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Diagnostics

Host and MTB genome encoded miRNA markers for diagnosis of tuberculosis

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A B S T R A C T

MicroRNAs (miRNAs) are a class of noncoding RNA molecules which are involved in various cellular and physiological processes. Previously, studies have identified several miRNAs that are potential diagnostic biomarkers for various infectious diseases including tuberculosis. We have performed small RNA sequencing using the Ion Torrent PGM platform in extra pulmonary tuberculosis (EPTB) subject's serum samples to identify circulating miRNAs during mycobacterium tuberculosis (MTB) infection. Our analysis identified 20 circulating miRNAs upregulated and 5 miRNAs downregulated during MTB infection in patient's serum. In addition, we have identified 6 MTB genome encoded miRNAs upregulated in EPTB patient's serum samples. Taqman based qRT-PCR analysis of host-genome encoded (hsa-miR-146a-5p and hsa-miR-125b-5p) and MTB genome encoded (MTB-miR5) miRNAs showed increased expression in a cohort of 52 healthy, pulmonary tuberculosis (PTB) and extra pulmonary tuberculosis (EPTB) patients serum samples. Our study identified for the first time a panel of host and MTB genome specific differentially expressed circulating miRNAs in serum samples of an Indian patient cohort with tuberculosis infection with a potential as a non-invasive diagnostic biomarker for tuberculosis infection.

1. Introduction

Despite advancement in pulmonary medicine in past few decades, tuberculosis (TB) globally continues to be the major cause of mortality and morbidity due to an infectious agent. According to World Health Organization (WHO) report 10.4 million people fell ill with TB in 2016; 6.3 million among them were new cases and 0.49 million had multidrug resistant TB. In 2016, TB accounted for 1.3 million deaths alone and 0.37 million deaths among HIV co-infected people [1]. Although, early diagnosis and appropriate treatment can prevent most TB related deaths, limited availability of a reliable tool for diagnosis of TB remains one of the major obstacles in controlling the spread of this epidemic. Current standards of TB diagnosis are based on detection of pathogens in the sample thus rely heavily on bacterial load which makes the diagnosis even challenging especially in smear negative pulmonary TB cases, extrapulmonary TB and paediatric TB cases where bacterial load is often low [2–4]. Various methods are employed for the diagnosis of EPTB such as radiology, smear microscopy, culture identification, histopathology, tuberculin skin test (TST), serological assays, all these methods have limitations such as suboptimal sensitivity or specificity and time delays whereas, tests based on DNA amplification or interferon-γ release are expensive. MTB is known to modulate host cellular microenvironment including immune mechanism and apoptosis. Owing to the limitations in conventional methods for diagnosis of TB, researchers in the past have emphasized the need for development of diagnostic tests based on host biomarkers that can be used for assessment of disease status and monitoring of treatment outcomes [5,6]. Major challenge for developing an ideal biomarker is to ensure that the discovered biomarker is a stable molecule, abundantly present in body fluids, can be easily recovered, offers high sensitivity and specificity with translational potential as a point of care test. In the above context, human MicroRNAs (miRNA), a class of non-coding highly conserved single stranded small RNAs of approximately 18–25 nucleotides in length that modulate the expression of genes involved in development, cell differentiation, proliferation, and apoptosis are promising candidates for use as a biomarker [7–10]. Recently, researchers have highlighted the importance of miRNAs in diagnosis of infectious diseases [11–13]. A preliminary study on circulating miRNAs in patients with active pulmonary TB reported 59 upregulated and 33 downregulated signatures when compared to non-TB controls [14]. Another study identified miR-3179 and miR-147 overexpression during TB infections in patients sputum as compared to controls [15]. It has been reported that miRNA-144 is highly expressed in peripheral blood mono nuclear cell while miRNA-361-5P, miRNA-889, and miRNA-576-3p were significantly upregulated in serum samples of active TB patients [16]. Further, six serum micro RNAs hsa-miR-378, hsa-miR-483- 5p, hsa-miR-

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22, hsa-miR-29c, hsa-miR-101 and hsa-miR-320b have been identified as specific markers to discriminate pulmonary TB from healthy controls [17]. Thus, it is evident that further exploration of miRNAs may be useful to unravel information on pathophysiology of TB and discovery of specific markers for its diagnosis. Recent reports have suggested that serum derived miRNAs are extremely stable under harsh conditions and useful as non-invasive biomarkers for early diagnosis of various infectious diseases [17]. In the present study, we performed small RNA sequencing and Taqman based qRT-PCR based serum miRNA profiling of subjects with pulmonary and extrapulmonary tuberculosis in comparison with healthy control. Our study identified a panel of serum derived circulating host as well as pathogen derived miRNAs which are differentially expressed during tuberculosis infection.

2. Materials and methods

2.1. Ethical statement

Our study was approved by Kasturba Medical College and Kasturba Hospital Institutional Ethics committee, Manipal Academy of Higher Education, Manipal, India. Written informed consent was obtained from all subjects prior to their enrolment in the study.

2.2. Study groups

The present study was conducted on 52 individuals divided into three groups. Group 1 included 15 TB patients diagnosed based on clinical signs, radiological findings smear microscopy, culture and nucleic acid amplification tests.

Group 2 included 22 Extrapulmonary TB patients diagnosed based on clinical signs, radiological/CT findings, MRI, smear microscopy, culture, histopathological findings and nucleic acid amplification tests. Group 3 included 15 normal healthy individuals without any symptoms of Tuberculosis.

2.3. Sample collection

Samples from TB and extra pulmonary TB patients were collected prior to anti-tubercular therapy. Five millilitres of venous blood was withdrawn in a sterile vacutainer from each participant enrolled in the study after obtaining written consent. Blood samples were kept at room temperature for 20 min and then were centrifuged at 3000 rpm for 10 min. Serum was collected, aliquots were stored immediately at −80 °C deep freezer until further analysis.

