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Utility of microRNAs in exhaled breath condensate as diagnostic biomarkers to differentiate pulmonary sarcoidosis from pulmonary tuberculosis

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Abstract. *Background and aim:* Pulmonary sarcoidosis and pulmonary tuberculosis (PTB) are two granulomatous diseases with similar clinical presentations. This study explored the potential of microRNAs (miRNAs) in exhaled breath condensate (EBC) as non-invasive diagnostic biomarkers to distinguish between pulmonary sarcoidosis and PTB. *Methods:* EBC samples were collected from 46 participants (20 with PTB, 26 with pulmonary sarcoidosis and 20 healthy controls) and miRNA profiling was done. Differentially expressed miRNAs were further validated using qRT-PCR in independent cohorts in EBC (17 PTB, 18 pulmonary sarcoidosis, and 25 controls) and blood (10 PTB, 10 pulmonary sarcoidosis, and 10 controls). *Results:* Initial profiling identified 19 miRNAs differentially expressed between pulmonary sarcoidosis and PTB, one between Pulmonary sarcoidosis and healthy controls, and 22 between PTB and healthy controls. The top five upregulated miRNAs (miR-132, miR-362-5p, miR-181c, miR-181a, and miR-512-3p) and two downregulated miRNAs (miR-454 and miR-139-5p) in Pulmonary sarcoidosis compared to PTB were selected for validation. Validation showed significant downregulation (p value <0.05) of only miR-454 in EBC of sarcoidosis patients compared to PTB patients with an optimal AUC of 0.663. miR-150* discriminated pulmonary sarcoidosis patients from healthy controls with an AUC of 0.982. When PTB were compared to healthy controls, miR-454 and miR-139-5p were discriminating both groups with AUCs of 0.991 and 0.994 respectively in EBC. *Conclusions:* These findings suggest that while miR-454 differentiated pulmonary sarcoidosis from Pulmonary Tuberculosis, their clinical utility in EBC is limited. Further investigation is needed to explore their diagnostic potential, and to improve diagnostic accuracy in EBC.

Key words: exhaled breath condensate, microRNAs, biomarker, sarcoidosis.

Introduction

Pulmonary tuberculosis (PTB) and pulmonary sarcoidosis are closely mimicking granulomatous diseases. TB is an infectious disease caused by *Mycobacterium tuberculosis* (MTB), transmitted primarily via respiratory droplets (1). The hallmark of TB pathology is the development of necrotizing granulomas composed of macrophages, T cells, and other

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immune cells, with caseous necrosis at their center (2). Sarcoidosis, on the other hand, is a systemic disease of unknown etiology characterized by the formation of non-necrotizing granulomas in affected tissues (3). Both pulmonary TB and pulmonary sarcoidosis share features such as pulmonary involvement, granuloma formation, and similar clinical symptoms, which can lead to diagnostic confusion. Furthermore, the demonstration of granulomas requires invasive tissue sampling, which has its complications and is not readily available. Therefore, reliable biomarkers in easy-to-access specimens are needed to differentiate these two conditions with reasonable confidence to provide appropriate therapy. Exhaled breath condensate (EBC) is a non-invasive technique for collecting airway lining fluid (ALF) (4). EBC is obtained during tidal breathing via mouth by cooling and condensing the aerosol present in exhaled air. It contains various molecules that can serve as biomarkers, providing insights into disease processes in the lungs or throughout the body (5). MicroRNAs (miRNAs) are 18-25-nucleotide long noncoding RNAs that are endogenous, stable, and highly conserved, playing crucial roles in post-transcriptional gene regulation under both normal and pathological conditions (6). miRNAs may significantly influence inflammation and granuloma formation (7). Studies on these noncoding RNAs have also shown potential as diagnostic and prognostic biomarkers for various respiratory diseases (8). Despite substantial research on the roles of miRNAs in TB and sarcoidosis individually, direct comparisons between the miRNA profiles of these diseases are scarce. Given the clinical similarities in their pulmonary manifestations, miRNAs could offer a valuable tool for differential diagnosis, especially when evaluated in novel specimens such as exhaled breath. This study aims to explore the differences in miRNA expression between pulmonary TB and pulmonary sarcoidosis, with a focus on identifying specific miRNAs that could serve as reliable biomarkers and therapeutic targets.

Methods

Study population

This cross-sectional study included 46 treatment-naive subjects in the exploratory group: 20 with pulmonary TB and 26 with pulmonary sarcoidosis, and 20 age- and sex-matched healthy controls. For validation, new 35 patients (18 with pulmonary sarcoidosis and 17 with PTB) and 25 healthy controls were recruited and their EBC and blood samples were collected. 10 subjects each from the healthy controls, Pulmonary sarcoidosis, and PTB groups were kept for blood-based validation. The schematic diagram of study design is shown in the figure 1. The diagnosis of PTB was made based on the presence of acid-fast bacilli positivity or mycobacterial TB detection by nucleic acid amplification testing of sputum or bronchoalveolar lavage. Sarcoidosis was diagnosed based on pathological demonstration of non-caseating granulomas on a lung biopsy obtained by bronchoscopy. The study was initiated after obtaining ethical approval from the institute (IEC-661/07.12.2018, dated 16.01.2019). Written and informed consent was obtained from all participants before sample collection, ensuring adherence to ethical standards in medical research.

Age-and sex-matched control subjects were chosen as those who did not exhibit any respiratory symptoms.

