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Prospective Study

Diagnostic utility of microRNA profiles in cavitatory and non-cavitatory pulmonary tuberculosis: Research protocol

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Abstract

BACKGROUND

Tuberculosis (TB) is a common infection causing huge morbidity and mortality to mankind. The analytical methods used in diagnosing TB are not sensitive in paucibacillary infections and also require trained technical personnel. MicroRNAs are stable in serum and other body fluids, and hold great potential in the diagnosis of TB.

AIM

To analyze the dysregulated microRNA profiles among patients with cavitatory and non-cavitatory pulmonary TB.

METHODS

The prospective study will be conducted in a tertiary care center in India. Adult patients with newly diagnosed pulmonary TB will be included. There will be two groups: Patients with sputum positive pulmonary TB with cavity and without cavity (group1), and apparently healthy individuals (group 2). The participants will undergo sputum examination, Xpert *Mycobacterium TB* complex/resistance to rifampin (*Mtb*/RIF) assay, chest X-ray, and blood investigations and serum microRNA detection. Ethics approval has been obtained. Written informed consent will be obtained. Appropriate statistical analyses will be used.

RESULTS

MicroRNAs will be correlated with sputum positivity, Xpert *Mtb*/RIF assay, radiological involvement, inflammatory markers, and course of the disease among

cases and controls.

CONCLUSION
MicroRNAs could serve as potential diagnostic biomarkers in diagnostically challenging TB patients.

Key Words: Imaging; Inflammatory marker; MicroRNA; Molecular diagnosis; Pulmonary tuberculosis; CBNAAT

Core Tip: Tuberculosis (TB) is a multisystem infectious disease. The route of entry of *Mycobacterium tuberculosis* is *via* the respiratory system, hence the commonest presentation is lung TB. It has various presentations from subtle lesions to cavitation in the lung. If not treated in time, it spreads to various organs which can increase morbidity and mortality. Current diagnostic tools lack sensitivity and are time-consuming. Identification of the microRNA profiles in TB could help in devising point-of-care testing which may be used at bed side or physician consulting rooms.

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INTRODUCTION

Tuberculosis (TB) is a leading cause of disability, ranking thirteenth among the various causes which lead to mortality[1]. In countries where TB is common, the prevalence is 3.54%, while in other countries the prevalence is 1.43%[2]. In 2020, many deaths were due to TB, which could either be due to under-diagnosis or inappropriate management[3]. In 2016, 1.3 million deaths were due to TB alone and 0.37 million deaths were observed among human immunodeficiency virus-TB co-infection[4]. In 2021, more than 10 million individuals were found to be infected with *Mycobacterium tuberculosis* (*Mtb*), and the number of deaths due to TB was around two million[1]. According to the meta-analysis by Placeres *et al*[2], the prevalence of latent TB is 51.61% and 40.24% in high- and low-burden countries, respectively.

The BRICS countries, including India, China, South Africa, Russia and Brazil, are considered to be high TB-burden regions. Among these countries, India and China are affected more than other countries due to the large population. Due to the lack of awareness about the early stage of the disease, there is an increasing spread among the community. In spite of implementation of extensive measures for an early diagnosis and treatment, there is a continued gap between the clinical manifestation of the disease and treatment[5]. The United Nations Sustainable Development Goals has decided to control TB epidemic by the year 2030[1]. India marks the year 2022 as a milestone for the TB surveillance by the TB Elimination Program. It recorded a 13% increase in case notifications compared to the year 2021[6]. The clinical presentation and diagnosis of TB are complex due to the increase in the aging population, along with the increase in the prevalence of drug-resistant strains of *Mtb*[4].

