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Integrating exosomal microRNAs and electronic health data improved tuberculosis diagnosis

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ABSTRACT

Background Tuberculosis (TB) is difficult to diagnose under complex clinical conditions as electronic health records (EHRs) are often inadequate in making an affirmative diagnosis. As exosomal miRNAs emerged as promising biomarkers, we investigated the potential of using exosomal miRNAs and EHRs in TB diagnosis.
Methods: A total of 370 individuals, including pulmonary tuberculosis (PTB), tuberculous meningitis (TBM), non-TB disease controls and healthy state controls, were enrolled. Exosomal miRNAs were profiled in the exploratory cohort using microarray and miRNA candidates were selected in the selection cohort using qRT-PCR. EHRs and follow-up information of the patients were collected accordingly. miRNAs and EHRs were used to develop diagnostic models for PTB and TBM in the selection cohort with the Support Vector Machine (SVM) algorithm. These models were further evaluated in an independent testing cohort.
Findings: Six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, miR-486) were differentially expressed in the TB patients. Three SVM models, "EHR+miRNA", "miRNA only" and "EHR only" were compared, and "EHR + miRNA" model achieved the highest diagnostic efficacy, with an AUC up to 0.97 (95% CI 0.80–0.99) in TBM and 0.97 (0.87–0.99) in PTB, respectively. However, "EHR only" model only showed an AUC of 0.67 (0.46–0.83) in TBM. After 2-month anti-tuberculosis therapy, overexpressed miRNAs presented a decreased expression trend ($p = 4.80 \times 10^{-5}$).
Interpretation: Our results showed that the combination of exosomal miRNAs and EHRs could potentially improve clinical diagnosis of TBM and PTB.
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1. Introduction

Tuberculosis (TB) has exceeded HIV/AIDS to become the deadliest single infectious disease [1], mainly due to the difficulties in achieving an early and definitive diagnosis and subsequent timely treatment of TB patients. Among active TB cases, pulmonary tuberculosis (PTB) accounts for 80% of all forms of TB, while tuberculosis meningitis (TBM) represents 1.6% of all TB cases. However, TBM is the most severe form of TB and accounts for up to 50% of TB-associated mortality [2]. Accurate early diagnosis of TB disease remains challenging because the symptoms and radiological features of TB overlap with those of many other diseases. Currently available diagnostic methods for TB have inherent limitations, such as long incubation times (weeks using culture-based methods), high financial cost, inaccessibility in resource-poor settings (*e.g.*, use of GeneXpert), and low sensitivity (only 10%–20% when using microscopy for paucibacillary TB) [1–3], and thus often do not meet clinical requirements [4]. These situations highlight the pressing need for new biomarkers or clinical approaches to improve TB diagnosis, especially for the most frequent form, PTB, and the most lethal form, TBM.

In 2014, WHO (World Health Organization) announced that non-sputum based diagnostic biomarkers for TB should be given a high priority [5]; subsequently a number of TB biomarkers had been reported by using blood transcriptomic analyses [6]. These published TB signatures

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Research in Context

Evidence before this study

We systematically searched PubMed and Google, without date restrictions or limitations to English language publications, using the terms "exosomal micorRNA or exosomal RNA" and "tuberculosis or pulmonary tuberculosis or tuberculous meningitis". This search identified five relevant studies, two of which reported the differential exosomal miRNA profiles isolated from MTB-infected macrophages; and three of which were clinical studies that indicated the role of exosomal miRNAs in the differentiation between TB patients and healthy state controls or asthma patients. However, current exosome miRNA research does not reflect the complex clinical conditions that are highly similar to TB disease but are difficult to differentiate. Furthermore, no studies have evaluated the diagnostic value of exosomal miRNAs in the tuberculous meningitis diagnosis.

Added value of this study

We performed a prospective multi-stage study to assess the performance of exosomal miRNAs and EHR data in the TBM and PTB diagnoses. The results indicated that exosomal miRNAs had the potential as diagnostic biomarkers for TBM and PTB disease. To the best of our knowledge, our report is the first to assess the diagnostic value of exosomal miRNAs in TBM. Notably, we reported an innovative machine learning framework combining exosomal miRNAs with patient's EHR data, with a superior performance in differentiating TBM and PTB patients from those highly suspected cases. The strengths of our study include its prospective and multiple phase design, disease control and healthy control setting, and the use of machine learning modeling.