Sample collection

EBC samples were collected from each participant using a standardized procedure designed to ensure consistency across subjects. The samples were collected with the R-Tube (Respiratory Research, Austin, USA) over a 10-minute protocol (9). Blood samples were collected from each participant of another independent group using standard venipuncture techniques.

Following collection, the samples were promptly stored at -80°C to prevent degradation of the miRNA content.

MiRNA isolation and profiling

miRNA from EBC (600 microlitre) samples of healthy controls, pulmonary sarcoid and pulmonary TB of exploratory group was isolated using the miRvana small RNA extraction kit (Ambion, ThermoFisher Scientific, Massachusetts, USA). The concentration of isolated miRNA was quantified using the Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific, Massachusetts, USA).

For miRNA profiling, 60ng of RNA was reverse transcribed into cDNA using the QuantiMir cDNA synthesis kit (System Biosciences, Palo Alto, CA,

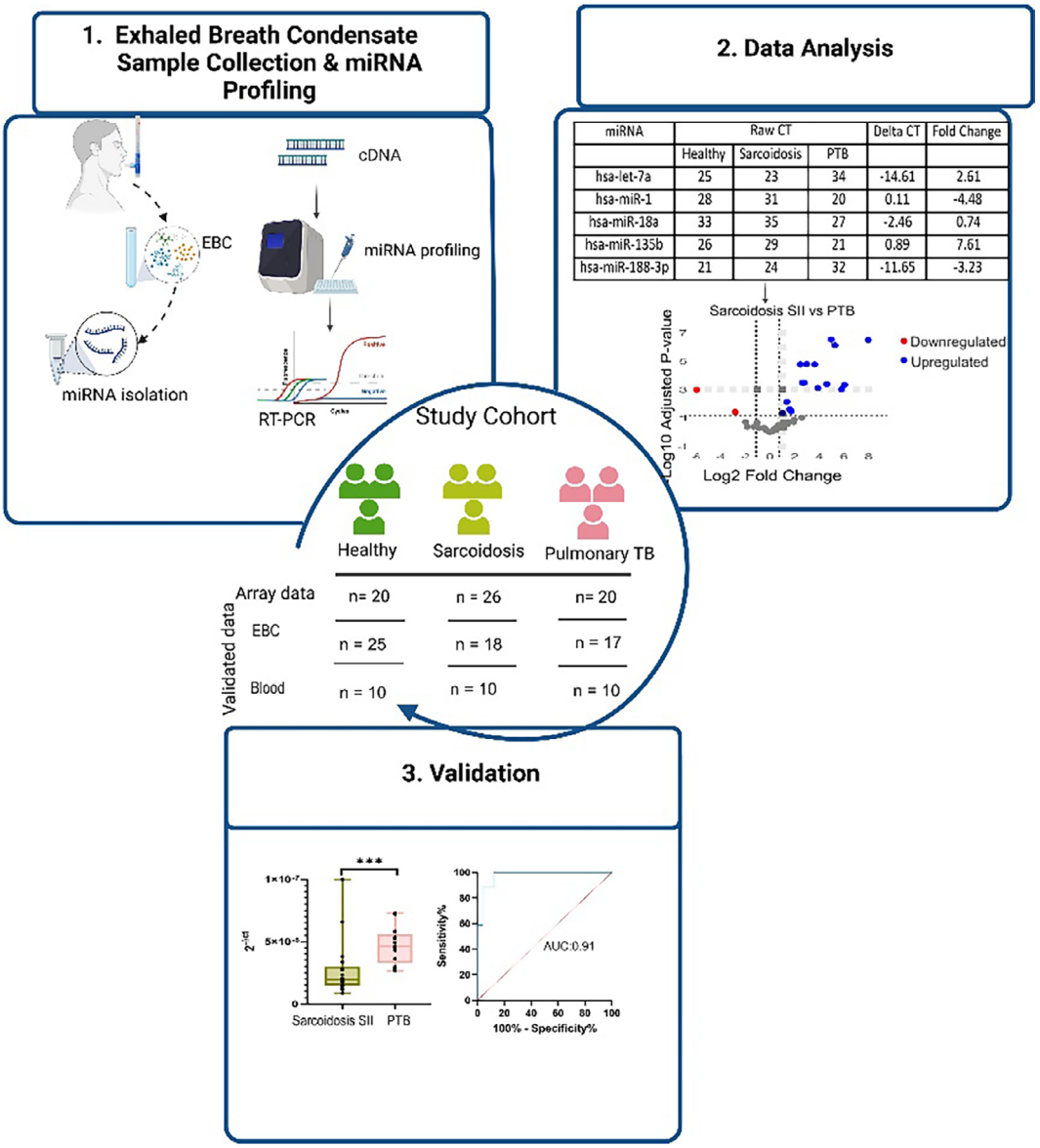

Figure 1. Schematic diagram of the study design.

USA). Finally, 5µL of cDNA was used for profiling of 905 miRNAs by using the human miRNome qPCR array platform (System Biosciences). Real time PCR was performed using the LightCycler 480 II (Roche, Basel, Switzerland).