Pulmonary TB

Mtb generally enters the human body through the lungs and starts replicating inside the macrophages, thus forming granulomas. The bacteria have the ability to evade the immune system in the lungs, and continue to multiply in the macrophages. The bacilli in the lungs have three fates: In 70%-80% of individuals, the infection is latent and is unable to infect others, which is called latent TB infection; in 10%-20% of individuals, caseous granuloma opens up disseminating infection through the breath of the infected person to other members in the family as well as neighborhood; and in the rest 10%, the bacteria spread beyond the lungs to establish extrapulmonary TB[7,8]. Most TB related deaths can be prevented with early diagnosis and appropriate treatment. However, the limited availability of a reliable diagnostic tool poses a major obstacle in the control of this epidemic. The currently available standard methods of TB diagnosis rely majorly on the adequacy of bacterial load. Owing to this limitation, emphasis has been laid on the need for the development of diagnostic tests which could be biomarkers of host responses. These tools can be used for diagnosis of the disease and monitoring the treatment outcomes[9,10].

Diagnosis of pulmonary TB

According to the World Health Organization 2013, pulmonary TB refers to the confirmation of TB based on the evidences from either a clinical or lab diagnosis of TB that involves the lower respiratory tract. In a person with latent inactive TB, the bacteria may be present in the body without causing disease[11]. Routinely done chest X-ray is not sensitive or specific, and shows a normal picture in spite of the disease presence. Chest computerized tomography can be sensitive in detecting microscopic or undefined lesions, which cannot be detected by chest X-ray. Further confirmation is made possible by detecting the bacilli in the sputum specimens. However, not all patients with active TB will be able to cough out enough sputum for laboratory analysis[11].

Sputum in diagnosis of pulmonary TB

For the diagnosis of TB, two sputum specimens have to be examined by microscopy. Microscopic examination of sputum smear is quick, easy, and cost-effective for detecting TB. However, at least 5000-10000 bacilli/mL of sputum must be present and the report is obtained within a day. Examination of Ziehl-Neelsen staining of sputum smears by light microscopy is widely used. Based on the number of bacilli in the smears, they are graded and thus the infectivity of the person. However, smears are not fool-proof in that patients with acid fast bacilli smears which are negative have been shown to have cultures positive for acid-fast-bacilli[11]. The laboratory test which is used for substantiating TB is culture. Also, it is mandatory to perform drug-sensitivity testing and genetic makeup of the organism to improve diagnostic accuracy and management. *Mtb* is grown on solid media, which can be the Lowenstein-Jensen slope or broth media. Liquid media are better, since the results will be available within two weeks[11]. In the recent times, diagnosis is made possible in early TB infection, with the advent of molecular assays such as nucleic acid amplification (NAA) test. A positive NAA test is considered to be diagnostic, especially for those who have an increased risk for the disease. Hence, public health TB programs at the community level, have the access to NAA testing for quicker diagnosis of TB. NAA testing has high positive predictive value and has the ability to rapidly identify the presence of *bacilli* in most smear-negative but culture-positive specimens[11].

Other investigations

The tuberculin skin test and interferon-gamma release assays are the immunology-based assays. But, the limitation with these assays is that they fail to detect the infection in the early stages. Since immune response takes at least eight weeks to get established, the test becomes positive only after this period[12].

MicroRNAs in pulmonary TB

There is a growing interest in identifying relevant microRNAs in the blood of patients using a PCR-based assay[13-15]. This could further enable identification of latent and active TB, as well as extrapulmonary TB. Circulating microRNAs play regulatory roles in various metabolic pathways and serve as ideal markers to detect *Mtb*[16]. MicroRNAs are 18-25-nucleotide-long non-coding RNAs and are stable in the body fluids[17-20]. MicroRNAs are considered to be ideal biomarkers, since they are easily accessible in the peripheral circulation, and have high specificity, sensitivity, and stability. MicroRNAs as biomarkers of disease, have been demonstrated in many malignancies and common infectious diseases[21-23]. During the disease process, certain microRNAs get up-regulated while few get down-regulated compared to healthy individuals. MicroRNAs such as miR-146, -31, and -150 are down-regulated while miR-16, -20, -21, -29, -30, -99, -155, -193, -223, -299, -365, -486, and let-7 family are upregulated in various stages of pathogenesis of the disease[8]. There are limited studies on the dysregulated microRNAs among the specific clinical subtypes of TB.