2.4. RNA isolation

RNA was extracted from each serum sample using *mir*Vana™ PARIS™Kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) following manufacturer's instructions. Briefly, 600 µl of serum sample was mixed with an equal volume of denaturing solution at room temperature. Then 1200 µl of Acid-phenol: Chloroform solution was added and the tube was vortexed for 30–60 s to mix the contents properly and was centrifuged at 10,000*g* for 5 min to separate the mixture into aqueous and organic phase. The upper aqueous phase was transferred to a fresh tube and was mixed with 1.25 vol of 100% ethanol and was transferred to the filter cartridge with collecting tube and was centrifuged briefly to pass samples through the filter cartridge, the flow through was discarded and 700 µl miRNA wash solution 1 was applied to the filter cartridge and was centrifuged for 15 s the flow through was discarded and then filter cartridge was washed twice by applying wash solution 2/3 followed by centrifugation for 15 s. Then filter cartridge was transferred to fresh collecting tube and RNA was eluted by applying 100 µl of nuclease free water followed by centrifugation for 30 s. Elute containing RNA was collected and stored at −80 °C deep freezer until use.

2.5. Small RNA sequencing

The small RNA sequencing was performed in pooled control (n = 2) and pooled extra pulmonary tuberculosis (EPTB) serum samples (n = 2). In brief, total RNA from the samples were enriched for small RNAs using Total RNA-Seq Kit v2 (ThermoFisher Scientific). The quality and quantity of samples enriched for small RNA were analyzed using the Agilent small RNA kit (Agilent Technologies, Santa Clara, California, USA) in 2100 Bioanalyzer Instrument. Samples were then barcoded and used for library construction using Total RNA-Seq Kit v2. Amplification of the templates was performed using Ion OneTouch™ 2 system and Ion OneTouch™ ES (ThermoFisher Scientific). Enriched products were loaded onto an Ion 318 chip and run on an Ion PGM system™ (ThermoFisher Scientific).

2.6. Computational and statistical analysis

BAM files of pooled control and pooled EPTB samples were uploaded in CAP-miRSeq pipeline for read pre-processing, alignment, distribution of RNA types, and differential miRNA expression analysis [18]. LogFC (log2fold change) > 1 and < -1 were assigned to up- and down regulated miRNAs with FDR (False Discovery Rate) < 0.05. Targets were predicted using miRDB (Score ≥ 80) [19], PITA (Score ≤ -10) [20], and DIANA microT v4 (Score ≥ 0.9) [21]. Pathway analysis was performed using DIANA miRPath v3.0 [22]. ROC analysis was performed for miRNA expression to analyse the diagnostic accuracy, against pulmonary TB and extra pulmonary TB using MedCalc software.

2.7. MTB genome encoded miRNA analysis

The unmapped reads after aligning to the human genome (GRCh37/hg19) were extracted and mapped to *Mycobacterium tuberculosis* (MTB) (H37Rv). We further identified all small RNA sequencing reads that aligned to MTB genome as potential miRNAs. To validate the same, we extracted 500 flanking upstream and downstream bases from the mapped coordinates and used a SVM based method to predict pre-miRNAs in the fasta sequence ab-initio. Pre-miRNAs residing between 450 and 550 were identified and with a criterion of minimum free energy less than −25 kcal/mol was used to filter the identified pre-miRNAs for better stability. The predicted pre-miRNAs were further evaluated with miREval 2.0 [23] to strengthen the results of our analysis. MatureBAYES was used to predict the mature miRNAs from the novel pre-miRNAs [24]. Lastly, we aligned the mature sequence against MTB genome to obtain the genomic location of the same.

2.8. Reverse transcription and real time PCR quantitation

cDNA was synthesized from total RNA using miRNA-specific primers according to the Taqman microRNA assay protocol (ThermoFisher Scientific, USA). Real-time PCR was performed in Applied Biosystems 7500 Fast cycler (Applied Biosystems, USA). All experiments were performed in triplicates. We have used miR-16 as endogenous control for all the serum samples. The relative expression level of each miRNA in an individual samples were calculated based on equation $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{miRNA} - Ct_{endogenous\ control}$ as published earlier [25].

3. Results

3.1. Clinical data

A total of 52 participants were recruited for the present study including 15 pulmonary TB patients, 22 EPTB patients and 15 healthy controls. Mean age of the participants was 43.11 (SD 15.95, Range 19–86 years). Twenty-nine among them were males whereas 23 were females. Among pulmonary TB cases 14, were smear positive whereas

Table 1
A detailed description of demographic details and clinical features of participants enrolled for the present study.

Characteristics	Pulmonary TB (15)	Extrapulmonary TB (22)	Healthy controls (15)
Age, Mean (SD, range in years)	47.67 (15.75, 22–73)	38.5 (17.38, 19–86)	45.33 (12.88, 24–72)
Gender			
Male (%)	11(73.33)	10(45.45)	8(53.33)
Female (%)	4(26.670)	12(54.54)	7(46.67)
Category, New case (%)	13(86.67)	21(95.45)	–
Previously treated case (%)	2(13.33)	01(4.54)	–
HIV status			
positive (%)	01(6.67)	03(13.63)	–
Negative (%)	14(93.33)	19(86.36)	15(100)
Diabetes			
Positive (%)	06(40.00)	01(4.54)	–
Negative (%)	09(60.00)	21(95.45)	15 (100)
Smear			
Positive (%)	14(93.33)	7(31.81)	–
Negative (%)	1(6.670)	15(68.18)	15(100)
Fever			
present (%)	10(66.67)	8(36.36)	–
Absent (%)	5(33.33)	14(63.63)	15(100)
Cough			
Present (%)	11(73.33)	8(36.36)	–
Absent (%)	04(26.67)	14(63.36)	15(100)
Weight loss			
Present (%)	05(33.33)	05(22.72)	–
Absent (%)	10(66.67)	17(77.27)	15(100)