Validation of miRNome results in fresh EBC and blood samples

The shortlisted miRNAs from the differentially expressed miRNA profiling data were further validated in EBC and blood samples collected from another independent cohort (Validation cohort). Total RNA was extracted from EBC and blood samples via Trizol method (10, 11). For qPCR validation, cDNA followed by qPCR amplification using KAPA FAST SYBR Green (KapaBiosystems, Sigma-Aldrich, St. Louis, MO, USA) was performed. For validation of miRNAs, specific stem loop RT primer and forward primer for each miRNA was used. To normalize the technical variation during validation, synthetic spike-in Cel-miR-39 was added to each sample after RNA extraction and used as the reference control. Endogenous housekeeping miRNAs

were not used, as consistent expression across EBC samples was not observed due to inherently low RNA content and biological variability in disease states.

Data processing and normalization

In this study, the expression levels of 905 miRNAs were measured for each EBC sample using the array-based qPCR platform. The raw data were initially assessed based on the cycle threshold (Ct) values. For quality control, only Ct values greater than 18 and less than or equal to 35 were included. Missing values were replaced with 36. Only miRNAs that were expressed in at least 50% of the samples were selected for statistical analysis. The data was normalized using the global mean normalization method. The –ΔCt values were calculated by subtracting each Ct value of the sample from the global mean of the Ct values of the same sample (12). This step facilitated the comparison of miRNA expression levels across the different subject groups. This method is widely used in high-throughput qPCR studies, especially when stable endogenous controls are not available. In the case of qRT-PCR validation, Ct values less than or equal to 35 were considered.

Differential expression analysis

To identify miRNAs that were differentially expressed between the pulmonary sarcoidosis and pulmonary tuberculosis groups, the normalized -ΔCt values of each miRNA were analyzed statistically. Differences in miRNA expression between the two groups were assessed for significance using Student's t-test or Mann Whitney U test. To control false discovery rates, the Benjamini-Hochberg correction was applied to the p-values generated by the t-test. An adjusted p-value (padj) < 0.05 was used as the threshold, indicating a statistically significant difference in miRNA expression between two groups.

The fold change was calculated as 2^–Δ ΔCt and volcano plot was generated to visually represent the differentially expressed miRNAs which plots the log2 fold change (logFC) against the -log10 of the adjusted p-value for each miRNA, providing a clear graphical representation of the relationship between the magnitude of the expression change and its statistical significance. miRNAs with a logFC greater than 1 or less than -1 and an adjusted p-value<0.05 were considered to be statistically significant and differentially expressed. For identifying individual expression of miRNAs, Ct values were standardized against the Cel-miR-39 reference control. The -ΔCt values were calculated as follows:

[-ΔCt = - (Ct miRNA - Ct Cel-miR-39)]

The fold change between disease and healthy groups was calculated as 2^ (– (ΔCt_disease – ΔCt_healthy)). Box plots were generated to visualize the relative expression levels of selected miRNAs across the groups.

Quantitative and statistical evaluation

Statistical significance was assessed using the student t test, two-tailed Mann-Whitney U test, Fisher's exact test, and Chi-square test. Statistical analyses and data visualization were conducted with the licensed version of GraphPad Prism 10 (13) and R studio version 4.3.2 (14). Receiver operating characteristic (ROC) curves and areas under the ROC curve (AUCs) were performed to assess the diagnostic ability of the miRNAs. All the statistical tests with a p-value < 0.05 were considered significant.

Results

Study population and clinical parameter distribution

46 patients were included in the initial exploratory group, 26 with pulmonary sarcoidosis, 20 with PTB along with 20 age and sex-matched healthy individuals as control. For the validation in EBC, 35 patients (17 with PTB and 18 with sarcoidosis) were included, along with 25 age and sex-matched healthy controls. Ten subjects from each group (healthy, pulmonary sarcoidosis, and PTB) were selected for validation in blood. The demographic details are provided in Table 1. The distribution and correlation of clinical parameters are given in Figure 2.

The expression of miRNAs is deregulated in the EBC of patients with pulmonary sarcoidosis and TB

In this study, we used miRNA profiling to quantify the expression levels of 905 miRNAs in the EBC collected from patients with pulmonary sarcoidosis, PTB, and healthy controls. We used differential

Table 1. Demographic details of initial exploratory group and validation group

	Initial exploratory group			Validation group					
				EBC			Blood		
Parameter	Healthy control	PS	PTB	Healthy control	PS	PTB	Healthy	Sarcoidosis	TB
Number of patients	20	26	20	25	18	17	10	10	10
Age	47.3(17.3)	41.88(10.4)	30.2(12.4)	35.9(14.4)	42.3(10.9)	28.64(14.9)	29.8(±7.29)	41.1(±9.6)	30.5((±14.82)
Gender									
Male	13(65%)	15(57.6%)	9(45%)	21(84%)	10(55.5%)	8(47%)	9(90%)	5(50%)	4(40%)
Female	7(35%)	11(42.3%)	11(55%)	4(16%)	8(44.5%)	9(53%)	1(10%)	5(50%)	6(60%)
BMI	26.3(4.6)	26.7(4.4)	18.3(4.3)	25.8(5.2)	26.5(3.5)	17.01(2)	23.7(±5.3)	27.6(±3.1)	19.74(±4.96)
Smoking status									
Non smokers	17(85%)	22(85%)	16(80%)	23(92%)	16(89%)	15(88.2%)	8(80%)	8(80%)	9(90%)
Smokers	3(15%)	4(15%)	4(20%)	2(8%)	2(11%)	2(11.7%)	2(20%)	2(20%)	1(10%)
Smoking index	300(212.3)	110(132.16)	260(360.4)	19(1.41)	90(14.14)	128(172.5)	17.5(±17.6)	90(±14.1)	60
Comorbidities									
Diabetes	-	3(11.5%)	3(15%)	-	3(16.6%)	0	-	1(10%)	2(20%)
Hypertension	-	4(15%)	1(5%)		2(11%)	1(5%)	-	1(10%)	2(20%)
Past history of TB	-	6(23%)	2(5%)		2(11%)	2(11.7%)		1(10%)	0
Family history of TB	-	2(10%)	5(12%)		1(5%)	6(35.2%)	-	1(10%)	1(10%)
Exposure to active TB	-	0	2(5%)		0	4(23.5%)	-	0	0
Diagnosis of TB									
Microbiological	-	-	20(100%)	-	-	17(100%)	-		10(100%)
Clinico-radiological	-	-	0	-	-	0	-	-	-
Serum ACE levels	-	64.18(51.25)	-	-	56.78(18.7)	-	-	52.37(±20.76)	-

