

miRNA-21 Signature in the Plasma Samples of Patients with Extrapulmonary Tuberculosis

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ABSTRACT

Tuberculosis is an infectious disease causing more than 1.6 million deaths worldwide every year. This morbidity is due to the lack of early diagnosis of tuberculosis especially in the Extrapulmonary Tuberculosis. In this study, we assess the regulation of miRNA-21 in the plasma samples of Extrapulmonary Tuberculosis infected patients. This study employed 20 Extrapulmonary Tuberculosis plasma samples and 10 Healthy control plasma samples. After evaluating the expression of miRNA-21 by Quantitative Real-Time PCR [qRT-PCR], miRNA-21 was found to be up-regulated in plasma samples of Extrapulmonary Tuberculosis as compared to the plasma samples of Healthy control. The expression analysis was mainly done by using $2^{-\Delta\Delta C_T}$ method. The ROC curve analysis revealed the diagnostic potential of the miRNA-21 in Extrapulmonary Tuberculosis with a good AUC value of 0.95. Our results conclude that the miRNA-21 have a potential application and can act as a biomarker in the diagnosis of Extrapulmonary Tuberculosis.

Keywords: Pulmonary tuberculosis, Extrapulmonary tuberculosis, Circulating miRNA-21, Biomarker, Up-regulation, Stop TB Program, ROC

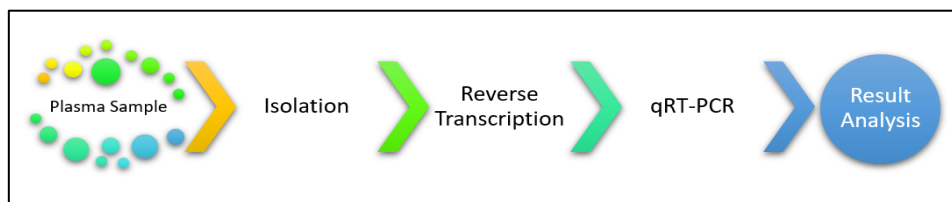


Fig. 1: Flowchart of the procedure followed in this study

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INTRODUCTION

Tuberculosis (TB) is an infectious and communicable disease which is caused by bacillus, *Mycobacterium tuberculosis*. The bacteria of tuberculosis spreads through the air by cough, sneezing, etc., as a medium by which it infects the individual with tuberculosis. According to the World Health Organization (WHO), tuberculosis remains in one of the top ten lethal diseases causes most deaths worldwide. By the year of 2017, 10

million peoples were infected with tuberculosis and around 1.6 million were died due to this lethal disease. The patients with positive HIV were mainly dying due to tuberculosis. In 2017, India has been recorded the highest number of tuberculosis positive rate worldwide which is more than 2 million. The World Health Organization (WHO) took an oath of ending the tuberculosis endemic by 2030 (WHO Global Tuberculosis Report 2018).