Several studies have been conducted over the last decade, for categorizing microRNAs as biomarkers of TB. Since TB at the latent stage is very inconclusive, these markers should be able to differentiate latent disease form the active one[24-26]. Pro-apoptotic microRNAs are found to be down-regulated in TB. Few microRNAs are activated by the toll-like receptor (TLR) pathway, which regulates inflammation by targeting interleukin-1α. MicroRNAs, by negatively regulating the insulin-like growth factor (IGF) pathway, target cell differentiation. In the macrophages, the IGF pathway activates lipopolysaccharide induced nuclear factor kappa B with release of inflammatory mediators[8]. The observed link between dysegulated microRNAs and active TB paves the way for better understanding of the pathogenic mechanisms[27].

TB is ranked the second leading cause of mortality among all the infectious diseases. This could be due to inadequacy in performance of the existing biomarkers to differentiate the varied presentations of pulmonary TB. MicroRNAs could serve as ideal diagnostic biomarkers of pulmonary TB. MicroRNAs could differentiate cavitatory from non-cavitatory pulmonary TB so that targeted therapy can be initiated according to the type and extent of the disease. MicroRNAs being very stable and can be implemented as point-of-care testing in diagnostically challenging groups of TB patients. These tests can be effectively utilized by the clinicians in the outpatient department and in the patient wards, and can also be used by community health workers in the society. Thus, this may offer hope on the eradication of TB in a planned and strategic way.

Therefore, circulating microRNAs could be a promising diagnostic tool which shall address the different aspects of the disease. It has been well established that the cavitatory TB has higher prevalence rates of multi-drug-resistant TB, higher relapse rates, and more complications in the long run. Hence, identifying the microRNAs specific for the group, would help in prognosticating the patients.

MATERIALS AND METHODS

Study design

This is a proof-of-concept study, so only a convenient sample has been chosen. MicroRNAs will be altered in all the TB infected patients. So, the expected percentage of microRNA positivity among the cases is 100%. This sample size is calculated based on the assumption that the expected percentage of microRNA positivity among the patients affected with TB is 100%. Studies of microRNAs in TB are available. But they have not been associated with the type and extent of lesions in the lungs. The existing diagnostic tools have a sensitivity and specificity both up to 90%. The microRNA profiles could be better than the existing diagnostic tests, and they have the potential to offer a more than 90% sensitivity as well as specificity. However, no reproducible data is available in the published literature on the difference in proportion of upregulation between cases and non-cases, as it is highly variable across the published studies. So,

computing sample size based on this might not be feasible. The protocol aims to study the upregulated and downregulated microRNAs and their association with the clinical subtypes of pulmonary TB.

Primary objectives: (1) To generate microRNA profiles specific to clinical subtype of tuberculosis (*i.e.*, cavitatory and non-cavitatory variants); (2) To correlate the microRNA profiles with sputum positivity in pulmonary TB patients; (3) To compare microRNA profiles with Xpert *Mtb*/resistance to rifampin (RIF) assay, inflammatory markers, and imaging techniques in patients with pulmonary TB; and (4) To compare the clinical course of the disease at the end of three months and six months with the baseline microRNA patterns in respective sub-groups of pulmonary TB.

Null hypothesis: MicroRNA profiles shall not be significantly altered to diagnose cavitatory and non-cavitatory TB.

Alternate hypothesis: Altered profiles of microRNAs could assist in the diagnosis of cavitatory and non-cavitatory tuberculosis.

With regard to sample size in convenient sampling, there are studies saying that a minimum of 30 could be the adequate sample size. Some studies say that 20 is adequate or 10 could be adequate, and there is no adequate information with regard to this sample size. However, the most important anticipated drawback could be bias. But the chances for bias in this study could be less since we are trying to identify profiles (set of upregulated and downregulated) of microRNAs specific to cavitatory and non-cavitatory TB. Moreover, we have stringent inclusion and exclusion criteria for inclusion of study participants.

We have considered geographical proximity, availability at a given time, or availability of financial support. Probably this study could facilitate us in conducting an in-depth study in future.

Inclusion criteria

Clinical, radiological, smear and culture proved new cases to whom treatment has not been initiated.