All values are expressed as mean ± SD or N (%)

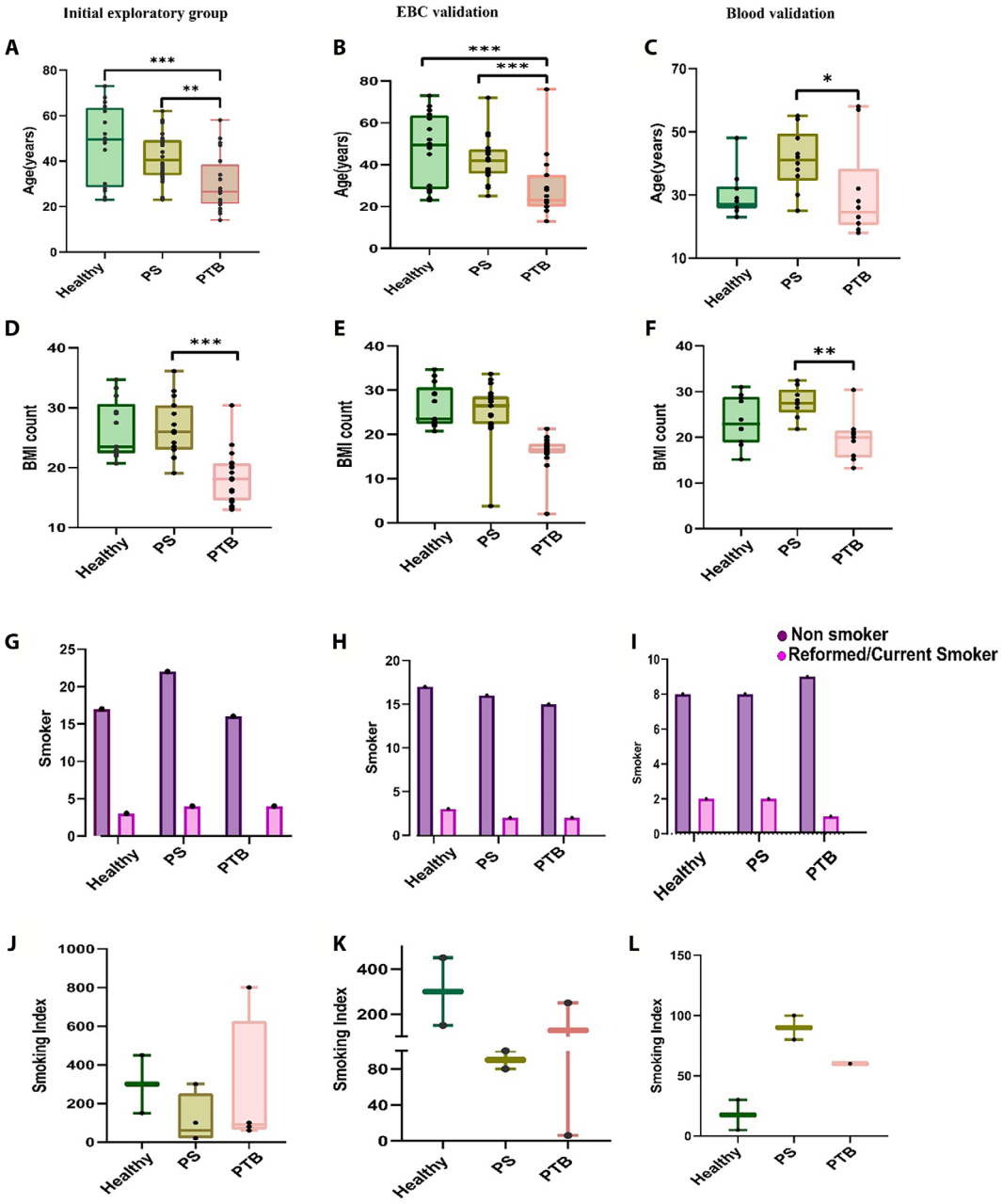

Figure 2. Comparison of various clinical parameters among patients with pulmonary sarcoidosis, pulmonary TB and healthy controls. (A–C) Age distribution: (A) Initial exploratory groups in EBC, (B) Validation groups in EBC, (C) Validation groups in blood. (D–F) BMI: (D) Initial exploratory group in EBC, (E) Validation group in EBC, (F) Validation group in blood. (G–I) Smoking status: (G) Initial exploratory group in EBC, (H) Validation group in EBC, (I) Validation group in blood. (J–L) Smoking index: (J) Initial exploratory group, (K) Validation group in EBC, (L) Validation group in blood. Significance: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. *Abbreviations:* PS: Pulmonary sarcoidosis; PTB: Pulmonary tuberculosis.