one was smear negative but was positive by NAAT (Nucleic Acid Amplification Test). Among EPTB patients, 4 were diagnosed for tubercular meningitis, 6 for tubercular lymphadenitis, two each for tubercular spondylodiscitis, tuberculous pleuritis, Potts spine and chest wall abscess whereas, one each was diagnosed for tubercular cystitis, psoas abscess, tubercular osteomyelitis and disseminated TB. A detailed description of demographic details and clinical features of the participants enrolled in the present study is shown in Table 1.

3.2. Small RNA sequencing identified host genome specific miRNAs

Small RNA sequencing analysis in pooled control (n = 2) and pooled extra pulmonary tuberculosis (EPTB) serum samples (n = 2) identified host genome encoded circulating miRNAs differentially expressed in EPTB patients' serum samples (Fig. 1A). Among the differentially expressed miRNAs, 20 miRNAs were 2-fold upregulated and 5 miRNAs were 2-fold downregulated in EPTB patients' serum samples (Table 2). We identified that several miRNAs upregulated in our study were previously reported in MTB infection (Table 2). To validate our findings in small RNA sequencing data, we have performed Taqman based qRT-PCR analysis of two host genome specific miRNAs in an independent cohort of healthy control, PTB and EPTB patients. Statistical analysis of selected miRNA expression between healthy control and extra-pulmonary TB (EPTB) and healthy and pulmonary TB (PTB), was performed using GraphPad Prism 7.03 software. Nonparametric analysis was carried out using Wilcoxon matched pairs signed rank test for hsa-miR-146a expression in healthy vs PTB patients sample. We observed that hsa-miR-146a expression showed significant upregulation in PTB serum samples (p = 0.006) when compared with healthy controls (Fig. 1B). Nonparametric analysis was carried out using Mann–Whitney test which showed significant upregulation of hsa-miR-125b in EPTB serum samples (p = 0.009) when compared with healthy control samples. (Fig. 1B).

3.3. MTB genome encoded miRNAs in EPTB patients

Analysis of MTB genome encoded miRNAs identified 6 novel MTB miRNAs expressed in EPTB patients' serum samples (Fig. 2). Target gene prediction identified several genes involved in cell cycle, transcriptional regulation, apoptosis, mRNA processing, lipid metabolism, cell to cell communication (Table 3). We have selected MTBmiR-5 for the clinical validation in EPTB and PTB samples based on the 1)

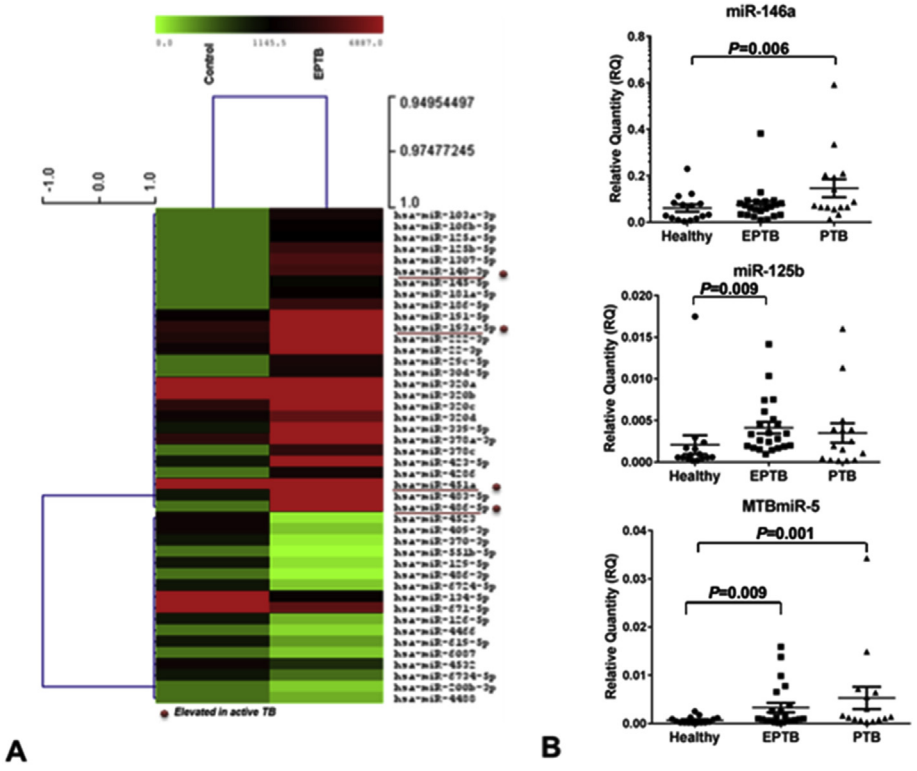

Fig. 1. Heatmap diagram showing clustering of differentially expressed miRNAs in EPTB serum samples. (A) Each row represents a miRNA and each column represents pooled control (n = 2) and pooled EPTB (n = 2) sample. The colour scale shown at the top illustrates the relative expression of miRNA. Red colour represents high expression and green colour represents low expression of identified miRNAs. (B) Taqman qRT-PCR based expression analysis of host genome encoded (hsa-miR-146a-5p and hsa-miR-125b-5p) and MTB genome encoded (MTBmiR-5) miRNAs in healthy controls (n = 15), pulmonary tuberculosis (n = 15) and extra pulmonary tuberculosis (n = 22) samples. Data represents two independent experiments with miRNA expression analysis was performed for each patient samples in duplicate reaction in Taqman qRT-PCR. Error bars reflect ± SEM. *P*-value of less than 0.05 indicates statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Differentially expressed miRNAs identified in EPTB serum samples in the present study.