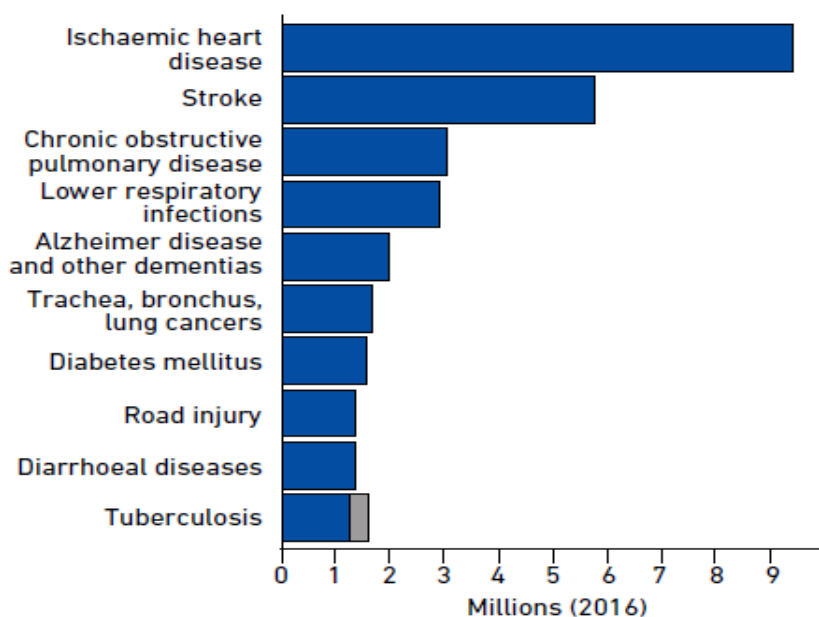


Fig. 2: Top causes of death worldwide. Deaths from TB among HIV positive people are shown in grey (WHO Global Tuberculosis Report 2018)

Tuberculosis generally occurs in the lungs of the host which is known as Pulmonary Tuberculosis (PTB) but the tuberculosis can also be occur other than the lungs such as in lymph node, CNS, pleura, bones and joints, urogenital, meninges, etc., which is known as Extrapulmonary Tuberculosis (EPTB). In 2017, the Extrapulmonary Tuberculosis accounts about 16% of the total tuberculosis reported cases. Usually, tuberculosis has these symptoms – a bad cough which last 3 weeks and more than 3 weeks, chest pain, blood in sputum during coughing, Weight loss, fatigue, fever and chills (Pang et al., 2019).

Generally, the diagnosis of tuberculosis is done by culturing the sample

suspected of tuberculosis infection which is the gold standard in the tuberculosis diagnosis. But this diagnosis procedure takes detection time of about 4-6 weeks causing delay in the tuberculosis diagnosis. Another technique for the tuberculosis diagnosis is based on molecular biology which is rapid. In this technique, the diagnosis of tuberculosis depends upon the detection of bacterial nucleic acid the sample. But this technique is expensive which require high skills, also the chances of cross-contamination are higher (Ryu et al., 2015). So there is a need of advanced diagnosis of tuberculosis which is less time consuming, in-expensive and highly specific.

Nowadays, microRNAs are getting much attention due to their availability and stable nature in body's circulating fluid. microRNAs are short, small and non-coding 21-25 nucleotides long molecules. Ongoing researches reveals the potential role of these microRNAs as biomarker in the diagnosis of tuberculosis and other diseases as well

(Latorre et al., 2015, Wagh et al., 2017 & Barry et al., 2018). Therefore, the present study was aimed to identify the altered level of miRNA-21 in Extrapulmonary Tuberculosis.

MATERIALS AND METHODS

The analytical flow of the present study was provided in the figure 3.

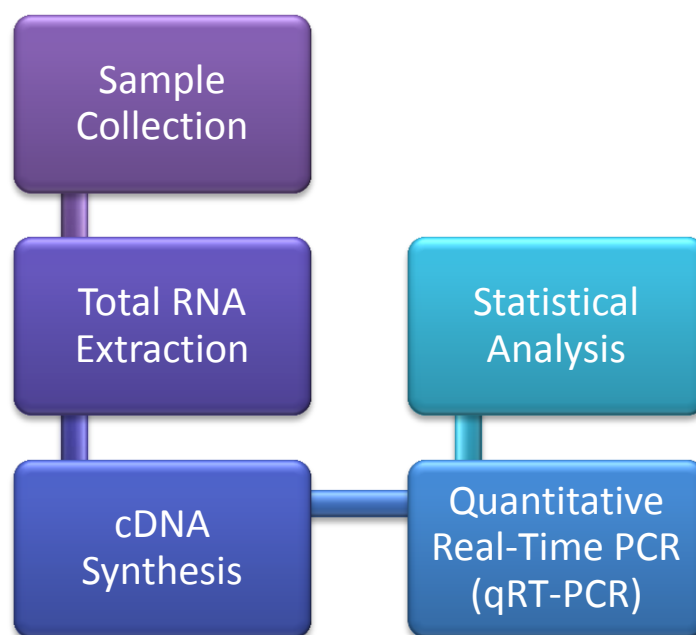


Fig. 3: Analytical flow of the present study showing the first step starting with Sample collection to the Statistical analysis