Implications of all of the available evidence

Our findings, together with existing evidence, show that exosomal miRNAs are potential biomarkers to promote the TB diagnosis, especially for TBM disease. The combination of miRNAs and EHR data through machine learning algorithm could serve as a feasible approach for improving the clinical diagnoses of the TBM and PTB diseases. However, further validation is needed.

often consist of dozens or hundreds of gene loci, such as the 393-transcript signature reported by Berry and colleagues [7]. These signatures have reasonable performances but are often expensive, technically demanding and difficult to deploy in the clinic [6]. A minimal set of gene loci with high diagnostic accuracy is urgently needed in the field [6], and several alternative approaches have been explored recently [8,9]. Sweeney and colleagues described a three-gene set (*GBP5*, *DUSP3*, and *KLF2*) that effectively discriminated active TB from other diseases with an area under the curve (AUC) of 0.83 [8]; however, their cohorts originated from public GEO datasets, and qPCR validation was not performed. Maertzdorf and colleagues demonstrated the diagnostic potential of a 4-gene set (*GBP1*, *ID3*, *P2RY14*, and *IFITM3*) for TB disease [9]; this 4-gene signature had variable performances in different datasets (AUC ranged of 0.58–0.94), and its performance for extrapulmonary TB remains unclear.

Recently, exosomes have emerged as a powerful tool for biomarker exploration in a number of diseases [10–14]. Exosomes are microvesicles released from living cells into the circulation, typically with a diameter of 30–100 nm and containing RNA and protein molecules. Exosomes are thought to participate in cell-to-cell communication and immune modulation by transporting and delivering their cargo molecules to target cells. Among different exosomal cargo molecules, microRNAs (miRNAs) are considered the most promising candidate biomarkers due to their high abundance, inherent stability, ease of sampling, and importance as global cellular regulators [11]. Indeed, the diagnostic potential of exosomal miRNAs has been the target of an extensive number of studies, especially in relation to cancer [10,12]. Exosomal miRNAs can pass through the blood-brain barrier [13] and traffic to the lung [11]; thus, they can be released from the site of infection into the circulation and serve as sentinels of focus pathophysiology and potential biomarkers. Until now, only a small number of TB studies focusing on exosomal miRNAs had been conducted. Singh et al. [15] and Alipoor et al. [16] reported differential profiles of exosomal miRNAs isolated from *Mycobacterium tuberculosis* (*MTB*)-infected macrophages, which showed that exosomal miRNAs had immunoregulatory and diagnostic potential during *MTB* infection. Two other studies also revealed that exosomal miRNAs could be candidates to discriminate TB patients from healthy state (HS, for short, including healthy controls and latent tuberculosis infection [LTBI] patients) controls [17] or asthma patients [18]. However, to the best of our knowledge, no published studies have explored whether exosomal miRNAs could be useful to distinguish TB from other diseases that had similar symptoms, which is more relevant and challenging than distinguishing TB from HS controls [19]. Furthermore, no published studies have explored the diagnostic value of exosomal miRNAs in TBM, the most severe form of TB. Thus, a more systematic and comprehensive study of exosomal miRNAs with regard to their potential as noninvasive TB biomarkers is still urgently needed.

In addition to exploring molecular and cellular biomarkers, researchers have also investigated various analytical models that can diagnose TBM based on electronic health records (EHRs) [2]. One example of such a model is the Thwaites' Vietnam model, which established a five-feature scoring scheme with reported 86% sensitivity and 79% specificity for TBM [2]. Despite these promising results, earlier models often showed inconsistent performance and were difficult to implement in different populations and settings. For example, the specificity of the Vietnam model reportedly dropped to 43% in a Malawi cohort [2] and only 5% in a Chinese cohort [20]. It is increasingly appreciated that, additional clinical data or approaches may be needed to improve the performance of current TB diagnostic methods.

The work described in this article consists of four sequential steps (Fig. 1). In the Exploratory Step, we identified 11 exosomal miRNAs that were significantly differentially expressed between TB cases (including both PTB and TBM) and HS controls, by using a microarray platform. In the Selection Step, by comparing PTB/TBM with their respective controls and using the qRT-PCR method, we further winnowed down to 6 miRNAs. In the Training Step, we trained machine learning Support Vector Machine (SVM) models combining exosomal miRNAs with EHR data by cross-validation to differentiate PTB/TBM patients from their disease controls or HS controls. Finally, in the Testing Step, we evaluated the performance of the models on new PTB/TBM cohort. The combined "EHR+miRNA" model performed better than using EHR data and miRNA data alone, which achieved a diagnostic sensitivity of 0.94 (95% CI 0.84–1.00) and specificity of 0.95 (0.86–1.00) for TBM, and 0.89 (0.84–1.00) for both sensitivity and specificity for PTB, respectively. In addition, to the best of our knowledge, this study represents the first time that exosomal miRNAs have been shown to be effective biomarkers for TBM disease.