Sl No.	miRNA ID	logFC	Previously reported in MTB infection	Our study
1	hsa-miR-425-5p	10.44		Upregulated
2	hsa-miR-146a-5p	8.26	Upregulated (Li et al., 2013, Li et al., 2016)	Upregulated
3	hsa-miR-486-5p	7.88	Upregulated (Meng et al., 2014)	Upregulated
4	hsa-miR-16-2-3p	7.00	Upregulated (Wagh et al., 2017)	Upregulated
5	hsa-miR-101-3p	6.49		Upregulated
6	hsa-miR-29c-3p	5.47		Upregulated
7	hsa-miR-378a-5p	5.44		Upregulated
8	hsa-miR-143-3p	4.31		Upregulated
9	hsa-miR-144-3p	4.31	Upregulated (Zhou et al., 2016, Lv et al., 2016, Liu et al., 2011)	Upregulated
10	hsa-miR-483-5p	3.58	Upregulated (Etna et al., 2018)	Upregulated
11	hsa-miR-378a-3p	3.52		Upregulated
12	hsa-miR-378d	3.48		Upregulated
13	hsa-miR-193a-5p	3.12	Upregulated (Etna et al., 2018)	Upregulated
14	hsa-miR-378f	3.12		Upregulated
15	hsa-miR-140-3p	3.11	Upregulated (Zheng et al., 2015)	Upregulated
16	hsa-miR-125b-5p	2.93	Upregulated (Wagh et al., 2017)	Upregulated
17	hsa-miR-378c	2.84		Upregulated
18	hsa-miR-29c-5p	2.36		Upregulated
19	hsa-miR-22-3p	1.91		Upregulated
20	hsa-miR-320a	1.63		Upregulated
21	hsa-miR-129-5p	−2.82		Downregulated
22	hsa-miR-409-3p	−3.07		Downregulated
23	hsa-miR-370-3p	−3.30		Downregulated
24	hsa-miR-551b-5p	−3.82		Downregulated
25	hsa-miR-4523	−5.05		Downregulated

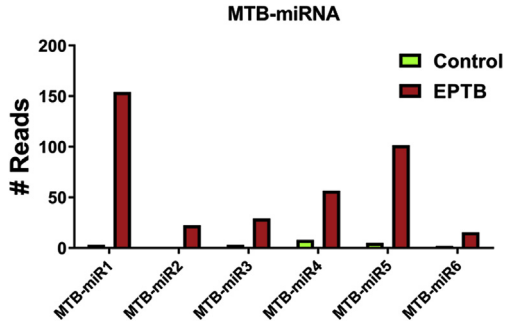

Fig. 2. Novel MTB genome encoded miRNAs in EPTB patients' serum samples identified by next generation small RNA sequencing.

secondary hair pin loop structure formation in the precursor miRNA sequence, 2) minimum free energy for the better stability from our analysis using miRNAFold and miREval 2.0 programs. To evaluate the diagnostic potential of MTB genome encoded miRNA expression, we designed a custom Taqman assay for MTB-miR5 for qRT-PCR validation study in PTB and EPTB serum samples. Nonparametric analysis using Wilcoxon matched pairs signed rank test showed significant upregulation of MTB-miR5 in EPTB serum samples ($p = 0.009$) when compared to healthy control samples. Nonparametric analysis using Mann–Whitney test analysis showed significant upregulation of MTB-miR5 in PTB serum samples ($p = 0.001$) when compared to healthy control samples (Fig. 1B).

3.4. Specificity and sensitivity analysis of miR-146a, mir-125b-5p and MTBmiR-5

Sensitivity and specificity levels of miR-146a, miR-125b-5p and MTBmiR-5 in order to analyse the diagnostic accuracy in healthy vs pulmonary TB and healthy vs extra-pulmonary TB provided area under curve (AUC) as follows: miR-125b-5p, healthy vs PTB was 0.889 (95% CI 0.661–0.985; $P < 0.001$) and healthy vs EPTB was 0.925 (95% CI 0.730–0.993; $P < 0.001$), miR-146a-5p, healthy vs PTB was 0.787 (95% CI 0.556–0.933; $P = 0.007$) and healthy vs EPTB was 0.753 (95% CI 0.497–0.922; $P = 0.038$). MTBmiR-5, healthy vs PTB was 0.985 (95% CI 0.779–1.00; $P < 0.001$) and healthy vs EPTB was 0.825 (95% CI 0.617–0.948; $P < 0.001$) (Table 4) and (Fig. 3). We performed the analysis with all three miRNA combined in healthy vs pulmonary TB provided area under curve (AUC) 0.799 (95% CI 0.670–0.894; $P < 0.001$) and healthy vs extra-pulmonary TB provided area under curve (AUC) 0.688 (95% CI 0.560–0.798; $P = 0.007$) (Table 4) and (Fig. 3).

3.5. Pathway analysis

Pathway analysis of upregulated host genome miRNAs specific target genes were performed using DIANA-miRPath v3.0 and DAVID v6.8 (https://david.ncifcrf.gov/) [22,26]. We identified several pathways including microRNA in Hippo Signaling, Cell Cycle, Fatty acid biosynthesis, Lysine degradation, Adherens junction, p53 signaling, and TGF-beta signaling pathway were targeted by upregulated miRNAs identified in mycobacterium tuberculosis infection (Fig. 4) and

Table 3
MTB miRNAs identified in EPTB serum samples in the present study.