expression analysis to compare miRNA expression in EBC in i) patients with pulmonary sarcoidosis compared to those with PTB, ii) patients with pulmonary sarcoidosis compared to healthy controls, and iii) patients with PTB compared to healthy controls. A total of 19 miRNAs were found to be differentially expressed (17 upregulated and 2 downregulated) in the EBC of patients with pulmonary sarcoidosis as

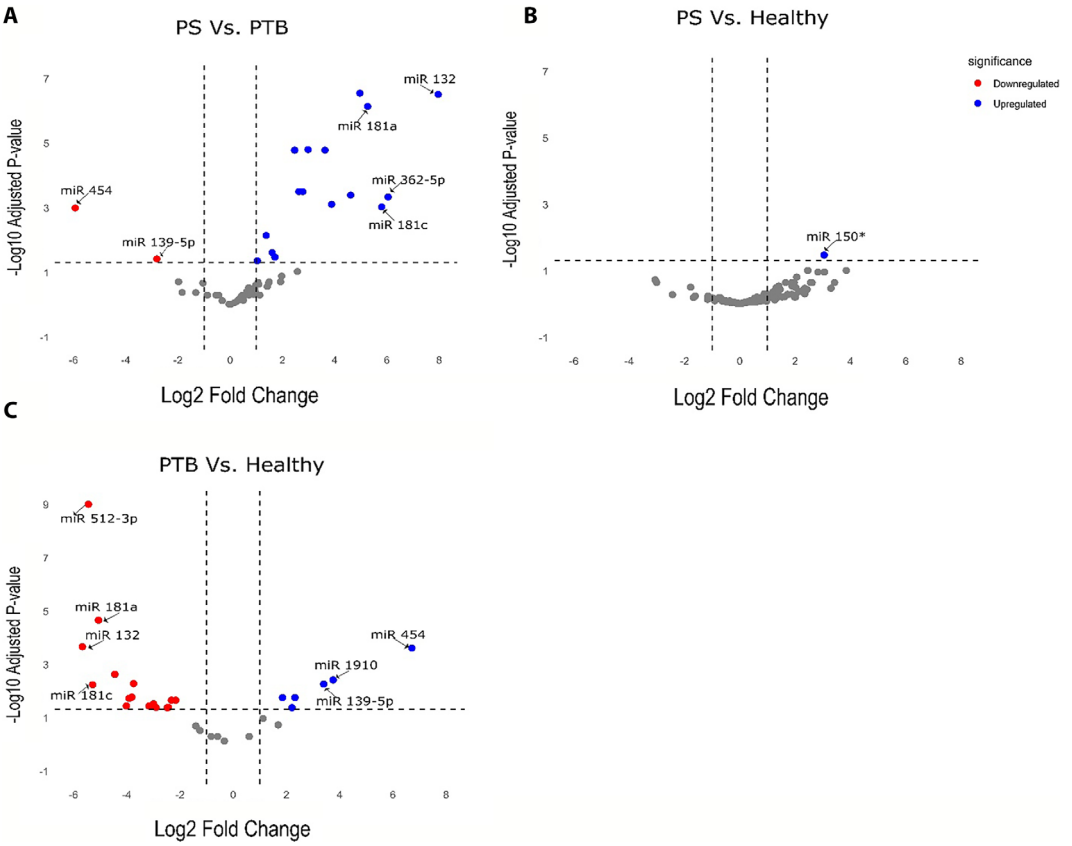

Figure 3. The expression of miRNAs is significantly altered in the EBC of patients with TB and sarcoidosis compared to healthy controls. Volcano plots depicting significantly differentially expressed miRNAs in (A) pulmonary sarcoidosis vs. pulmonary TB (B) pulmonary sarcoidosis vs. healthy controls, and (C) pulmonary TB vs. healthy controls. PS: Pulmonary sarcoidosis; PTB: Pulmonary tuberculosis.

compared to those with pulmonary TB (Figure 3A). From these, 19 differentially expressed miRNAs, we chose the top four upregulated miRNAs (based on a logFC greater than 5) miR-132, miR-362-5p, miR-181c, and miR-181a, and two downregulated miRNAs - miR-454 and miR-139-5p, for further validation by qRT-PCR in validation cohort. Interestingly, we found only one differentially expressed miRNA in the EBC of patients with pulmonary sarcoidosis compared to healthy controls. The expression of miR-150* was found to be significantly upregulated in the EBC of patients with sarcoidosis as compared to healthy controls (Figure 3B). Upon comparing the miRNA expression patterns in the EBC of patients with PTB to healthy controls, we identified 22 differentially expressed miRNAs, amongst which 6 were upregulated and 16 were downregulated in patients with TB (Figure 3C). The top 3 upregulated miRNAs were miR-454, miR-1910, and miR-139-5p, while the top 4 downregulated miRNAs were miR-132, miR-512-3p, miR-181c, and miR-181a. These miRNAs were selected for further validation in the EBC and blood samples using qRT-PCR.

