1+	2	59.12	42.47	28.25	P <0.001*
negative	43	60.15	26.96	52.99	
* t-test for equality of means was used; p<0.001 is statistically highly significant; AFB: Acid Fast Bacilli; IP: Intensive phase of treatment					

DISCUSSION

Baseline Characteristics

We found no significant difference between the age and sex of the patients and their respective baseline sputum miR-144 copy levels. A similar finding was observed by Yanaihara et al., in which, they observed no age or sex difference between the serum miRNA levels and survival of adenocarcinoma patients postoperatively [22]. In a study correlating active TB and miR-29a expression profile using PBMCs, male patients were shown to have higher expression as compared to females, at baseline, and after 2 months of IP [23]. Circulating miR-144 was found to be elevated in a few other conditions due to its diverse roles in the pathogenesis of human diseases, from hemolytic anemia to malignancy [24-33].

Sputum miR-144 in PTB Diagnosis and Prognosis

Several studies have reported the ability to circulate miRNAs to differentiate between controls, Latent Tuberculosis Infection (LTBI), and TB disease status [34-37]. In our study, a statistically significant difference in sputum miR-144 copy levels was found between controls and active PTB patients ($p<0.001$).

A recent study using PBMCs and pleural fluid showed that there are changes in the systemic effects of miRNAs upon therapy [38]. They assessed miR-424 and miR-164a at baseline and observed that the values returned to normal after two months of successful therapy. In a similar study assessing four serum miR profiles, miR-16 showed down-regulation while miR-155 showed up-regulation upon treatment completion for active TB [37]. Though circulating miR levels were found to be stable, correlation with simultaneously collected sputum AFB samples was not done. Since Indian National guidelines stipulate sputum assessment for PTB patients during treatment and at the end of treatment, simultaneous sputum miR sample collection throughout antimycobacterial therapy is a viable option given the difficulties in processing serum samples like expertise, specialized equipment, and training needed to isolate specific cell types from the blood.

A report by Lv et al., correlated the serum and sputum expression levels of miR-144 levels and compared it with the control group [16]. This study has shown that sputum and serum miR-144 can be interchangeably used in the diagnosis and follow up of PTB. To assess treatment response, they followed up for one month, collected serum and sputum samples, and observed that both showed significant down-regulation of miR-144 expression profiles among responders. While the ability of sputum miR-144 to differentiate between controls and disease population is well understood in this study, the potential of miR-144 as a prognostic marker needed further evaluation.

The importance of follow-up sputum examination as and when there is clinical and/or microbiological deterioration can never be overemphasized in this era of drug resistance in TB. We followed up with the patients for their entire treatment period (6 or 8 months, based on whether they were a new case or previously treated). The down-regulation of sputum miR-144 among patients who had sputum for AFB smear conversion (done as a part of RNTCP guidelines) at the end of IP was found to be statistically highly significant ($p<0.001$).

As a Surrogate Marker for Non-Responders

The potential of miR-144 as a surrogate biomarker for clinical and microbiological deterioration during treatment needs to be noted. The ends of CP/end of treatment miR-144 copy levels were comparable to that of controls. The miR-144 copy levels of two patients who had smear positivity at the end of IP were found to be down-regulated at the end of treatment. Their values were comparable to that of the control group (69.24 ± 7.99 vs 61.72 ± 3.46, mean $\pm$ SD). Also, no statistical difference was observed between their sputum miR-144 copy levels to that of the rest 43 patients who had achieved smear conversion within two months of therapy ($p=0.196$).

To summarize, sputum miR-144 copy levels were able to differentiate between controls and PTB patients. It exhibited a significant down-regulation, in line with the sputum AFB conversion-at the end of IP and the end of CP/end of treatment. Furthermore, the downregulation of miR-144 copy levels was less among the two patients who did not have smear conversion at the end of IP as compared to those who had smear conversion. For these non-responders, as the clinical and microbiological improvement was noted at the end of treatment, miR-144 copy levels also showed down-regulation and became comparable to that of the rest.

Limitations

Our study mostly included Tamilian South Indian population and thus cannot be extrapolated for other ethnic popula-

tions. Further, we limited our scope to study only drug-sensitive and PTB population as it was an exploratory approach towards treatment response for the entire therapy duration. Hence the impact of Extrapulmonary TB (EPTB), Human Immunodeficiency Virus (HIV)-TB, and Drug-Resistant TB (DR-TB) subpopulations on sputum miR-144 copy levels were not studied. Though two patients were sputum AFB positive at the end of IP, none developed drug resistance, and hence the analysis of sputum miR-144 copy levels among the DR-TB subpopulation could not be performed.

Directions for Future Research

Similar longitudinal studies among multi-ethnic cohorts including the challenging DR-TB, HIV-TB subpopulations will provide more insight into the usage of miR-144 copy levels. Nevertheless, in our quest for better diagnostic and prognostic biomarkers to achieve global targets envisioned in the WHO's END TB strategy, molecular tests like this requires due consideration for their simple and non-invasive nature.

DECLARATION

Acknowledgments

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Declaration of Conflict Of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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