MTB miRNA	Start	End	Mature miRNA Sequence	Target Genes
MTBmiR-1	1385076	1385097	GCCGCCGCCGCCCGCGCCGCCG	*NPPC, NKX2-3, MGAT3, C6orf223, GPR114*
MTBmiR-2	1979452	1979473	CGGGCCGCCGACUCCGCCAGGU	*PYROXD1, CELF5, SLC12A5, PRF1, MIER2*
MTBmiR-3	4046796	4046817	AGCGGCGGGGGCCGGUGGCGCG	*SLC12A5, PCDHAC2, EN1, LBX1, HOXB8*
MTBmiR-4	340622	340643	CGGCGGCGGCGGCCGCGGCGGC	*FAM104A, BRSK2, MPDU1, LBX1, EMILIN1*
MTBmiR-5	1472988	1473009	UGAGAGACUGCCGGGGUCAACU	*KCNJ6, ADRA1B, DPYSL2, IRX6, PLXDC1*
MTBmiR-6	924161	924182	GGGUUCGAGUCCCGUCAGGGUC	*CHSY1, ZNF747, CGGBP1, NAAA, ZC3H12B*

Table 4
Sensitivity and specificity analysis of miR-146a, miR-125b-5p and MTBmiR-5.

miRNAs	Samples	Sensitivity	Specificity	P-value
miR-125b-5p	Healthy vs Pulmonary TB	100	70	< 0.001
	Healthy vs Extra Pulmonary TB	91.67	80	< 0.001
miR-146a-5p	Healthy vs Pulmonary TB	100	55.56	0.007
	Healthy vs Extra Pulmonary TB	100	55.56	0.038
MTBmiR-5	Healthy vs Pulmonary TB	100	90.91	< 0.001
	Healthy vs Extra Pulmonary TB	76.92	81.82	< 0.001
All three miRNAs	Healthy vs Pulmonary TB	100	56.67	< 0.001
	Healthy vs Extra Pulmonary TB	85.29	56.67	0.007

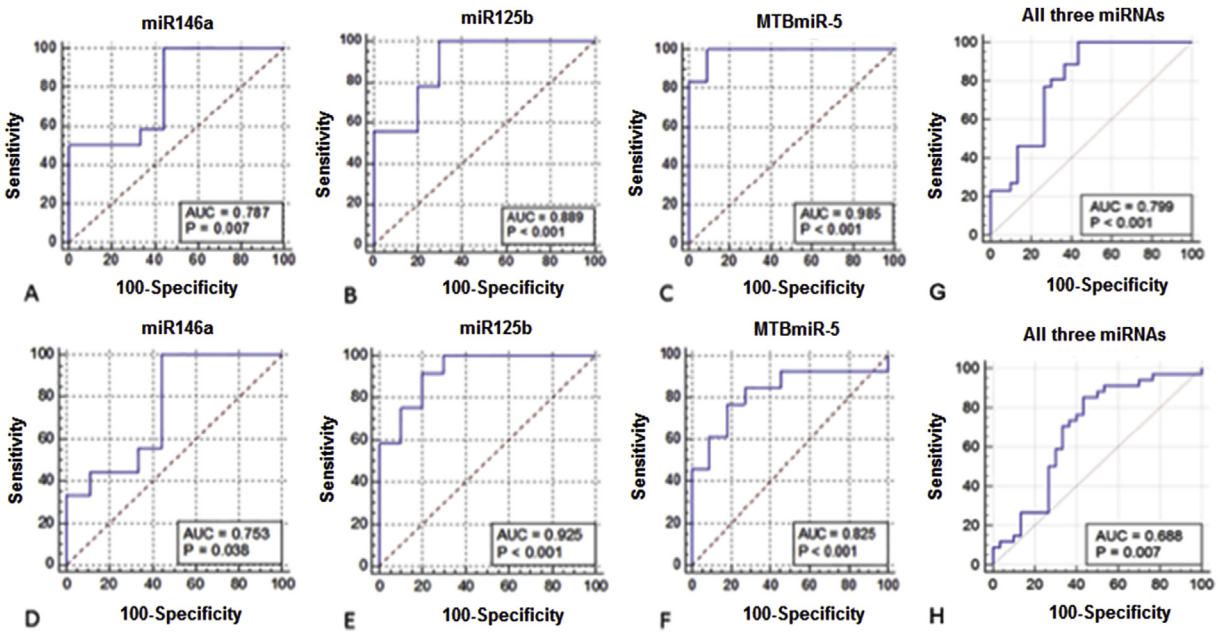

Fig. 3. ROC analysis of miR-146a-5p, miR-125b-5p and MTBmiR-5 from qRT-PCR expression analysis in healthy (n = 15), pulmonary tuberculosis (n = 15) and extra pulmonary tuberculosis (n = 22) samples. ROC analysis was performed using MedCalc software. (A, B and C): individual miRNAs in Healthy vs Pulmonary TB; (D, E and F): individual miRNAs in Healthy vs Extra-Pulmonary TB. (G) All three miRNAs in Healthy vs Pulmonary TB and (H) All three miRNAs in Healthy vs Extra-Pulmonary TB.

(Supplementary Table 1, Supplementary Figs. S1–S7).