Validation of the expression of differentially expressed miRNAs in the EBC and blood of patients with sarcoidosis compared to those with TB

Among the shortlisted miRNAs, the expression of miR-454 and miR-139-5p was downregulated in the EBC of patients with pulmonary sarcoidosis compared to those with pulmonary TB (Figure 4A & Figure 4C), but only miR-454 significantly differentiated between both groups. Interestingly, the expression of both the miRNAs, miR-454 and miR-139-5p, was significantly downregulated in the blood of patients with sarcoidosis compared to those with TB (Figure 5A & Figure 5C). Receiver

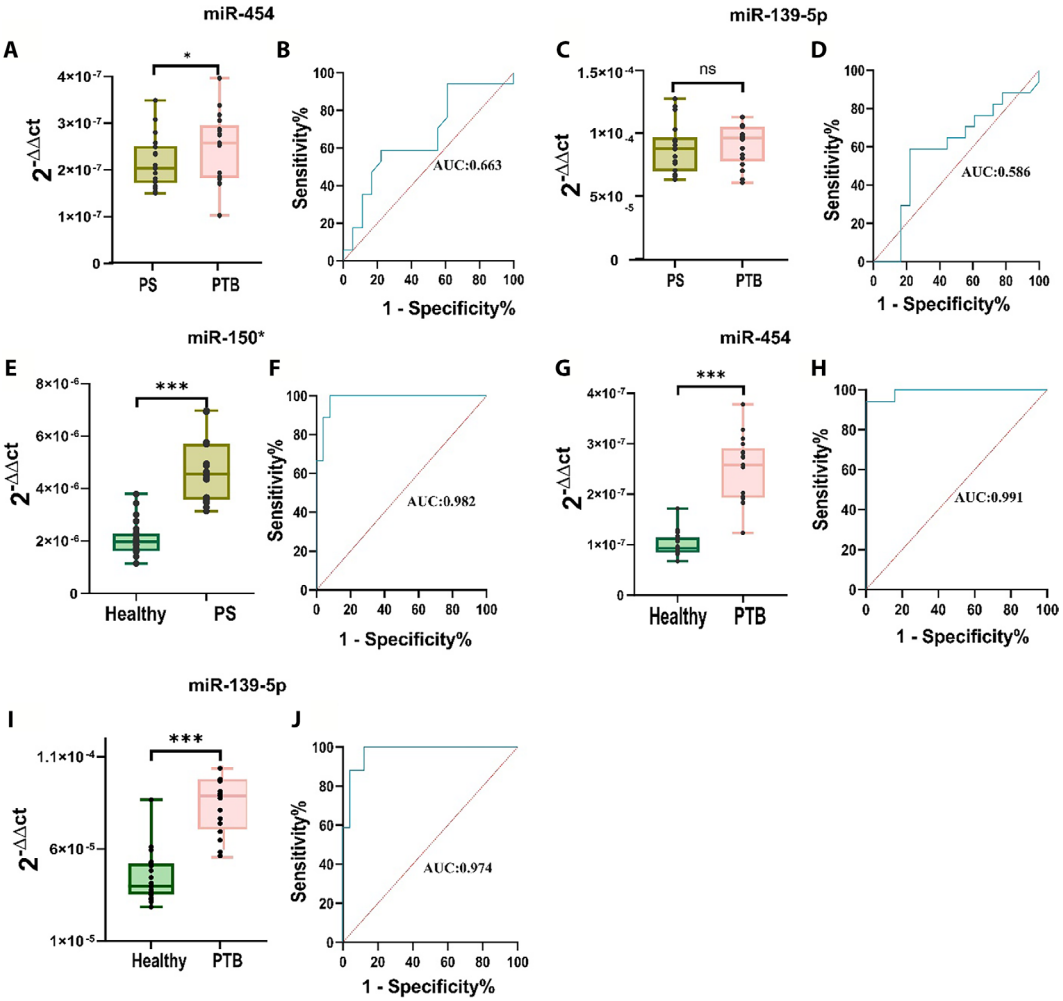

Figure 4. Validation of the expression of differentially expressed miRNA in the EBC in different comparison groups along with their diagnostic performance. (A–D) Pulmonary Sarcoidosis vs. PTB: (A) miR-454, (B) ROC for miR-454, (C) miR-139-5p, (D) ROC for miR-139-5p. (E–F) Pulmonary Sarcoidosis vs. Healthy: (E) miR-150*, (F) ROC for miR-150*. (G–J) PTB vs. Healthy: (G) miR-454, (H) ROC for miR-454, (I) miR-139-5p, (J) ROC for miR-139-5p. Significance: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.PS: Pulmonary sarcoidosis; PTB: Pulmonary tuberculosis.

operating characteristics (ROC) curves was plotted, which yielded AUCs of 0.663 and 0.586 in the EBC (Figure 4B and Figure 4D) and AUCs of 0.770 and 0.810 in blood (Figure 5B and Figure 5D) for miR-454 and miR-139-5p, respectively.

Validation of the expression of differentially expressed miRNAs in the EBC and blood of patients with sarcoidosis compared to healthy controls

We found a significantly higher expression of miR-150* in the EBC (Figure 4E) and blood of patients with sarcoidosis compared to those with healthy controls (Figure 5E). The diagnostic performance of miR-150 was found to be excellent in EBC with an AUC of 0.982 (Figure 4F), while in the blood, AUC was 0.780 (Figure 5F).