Group 1: This group will be composed of sputum-positive (acid-fast staining or CBNAAT) pulmonary TB patients. Sputum-positive (acid-fast staining or CBNAAT) pulmonary TB patients will be further classified as cavitatory (n = 20) or non-cavitatory type (n = 20) by chest X-ray or CT scanning (n = 20).

Group 2: This group will consist of age- and gender-matched persons who are free of acute illness and with no history of pre-existing chronic medical illness (n = 13).

Exclusion criteria

The exclusion criteria will be: (1) Age > 60 years and < 18 years; (2) Current smoking or alcoholism; (3) Presence of any prior chronic medical illness (diabetes mellitus, hypertension, liver disease, renal disease, endocrine disease, cerebro- and cardiovascular diseases, autoimmune disorders, haematological disorders other than iron deficiency anaemia, and recently cured cancer or active cancer); (4) Pregnancy; (5) Drug-resistant TB; (6) TB patients with human immunodeficiency virus co-infection; (7) Previously treated with anti-tuberculosis therapy, and other active lung infections like community acquired pneumonia; (8) and On drugs such as corticosteroids, anti-inflammatory drugs, anticonvulsants, and anticancer drugs.

The policies have changed in India. Most of the TB patients are being managed by the smaller government district hospitals. Hence, the number of TB patients approaching tertiary care hospitals like our institution is a slightly lesser compared to the scenario which existed few years back. Hence, the time taken to include participants also could take longer.

Ethics statement

The proposed study will be conducted at Sri Ramachandra Institute of Higher Education and Research, Chennai, India. The Institutional Ethics Committee (IEC) has approved the study (IEC number IEC/21/JUN/163/43). The outcome variables will be performed at baseline, and the patients will be followed as per the standard of care. The study is registered with Indian Council of Medical Research, India, CTRI/2023/08/056740 (https://ctri.nic.in/Clinicaltrials/Login.php).

Investigations to be done

All the patients will be subjected to analysis of sputum smear and culture, Xpert *Mtb*/RIF assay, chest X-ray, complete blood count, interleukin 6 (IL-6) and matrix metalloproteinase-1 (MMP-1), and serum microRNAs. Statistical analyses will be done. A p-value less than 0.05 will be considered statistically significant. The Xpert *Mtb*/RIF assay is a test that simultaneously detects *Mtb* complex and RIF. There is a chronic inflammatory state associated with TB which could be reflected by analysis of IL-6. One of the enzymes involved in cavity formation is MMP-1, measurement of which could help in identifying early cases of cavitatory TB. MMP-1 levels can be associated with microRNA profiles.

Isolation and analysis of microRNAs from serum samples

Five milliliter of venous blood is collected into a sterile vacutainer. Samples are centrifuged at 3000 rpm for 10 minutes, and the supernatant serum is aliquoted and stored immediately at -80°C until analysis. Total RNA from serum samples is then isolated using TRIzol and further purified using a RNeasy minikit according to the manufacturer's instructions. The concentration and quality of RNA are measured with a Nanodrop spectrophotometer and checked by gel electrophoresis. After RNA isolation from the samples, microRNA labelling and hybridization and microanalysis of the RNA (equal

amounts of RNA from 5 participants of each respective group is pooled for profiling) are done according to the standard guidelines.

Real-time PCR analysis

To confirm that the pattern of specific differentially expressed microRNAs, a validation study using independent samples is performed. Reverse transcription-PCR is performed to confirm the array results. Each sample is normalized on the basis of an appropriate endogenous control. The experiment is conducted in triplicate. Statistically significant occurrence is used to evaluate the diagnostic effect of the candidate microRNAs.

PureFast® microRNA mini spin purification kit (containing Carrier RNA, Lysis buffer, Wash Buffer-1, Wash Buffer-2, and Spin columns with collection tube and elution buffer) and microRNA real-time kit are procured from HELINI Biomolecules, Chennai, India.

cDNA synthesis protocol

cDNA Synthesis Detection Mix contains cDNA, microRNA-cDNA primer, RT enzyme, and purified microR. PCR vials are centrifuged briefly before placing into the thermal cycler. cDNA synthesis thermal profile takes place in two steps. qPCR Detection Mix contains probe PCR master mix, microR PP mix, PCR grade water, and cDNA.