4. Discussion

In the last decade, several studies have highlighted the importance of studying TB infection at the RNA level. Micro RNAs (miRNAs) are involved in post-transcriptional regulation of several protein-coding genes in tissue specific fashion with emerging role of critical regulators in major biological processes. Previous studies have demonstrated the role of miRNAs as a key player in the outcome of many infectious disease modulator of host immune system [27]. Recent evidences have shown that specific human miRNAs may have potential role in tuberculosis infection either by enhancing the immune response or by facilitating pathogen immune evasion [28,29]. Few studies have also identified intracellular pathogen encoded miRNAs [30,31]. Since, miRNAs can regulate the innate immune response against active TB infection, they provide a major advantage as a biomarker for the diagnostic and therapeutic monitoring of TB and may also help to differentiate between active and latent TB infection [32–34]. Identification of miRNAs involved in regulating biological processes such as autophagy and apoptosis during MTB infection presents an opportunity to employ miRNAs as therapeutic tool against TB infection [35,36]. Recent studies have shown that circulating miRNAs can be stably detected in body fluids and have potential to serve as diagnostic markers for infectious diseases including TB [34,37,38]. In the present study, we have performed next generation sequencing of circulating miRNAs in serum specimens from EPTB patients and healthy control and identified a panel of 20 host genome encoded miRNAs significantly upregulated and 5 miRNAs downregulated when compared with serum specimens from the healthy control group. Among the upregulated miRNAs identified in EPTB patients, 8 miRNAs were previously reported to be upregulated during MTB infection. Target prediction and pathway analysis of identified miRNAs showed significant enrichment in cell cycle, transcriptional regulation, apoptosis, Hippo signaling pathway, p53 signaling pathway lipid metabolism and cell to cell communication. We have identified 79 genes in Hippo signaling pathway and 43 genes in the P53 signaling pathway are targeted by differentially expressed miRNAs in the EPTB patients (Supplementary Table 1). Recent studies have shown that p53 and Hippo signaling pathway regulates host-mycobacterial interactions during active TB infection [39,40]. Previously, small RNAs have been identified by Arnvig and Young (2009) by screening cDNA libraries prepared from *M. tuberculosis* [30]. Recently, another study have shown the expression profile of Mycobacterial sRNA in bacterial cultural supernatant and plasma samples of TB patients [38]. Our study identified 6 MTB genome encoded miRNAs expressed in EPTB patients serum by small RNA sequencing.

To validate our findings from small RNA sequencing data, we have selected two host genome and one MTB genome encoded miRNA for qRT-PCR based expression analysis in an independent cohort of PTB, EPTB patients and healthy controls to evaluate their diagnostic

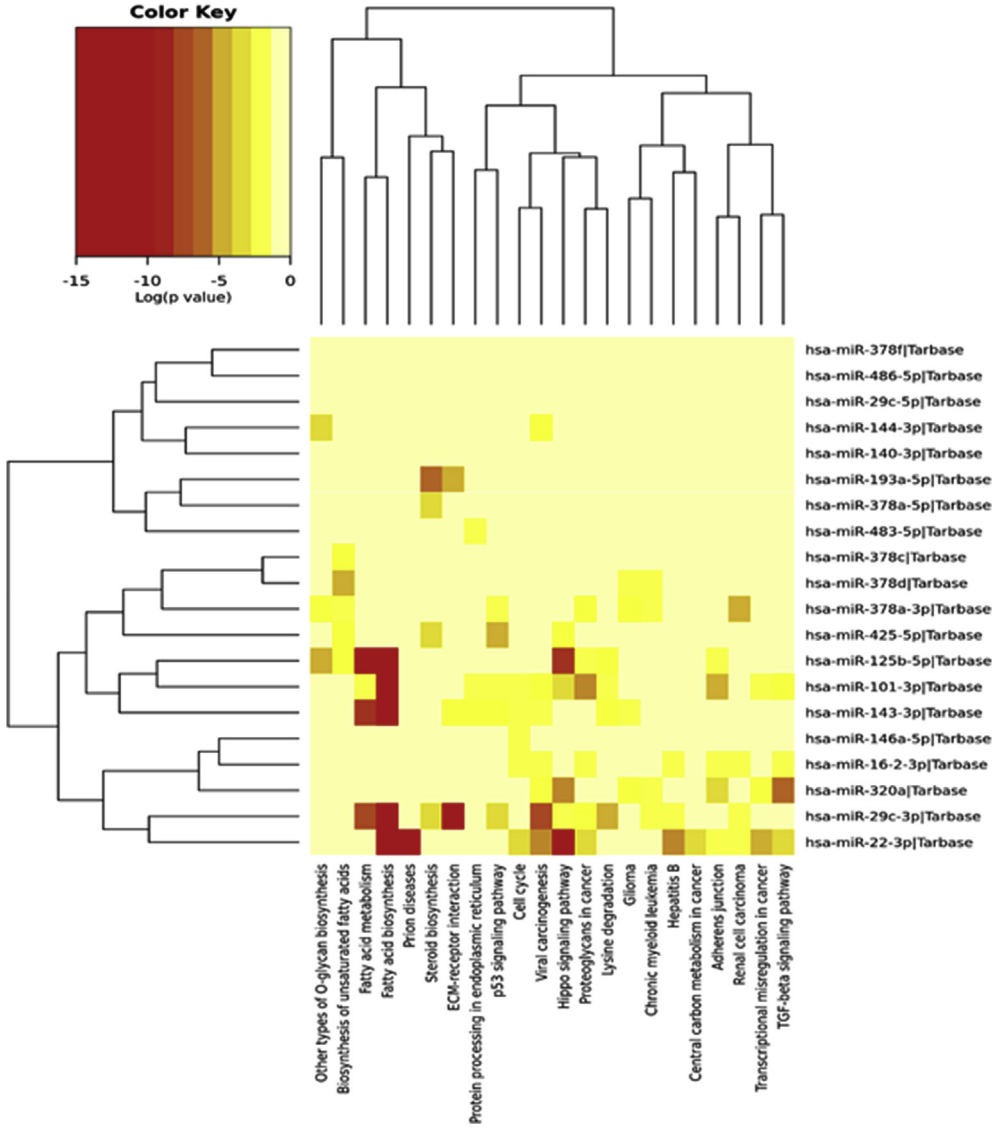

Fig. 4. Pathway analysis for host genome encoded miRNAs from small RNA sequencing identified 20 up regulated miRNAs in TB patients compared to healthy controls. Pathway analysis was performed using DIANA miRPath v3.0 (threshold P-value < 0.05).