Clinical Sample Collection: The Patient's leftover samples were used in this study from the collaborative research partners – Dr. B. Lal Clinical Laboratory Pvt. Ltd., Jaipur and TB Hospital, Jaipur. Plasma samples of clinically confirmed Extrapulmonary tuberculosis were taken in use for the study. The samples having haemolysis were excluded from the study. Before including the plasma samples of the patients for the study, their clinical and radiological findings were reviewed and then they were further categorized as healthy and a case of extrapulmonary tuberculosis.

Total RNA Extraction:

The Patient's leftover samples were used in this study from the collaborative research partners – Dr. B. Lal Clinical Laboratory Pvt. Ltd., Jaipur and TB Hospital, Jaipur. Plasma samples of clinically confirmed Extrapulmonary Tuberculosis were taken in use for the study. The samples having

haemolysis were excluded from the study. miRNAs from respective plasma samples were extracted by using the protocol of kit supplier (Qiagen's miRNeasy Serum/Plasma Kit having catalogue no. 217184). All extracted miRNAs were store at -80°C till further use.

cDNA Synthesis/Reverse Transcription:

12 μl RNA sample was utilized in the cDNA synthesis by Qiagen's miScript Reverse Transcription Kit. The kit contains Reverse Transcriptase Mix & RT Buffer. The Reverse Transcriptase Mix comprises of a poly[A] polymerase and a reverse transcriptase. The RT Buffer consists of Mg^{2+} , dNTPs, oligo-dT primers, and random primers. The reaction was then incubated at 37°C for 60 minutes and then at 95°C for 5 minutes.

Quantitative Real-Time PCR [qRT-PCR]:

The forward primer was synthesized by Qiagen. The reverse primer is a universal primer available in the Qiagen's miScript

SYBR Green PCR Kit. The PCR mastermix contained the buffer, polymerase enzyme, SYBR Green for detection. This reaction was incubated at 95°C for 15 minutes, 94°C for 15 seconds, 55°C for 30 seconds and 70°C for 30 seconds and run for 40 cycles. Tube without any cDNA was used as no template control [NTC]. All reactions were performed on QauntStudio 3.0 [Thermo Fisher]. The threshold was set manually at the lowest point to obtain a cycle threshold [Ct] value for each reaction. Data were analyzed by adopting the $2^{-\Delta\Delta C_T}$ method. The relative expression of a targeted gene was calculated using the formula $2^{-\Delta\Delta C_T}$; the folds [$2^{-\Delta\Delta C_T}$] showed the multiple proportions of the targeted gene expression in the experimental and control groups, and $\Delta\Delta C_T = [Ct_{\text{target gene}} - Ct_{\text{reference gene}}]_{\text{the experimental group}} - [Ct_{\text{target gene}} - Ct_{\text{reference gene}}]_{\text{the control group}}$. miRNA-39 was used for the normalization of the miRNA-21 expression data observed in infected and healthy plasma samples. miRNA-39 also acted as an endogenous control which ensured the quality of isolated miRNA throughout the protocol.

Statistical Analysis:

The data were analysed in GraphPad Prism 8 for the statistical analysis. Student's t-Test was applied on the data which gives the significant difference in the data at $p < 0.05$. The Receiver Operating Characteristics [ROC] curve analysis was done to evaluate the diagnostic potential. The Area Under the Curve [AUC] value revealed the degree of separability of our data i.e, how much the two groups [Healthy Control and the infected] are separated from each other. Results were considered statistically significant when the p value was observed less than 0.05.