Real-time PCR thermal profile

After 45 cycles, relative gene expression analysis is automatically done by the qPCR machine software and results are interpreted. Quantification of gene expression of interest is accomplished by measuring the fractional cycle number at which the amount of expression reaches a fixed threshold (Ct), which is directly related to the amount of product. The PCR cycle at which fluorescence measured by the instrument reaches a threshold value is called threshold cycle (Ct), which is set at a point that is above the background signal. The threshold cycle is inversely proportional to the log of the initial copy number. The amplification plot with amplification cycles *vs* fluorescence units is shown in Figure 1.

The dysregulated microRNAs in pulmonary TB patients are compared with those in healthy controls. Further the microRNAs will be correlated with sputum positivity, Xpert *Mtb*/RIF assay, radiological involvement, inflammatory markers, and the course of the disease.

Statistical analysis

Categorical variables will be analyzed by the χ^2 or Fischer's exact test. Continuous variables will be analyzed by one-way analysis of variance or the Kruskal-Wallis test. *Post-hoc* analysis using the least significant difference test will be used to analyze the results of the statistical comparisons. A p-value less than 0.05 will be considered statistically significant. Statistical analyses will be done with SPSS version 16.

RESULTS

During the progression of the disease, specific microRNAs will be selectively upregulated or downregulated. This phenomenon varies depending on the clinical presentation of the disease and whether it manifests as pulmonary TB with cavity formation or without cavity. This distinct modulation of microRNA expression acts as a pivotal determinant, offering unique signatures that can effectively indicate the pathogenic stage of the disease.

The inclusion criteria for patients participating in the study necessitate their identification *via* sputum assays, the Xpert *Mtb*/RIF test, and confirmation of radiological lung involvement. All patients will undergo comprehensive blood tests to evaluate parameters such as complete blood count and inflammatory markers. Subsequently, the identified microRNAs will be correlated with sputum test results, the Xpert *Mtb*/RIF findings, the extent of radiographic lung infiltration, and the levels of inflammatory markers.

Throughout the disease trajectory, all the patients will be consistently administered the standard treatment regimen appropriate for their diagnosis. The ongoing clinical progression of the disease will be closely observed and documented, juxtaposed with detailed laboratory assessments that monitor the microRNA profiles and the disease.

DISCUSSION

Biological indicators called microRNAs can be utilized to differentiate between various TB infection stages or therapeutic responsiveness. By attaching themselves to mRNA in the cytoplasm of the cell, they regulate the expression of genes. MicroRNAs are being explored as potential biomarkers for TB because they are sensitive, specific, and accessible. MicroRNA signals that can differentiate between individuals with active TB and healthy controls or those with latent TB have been found in a number of investigations. MicroRNAs are crucial in pathogen-host interactions, according to new research. Since they have been consistently and frequently found in the blood, circulating microRNAs have the potential to be used as molecular markers for a variety of physiological and pathological disorders. With a respectable level of sensitivity and specificity, upregulated miR-29a may distinguish TB patients from healthy controls. These circulating microRNAs are projected to influence a number of considerably enriched pathways, the majority of which are implicated in the regulation of the cytoskeleton, acute-phase response, and inflammatory response[28].