potential. The validation study showed that hsa-miR-146a was significantly upregulated in PTB patients serum samples ($p = 0.006$) and hsa-miR-125b was significantly upregulated in EPTB patients serum samples ($p = 0.009$) when compared with healthy controls. MTB genome encoded miRNA-5 was significantly upregulated in both EPTB patients serum samples ($p = 0.009$) and PTB patients serum samples ($p = 0.001$) when compared with healthy controls. The specificity of MTBmiR-5 was 90.91 to distinguish between healthy and pulmonary tuberculosis and 81.82 between health and extra pulmonary TB. We also found amplification of MTBmiR-5 (Ct value < 35) in two healthy control out of 15 which could be due to latent TB infection which is prevalent in Indian population. Previous studies have shown that miR146a and miR125b play significant role in regulating innate immune responses. It has been proven experimentally that miR146a level in the host regulates pro-inflammatory cytokines by targeting *IRAK1* and *TRAF6* [41]. During MTB infection, miR-146a inhibits NO production by targeting *TRAF6* and compromises host defense mechanism during bacterial infection [42]. Similarly, miR-125b targets TNF-α to suppress host defense mechanism during MTB infection [41,43]. Our study showed the diagnostic potential of two host genome encoded and one MTB genome encoded miRNAs in an Indian cohort of both PTB and EPTB patients serum samples. One of the major limitations of the present study is limited sample size which could be overcome by testing a panel of differentially expressed host and MTB genome encoded miRNAs in a larger Indian cohort of EPTB and PTB patients along with individuals with other lung pathologies. MTB genome encoded miRNAs identified by our study will provide an opportunity for a detailed study to understand its role in diagnosis and prognosis of active TB infection.

5. Conclusion

Several studies have identified host genome encoded miRNAs that are intricately involved in host-pathogen interaction during TB infection. Functional analysis of selected miRNAs have shown their role in both adaptive and innate immune response against MTB highlighting their potential as diagnostic and prognostic marker during active TB infection. Differentially expressed miRNAs can be detected in various body fluids such as serum, saliva and urine which makes them ideal candidate marker to monitor TB infection and also to distinguish between active and latent infection. Recent efforts have been made to exploit miRNAs as host directed therapy against TB infection in mouse models. We have identified a panel of host and MTB genome encoded miRNAs differentially expressed in both PTB and EPTB patients' serum. Our miRNA panel has the potential for non-invasive diagnostic

screening tool for active TB infection and can be extended to distinguish between active and latent TB infection in larger patient cohort.

Author contributions

SC designed and performed experiments, analyzed data and wrote the manuscript; AK and KR performed experiments, analyzed data; SM performed bioinformatics analysis and analyzed the data; KS and KC conceived and supervised the study, experimental design and manuscript writing.

Conflicts of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.04.002.