RESULTS

A total of 30 samples were utilized in this present study out of which 20 samples were Extrapulmonary tuberculosis and the rest 10 were Healthy controls. The level of miRNA-21 was significantly altered in extrapulmonary tuberculosis as compared to Healthy controls. The miRNA-21 was found to be up-regulated with a mean fold change of 2.06 in the plasma of extrapulmonary tuberculosis as compared to the healthy controls.

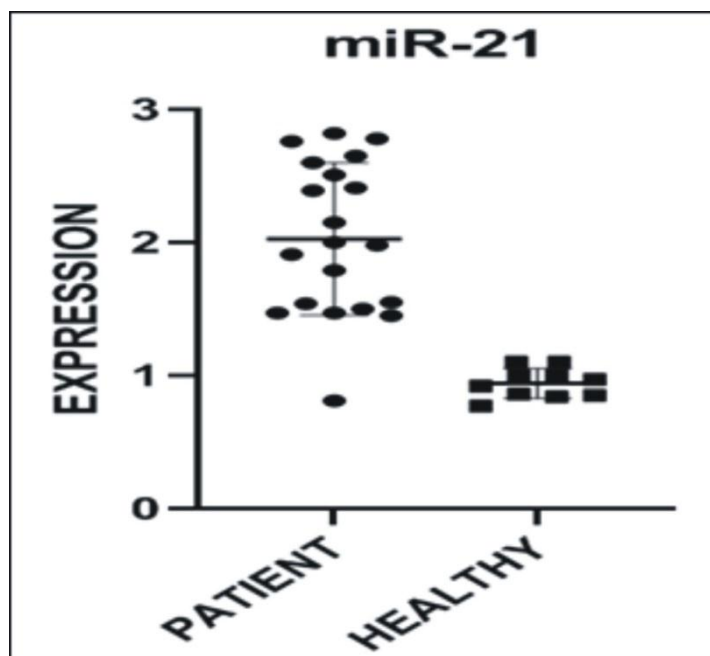


Fig. 4: Expression of miRNA-21 in EPTB & Healthy control samples in terms of Fold Change

● – Extrapulmonary sample; ■ – Healthy control

The Receiver Operating Characteristics [ROC] curve analysis revealed a significant

distinguishing efficiency and a good diagnostic potential with an Area Under Curve

[AUC] value of 0.95 for miRNA-21 with a statistically significant p-value of less than 0.05. Also, the t-Test applied on the above

data gave us a significant result with a t-value of 6.11 and the data were significant at $p < 0.05$.