Validation of the expression of differentially expressed miRNAs in the EBC and blood of patients with TB compared to healthy controls

Amongst the miRNAs chosen for validation, the expression of miR-454 and miR-139-5p was significantly upregulated in the EBC of patients with PTB compared to healthy subjects (Figure 4G and Figure 4I). miR-454 and miR-139-5p had excellent

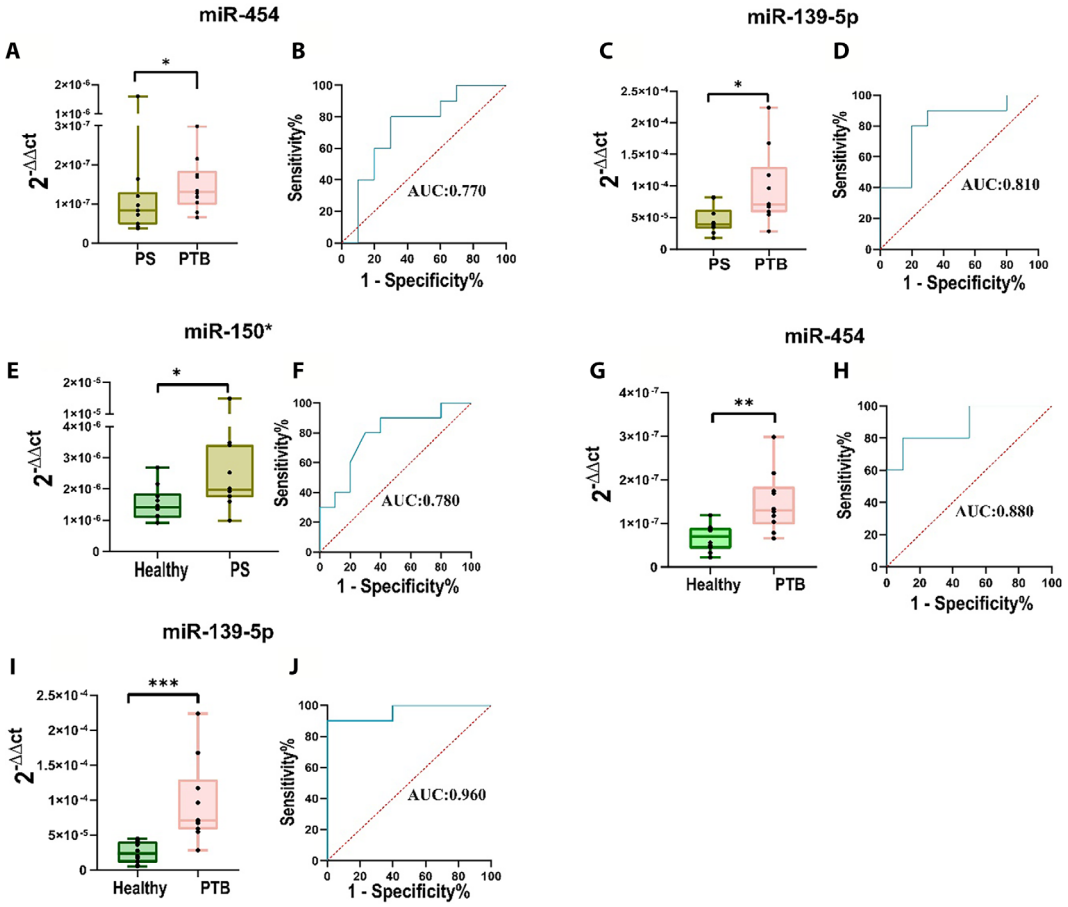

Figure 5. Validation of the expression of differentially expressed miRNA in blood in different comparison groups along with their diagnostic performance. (A–D) Pulmonary Sarcoidosis vs. PTB: (A) miR-454, (B) ROC for miR-454, (C) miR-139-5p, (D) ROC for miR-139-5p. (E–F) Pulmonary Sarcoidosis vs. Healthy: (E) miR-150*, (F) ROC for miR-150*. (G–J) PTB vs. Healthy: (G) miR-454, (H) ROC for miR-454, (I) miR-139-5p, (J) ROC for miR-139-5p. Significance: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.PS: Pulmonary sarcoidosis; PTB: Pulmonary tuberculosis.

diagnostic performance in EBC and blood, with AUCs of 0.991 (Figure 4H) and 0.974 (Figure 4J) in EBC and AUCs of 0.880 (Figure 5F) and 0.960 (Figure 5J) in blood, respectively.

DISCUSSION

This study explored miRNA expression profiles in the EBC of patients with PTB and pulmonary sarcoidosis, aiming to identify miRNAs that could serve as biomarkers for differentiating these closely mimicking granulomatous diseases. The shortlisted miRNAs after initial profiling were subsequently validated in EBC and blood samples. Among the miRNAs shortlisted for validation, two miRNAs, namely, miR-139-5p and miR-454, were found to be differentially expressed. The expression and clinical importance of miR-454 and 139-5p in sarcoidosis and tuberculosis in EBC have yet to be explored, and not much is known about miR-454's cellular functions and direct functional targets in Sarcoidosis and tuberculosis. In our investigation, we observed that miR-454 expression was considerably downregulated in both EBC and blood of sarcoidosis, with moderate accuracy (0.66) in EBC and good accuracy (0.77) in blood. miR-454 is known to inhibit the translation of CXCL12 mRNA by targeting its 3′-UTR in human lung epithelial cells. CXCL12 is a chemokine involved in recruiting inflammatory cells, and its overexpression is linked to enhanced