References

[1] World Health Organization. Global tuberculosis report 2017 available at: http://www.who.int/tb/publications/global_report/en/.
[2] Schaaf HS, Collins A, Bekker A, Davies PD. Tuberculosis at extremes of age. Respirology 2010;15:747–63.
[3] Perez-Velez CM, Marais BJ. Tuberculosis in children. N Engl J Med 2012;367:348–61.
[4] Pearce EC, Woodward JF, Nyandiko WM, Vreeman RC, Ayaya SO. A systematic review of clinical diagnostic systems used in the diagnosis of tuberculosis in children. AIDS Res Treat 2012;2012:401896.
[5] Wallis RS, Pai M, Menzies D, Doherty TM, Walzl G, et al. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet 2010;375:1920–37.
[6] Lawn SD, Zumla AI. Tuberculosis. Lancet 2011;378:57–72.
[7] Aigner A. MicroRNAs (miRNAs) in cancer invasion and metastasis: therapeutic approaches based on metastasis-related miRNAs. J Mol Med (Berl) 2011;89:445–57.
[8] Kim VN, Nam JW. Genomics of microRNA. Trends Genet 2006;22:165–73.
[9] Ambros V. The functions of animal microRNAs. Nature 2004;431:350–5.
[10] Liu A, Tetzlaff MT, Vanbelle P, Elder D, Feldman M, Tobias JW, Sepulveda AR, Xu X. MicroRNA expression profiling outperforms mRNA expression profiling in formalin-fixed paraffin-embedded tissues. Int J Clin Exp Pathol 2009;2:519–27.
[11] Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutat Res 2011;717:1–8.
[12] Verma P, Pandey RK, Prajapati P, Prajapati VK. Circulating MicroRNAs: potential and emerging biomarkers for diagnosis of human infectious diseases. Front Microbiol 2016;7:1274.
[13] Correia CN, Nalpas NC, McLoughlin KE, Browne JA, Gordon SV, MacHugh DE, Shaughnessy RG. Circulating microRNAs as potential biomarkers of infectious disease. Front Immunol 2017;8:118.
[14] Fu Y, Yi Z, Wu X, Li J, Xu F. Circulating microRNAs in patients with active pulmonary tuberculosis. J Clin Microbiol 2011;49:4246–51.
[15] Yi Z, Fu Y, Ji R, Li R, Guan Z. Altered microRNA signatures in sputum of patients with active pulmonary tuberculosis. PLoS One 2012;7:e43184.
[16] Qi Y, Cui L, Ge Y, Shi Z, Zhao K, Guo X, et al. Altered serum microRNAs as biomarkers for the early diagnosis of pulmonary tuberculosis infection. BMC Infect Dis 2012;12:384.
[17] Zhang X, Guo J, Fan S, Li Y, Wei L, et al. Screening and identification of six serum microRNAs as novel potential combination biomarkers for pulmonary tuberculosis diagnosis. PLoS One 2013;8:e81076.
[18] Sun Z, Evans J, Bhagwate A, Middha S, Bockol M, Yan H, Kocher JP. CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. BMC Genomics 2014;15:423.
[19] Wang X. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. RNA 2008;14:1012–7.
[20] Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. Nat Genet 2007;39:1278–84.
[21] Maragkakis M, Vergoulis T, Alexiou P, Reczko M, Plomaritou K, Gousis M, Kourtis K, Koziris N, Dalamagas T, Hatzigeorgiou AG. DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association. Nucleic Acids Res 2011;39:W145–8.
[22] Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, Dalamagas T, Hatzigeorgiou AG. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. Nucleic Acids Res 2015;43:W460–6.
[23] Gao D, Middleton R, Rasko JE, Ritchie W. miREval 2.0: a web tool for simple microRNA prediction in genome sequences. Bioinformatics 2013;29:3225–6.
[24] Gkirtzou K, Tsamardinos I, Tsakalides P, Poirazi P. MatureBayes: a probabilistic algorithm for identifying the mature miRNA within novel precursors. PLoS One 2010;5:e11843.
[25] Zhu Z, Qi Y, Ge A, Zhu Y, Xu K, Ji H, Shi Z, Cui L, Zhou M. Comprehensive characterization of serum microRNA profile in response to the emerging avian influenza A (H7N9) virus infection in humans. Viruses 2014;6:1525–39.
[26] Huang DW, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC, Lempicki RA. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol 2007;8:R183.
[27] Kumar R, et al. MicroRNA17-5p regulates autophagy in *Mycobacterium tuberculosis*-infected macrophages by targeting Mcl-1 and STAT3. Cell Microbiol 2016;18:679–91.
[28] Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, Hua M, Li N, Yao H, Cao X. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-γ. Nat Immunol 2011;12:861–9.
[29] Iwai H, Funatogawa K, Matsumura K, Kato-Miyazawa M, Kirikae F, Kiga K, Sasakawa C, Miyoshi-Akiyama T, Kirikae T. MicroRNA-155 knockout mice are susceptible to Mycobacterium tuberculosis infection. Tuberculosis (Edinb). 2015;95:246–50.
[30] Arnvig KB, Young DB. Identification of small RNAs in Mycobacterium tuberculosis. Mol Microbiol 2009;73:397–408.
[31] Gu H, Zhao C, Zhang T, Liang H, Wang XM, Pan Y, Chen X, Zhao Q, Li D, Liu F, Zhang CY, Zen K. Salmonella produce microRNA-like RNA fragment Sal-1 in the infected cells to facilitate intracellular survival. Sci Rep 2017;7:2392.
[32] Miotto P, et al. miRNA signatures in sera of patients with active pulmonary tuberculosis. PLoS One 2013;8:e80149.
[33] Wang C, Liu C, Wei L, Shi L, Pan Z, Mao L, et al. A group of novel serum diagnostic biomarkers for multidrug-resistant tuberculosis by iTRAQ-2D LC-MS/MS and Solexa sequencing. Int J Biol Sci 2016;12:246–56.
[34] Zhou M, Yu G, Yang X, Zhu C, Zhang Z, Zhan X. Circulating microRNAs as biomarkers for the early diagnosis of childhood tuberculosis infection. Mol Med Rep 2016;13:4620–6.
[35] Guo L, Zhao J, Qu Y, Yin R, Gao Q, Ding S, et al. MicroRNA-20a inhibits autophagic process by targeting ATG7 and ATG16L1 and favors mycobacterial survival in macrophage cells. Front Cell Infect Microbiol 2016;6:134.
[36] Duan W, Yang T, Zhou SF, Wang ZL, Zhou ZW, He ZX. Novel targeting of PEGylated liposomes for codelivery of TGF-beta1 siRNA and four antitubercular drugs to human macrophages for the treatment of mycobacterial infection: a quantitative proteomic study. Drug Des Dev Ther 2015;9:4441–70.
[37] Latorre I, Leidinger P, Backes C, Domínguez J, de Souza-Galvão ML, Maldonado J, et al. A novel whole-blood miRNA signature for a rapid diagnosis of pulmonary tuberculosis. Eur Respir J 2015;45:1173–6.
[38] Fu Y, et al. Detection of mycobacterial small RNA in the bacterial culture supernatant and plasma of patients with active tuberculosis. Biochem Biophys Res Commun 2018;503:490–4.
[39] Boro M, Singh V, Balaji KN. Mycobacterium tuberculosis-triggered Hippo pathway orchestrates CXCL1/2 expression to modulate host immune responses. Sci Rep 2016;6:37695.
[40] Liang S, Song Z, Wu Y, Gao Y, Gao M, Liu F, Wang F, Zhang Y. MicroRNA-27b modulates inflammatory response and apoptosis during Mycobacterium tuberculosis infection. J Immunol 2018;200:3506–18.
[41] Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signalling proteins of innate immune responses. Proc Natl Acad Sci U S A 2006;103:12481–6.
[42] Li M, Wang J, Fang Y, Gong S, Li M, Wu M, Lai X, Zeng G, Wang Y, Yang K, Huang X. microRNA-146a promotes mycobacterial survival in macrophages through suppressing nitric oxide production. Sci Rep 2016;6:23351.
[43] Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA, Croce CM. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 2007;179:5082–9.