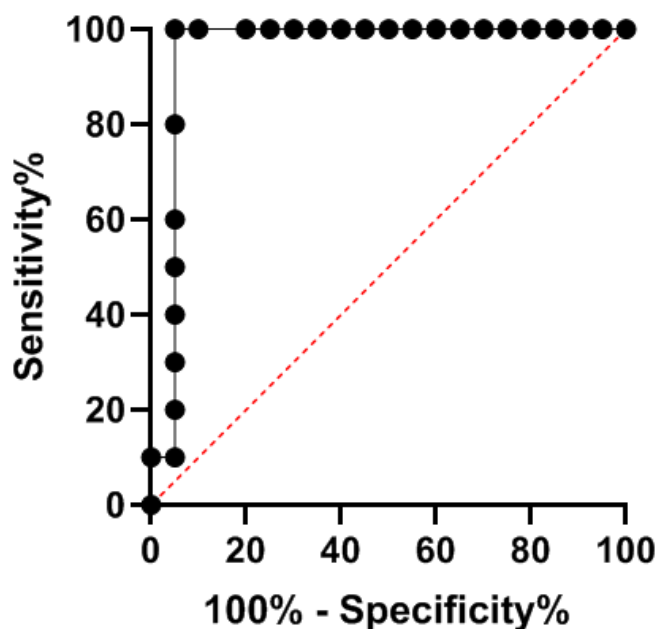


Fig. 5: ROC curve analysis of miRNA-21 in 20 EPTB samples and 10 Healthy samples

DISCUSSION

Mycobacterium tuberculosis is an air-borne pathogen which transmits from one host to another via air as a medium. The tuberculosis is a lethal disease in poor resources countries and also in India due to improper diagnosis and treatment. The methods available for tuberculosis diagnosis are somehow not adequate for the early diagnosis of tuberculosis. Hence, there is a need of new method that enables to diagnose the tuberculosis early in the host. To date, various microRNAs were found to have a potential role in disease diagnosis and can act as biomarkers. Few studies were conducted on plasma samples of tuberculosis infected patients (Wagh et al., 2017, Barry et al., 2018, Zhou et al., 2016, Kulshrestha et al., 2019 & Zheng et al., 2015). These results showed significant conclusions in the field of biomarkers. In consistent with these results, the present study was performed in the plasma of Extrapulmonary Tuberculosis and in this study, we observed a significant alteration in the expression level of miRNA-21.

The first ever identified mammalian microRNA was miRNA-21. After finding of this miRNA, no study was conducted on miRNA-21 which examined the role of miRNA-21 in Extrapulmonary tuberculosis. To uncover the functional role of miRNA-21, more studies will have to be conducted during tuberculosis. The results coming out from the present study implies the role of *Mycobacterium tuberculosis* in alteration of miRNA and their biogenesis in the human body. This alteration was mainly influenced by the MPT64 protein, which was secreted by tuberculosis bacteria in the host which regulates the NF κ B-bcl-2 pathway resulting in the alteration of miRNA-21 during tuberculosis infection. Apoptosis of macrophage was inhibited during tuberculosis infection. This happened due to the secretion of MPT64 protein by the *Mycobacterium tuberculosis* when it enters into the host. The MPT64 protein up-regulates the miRNA-21 that further enhances the expression of bcl-2. This up-regulation of bcl-2 inhibits the apoptosis of macrophages engulfing the

bacteria. Inhibition of apoptosis of macrophages let the bacteria survives on the host and the infection too (Wang et al., 2014). The ROC curve analysis revealed the diagnostic potential of miRNA-21 with a good AUC value of 0.95. The data were also found to be significant at p-value less than 0.05. We also applied Student's t-Test on the data and got the statistically significant p-value which is also less than 0.05.

A study by Wang and colleagues in 2011 examined PBMCs obtained from patients with proven *M. tuberculosis* and compared the expression of several miRNAs to healthy subjects. Similar to our findings, miRNA-21 was also significantly up-regulated in the macrophages obtained from the TB subjects (Wang et al., 2011). Another study conducted by Yuhui Xu et al. (2013) examined levels of miRNA-21 was significantly up-regulated in PBMCs obtained from tuberculosis infected patient (Xu et al., 2013).

To identify biomarkers, various researches and studies in present are going on utilizing plasma, serum, whole blood, PBMCs which will better diagnose the tuberculosis infection. The development of new biomarkers to diagnose TB is urgently required. This study has aimed to achieve this through the use of plasma miRNA. The appeal of using miRNA as a clinical biomarker is high, given their stability in the extracellular environment and their ease of measure in plasma and serum, which are readily obtainable.

CONCLUSION

In this present study, we observed that miRNA-21 was found to be up-regulated with a mean fold change of 2.06 in plasma samples of extrapulmonary tuberculosis patients in comparison to healthy population, indicating the potential of miRNA-21 in the diagnosis of extrapulmonary tuberculosis. However, further studies are required to be focus on their underlying mechanism in the extrapulmonary tuberculosis infection with a large number of sample size in the development of miRNA - 21as early diagnostic marker for extrapulmonary tuberculosis. Diagnosing

extrapulmonary tuberculosis would strengthen the STOP TB program.

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