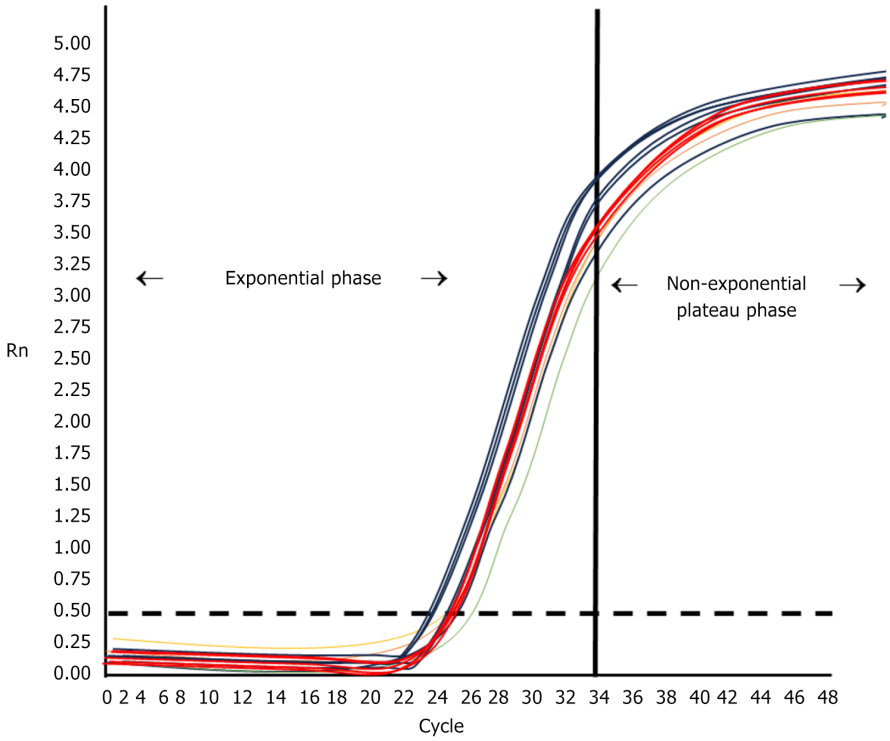

Figure 1 Amplification plot (Rn *vs* Cycle) of a microRNA.

It may be possible to distinguish between tuberculoma with and without decay using serum miR-155, miR-191, and miR-223. Serum levels of miR-26a, miR-191, miR-222, and miR-320 distinguish between fibrotic cavitary TB (FCT) and tuberculoma with degradation. Patients with FCT and those with tuberculoma without decay have different levels of serum expression of miR-26a, miR-155, miR-191, miR-222, and miR-223. As a result, the degree and direction of expression of the set of microRNAs might be used to characterize different TB course variations with varying levels of destruction and inflammatory process severity. Due to their ability to simultaneously regulate several genes, microRNAs are being investigated as potential treatment targets for TB. For instance, miR-155 can support the survival of *Mtb*-specific T lymphocytes while also providing protection against mycobacterial infection[29].

Altered microRNAs have shown promise as potential diagnostic biomarkers in the complex and challenging presentations of TB patients. These specialized RNA molecules could play a crucial role in identifying TB cases that are difficult to diagnose accurately through traditional methods. Additionally, microRNAs could not only aid in diagnosing TB but also serve as prognostic markers that are closely linked with the clinical outcomes and various laboratory investigations related to the disease. Moreover, the involvement of microRNAs in TB cases opens up exciting possibilities for personalized medicine approaches where treatment strategies could be tailored based on the unique microRNA profiles of individual patients. By understanding the intricate relationship between these altered microRNAs and disease progression, healthcare providers may be able to make more informed decisions regarding treatment plans and predict patient responses to specific interventions.

CONCLUSION

MicroRNA signatures offer a window into the pathophysiology of TB, shedding light on the molecular mechanisms underlying the disease's manifestation and progression. Through further research and validation studies, these microRNAs could potentially revolutionize TB management by providing clinicians with valuable tools to improve diagnostic accuracy, predict treatment outcomes, and monitor disease progression in real time. In conclusion, the emerging role of altered microRNAs as potential diagnostic and prognostic markers in TB patients represents a significant step forward in combating this infectious disease. By harnessing the power of these tiny but influential molecules, healthcare professionals can strive towards more effective and personalized care strategies for individuals affected by TB.

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FOOTNOTES

Author contributions: Moorthy S, Bhaskar E, Singh S, and Silambanan S designed the research study; Moorthy S, Bhaskar E, and Santhi S performed the research; Moorthy S and Santhi S contributed new reagents and analytic tools; Moorthy S, Bhaskar E, Singh S, and Silambanan S analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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Clinical trial registration statement: The study is registered with Indian Council of Medical Research, India, CTRI/2023/08/056740 (https://ctri.nic.in/Clinicaltrials/Login.php).

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