inflammatory responses. By suppressing CXCL12, miR-454 reduces the production of pro-inflammatory cytokines, such as IL-6 and TNF-α, which play critical roles in granulomatous inflammation. Furthermore, overexpression of miR-454 has been shown to improve alveolar epithelial barrier integrity, protecting the lungs from excessive permeability and damage during inflammatory responses (15). In the context of sarcoidosis, the observed downregulation of miR-454 may lead to reduced suppression of CXCL12, thereby increasing inflammatory cell recruitment and cytokine production. This could contribute to the chronic immune activation and granuloma formation characteristic of sarcoidosis. Also, the loss of miR-454's protective effects on the epithelial barrier may exacerbate lung tissue damage and leads to disease progression. Although miR-139-5p could not discriminated TB and sarcoidosis in EBC, however in blood, it discriminated between the two groups with an accuracy of 81%. Other studies have reported that miR-139-5p has downregulated in airway epithelial cells treated with Th2 cytokines and to play a role in enhanced Th2 mediator expression and antiviral responses (16, 17). Between sarcoidosis and healthy controls, only one miRNA, i.e., miR-150* significantly discriminated the two groups with good accuracy (0.98). This observation aligns with previous research on miRNA profiles in sarcoidosis; a study on bronchoalveolar lavage (BAL) cells of sarcoidosis patients identified miR-150, along with other miRNAs, as upregulated in sarcoidosis compared to controls. The study also highlighted miR-150's role in modulating the cytokine/chemokine receptor network, a critical pathway in pulmonary inflammation and granuloma formation (18). Moreover, miR-150 has been implicated in hematopoiesis and B-cell and T-cell development, suggesting that its upregulation in sarcoidosis might reflect immune system dysregulation and lymphocyte-driven granuloma formation characteristic of the disease (19). Recent research has demonstrated that miR-150 targets the AKT3 pathway, reducing pro-inflammatory cytokine levels such as IL-1β, IL-6, and TNF-α (20). This aligns with sarcoidosis's immune dysregulation and lymphocyte-driven granuloma formation. Upregulation of miR-150 in sarcoidosis may reflect an attempt to mitigate excessive inflammation. When the shortlisted miRNAs in pulmonary TB and healthy comparisons were validated in EBC and blood of another independent cohort, miR-454 and miR-139-5p were upregulated in both EBC and blood and demonstrated the highest discriminatory power, distinguishing TB from healthy controls. The distinct expression patterns of miR-454 and miR-139-5p in sarcoidosis and TB underscore their context-dependent regulatory functions in inflammation and epithelial integrity. miR-454's downregulation in sarcoidosis and upregulation in TB suggests it may serve as a critical marker for differentiating these two diseases. In summary, our findings indicate that miR-454 in EBC has modest diagnostic accuracy of 66% in differentiating sarcoidosis from PTB, which is encouraging for an initial exploratory biomarker study. However, further validation in bigger cohorts and the integration of additional biomarkers are required to improve the diagnostic capability.

Limitation

The limitation of this study is a relatively small cohort size. Larger and diverse cohort is needed to enhance the statistical power of the study to further validate the diagnostic utility of these findings and ensure their generalizability.

Conclusion

This pilot study showed the limited diagnostic utility of miR-454 expression in EBC for distinguishing pulmonary sarcoidosis from pulmonary TB. Given its suboptimal diagnostic performance, further studies involving larger cohorts and follow-up after treatment will further elucidate diagnostic potential and explore its clinical utility in differentiating these granulomatous lung diseases.

Abbreviations

ALF: Airway Lining Fluid, AUC: Area Under the Curve, BAL: Bronchoalveolar Lavage, cDNA: Complementary DNA, Ct: Cycle Threshold, EBC: Exhaled Breath Condensate, IL: Interleukin, logFC: Log2 Fold Change ,miRNA: Micro-ribonucleic acid, MTB: *Mycobacterium tuberculosis* ,PS: Pulmonary Sarcoidosis ,PTB: Pulmonary TB, qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction, ROC: Receiver Operating Characteristic, TB: Tuberculosis, TNF-α: Tumor Necrosis Factor alpha, UTR: Untranslated Region.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership,

equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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Authors' contribution: Divyanjali Rai: Conceptualization and design of the study, manuscript writing; **Adwika PP:** Formal analysis, Interpretation and visualization of the data, manuscript writing; **Jaya Tak**: Data Curation, Investigation, Validation; **Sunil Bangaru:** Investigation, Data Curation; **Bijay Pattnaik**: Conceptualization and study design, data interpretation; **Sunita Yadav**: Data curation, formal analysis and visualization of study; **Seetu Kumari**: Laboratory benchwork; **Jyoti Kumari**: Laboratory testing; **Umashankar Verma**: Data acquisition and Investigation; **Geetika Yadav**: Project Administration and Supervision; **R S Dhaliwal**: Project Administration and Supervision; **Naveen K. Bhatraju**: Data Curation, Formal analysis, Interpretation of the data, Writing of the manuscript; **Saurabh Mittal:** Supervision and design of the study, clinical recruitment; **Pawan Tiwari:** Supervision and design of the study, clinical recruitment; **Vijay Hadda:** Clinical recruitment, manuscript editing; **Karan Madan:** clinical recruitment; **Anurag Agrawal**: Conceptualization and design of the study, Supervision, analysis, final approval of the manuscript; **Randeep Guleria**: clinical recruitment, supervision; **Rohit Vadala**: Data Curation, Investigation; **Anant Mohan:** Conceptualization and design of the study, clinical recruitment, project supervision, writing and final approval of the manuscript.

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