2. Materials and methods

Fig. 1 shows the flow chart of the study.

2.1. Study participants

The exploratory cohort included 11 active TB patients (7 PTB and 4 TBM) and 8 controls. They were 10 males and 9 females from ages 25 to 58 years, with no significant age and gender difference between TB

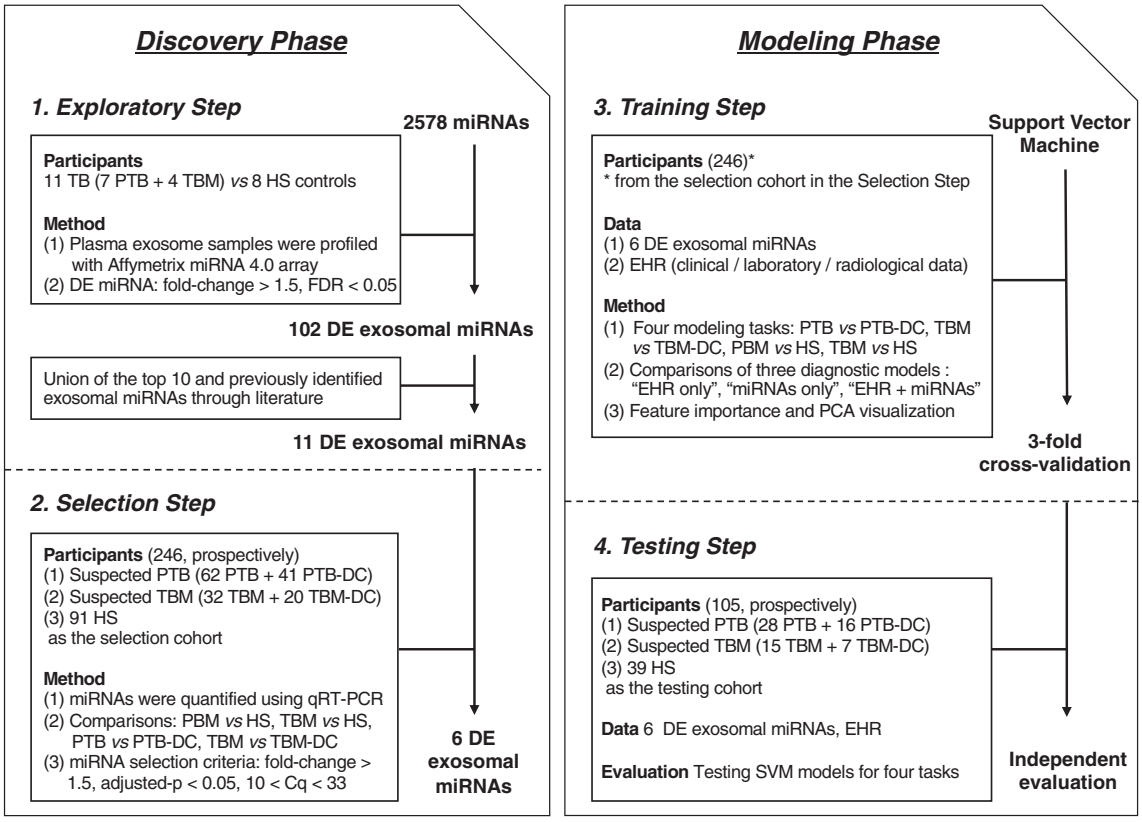


Fig. 1. Overview of the strategy for investigating exosomal miRNAs and diagnostic models for TBM and PTB A total of 407 individuals were recruited, and 370 individuals were finally included. PTB: pulmonary tuberculosis; TBM: tuberculosis meningitis; HS: healthy state; DE exosomal miRNAs: differentially expressed exosomal miRNAs; PTB-DC: non-PTB disease control; TBM-DC: non-TBM disease control; Cq: cycle of quantification; EHR: electronic health record; PCA: principal component analysis; SVM: support vector machine.

and control groups. The 11 TB patients were confirmed by *MTB*-culture, and HIV-negative, and without other infectious diseases. The 8 controls were household contacts, who were regarded as healthy after taking a physical examination and had no history of TB. We prospectively interrogated a selection cohort of 272 individuals and another independent testing cohort of 116 individuals, and finally enrolled 246 and 105 participants, respectively. The two cohorts were as follows: [1] The selection cohort consisted of 103 suspected PTB cases, 52 suspected TBM cases and 91 HS controls; [2] The testing cohort included 44 cases suspected of having PTB, 22 cases suspected of having TBM and 39 HS controls.

Inpatients who underwent investigation for persistent highly suspected PTB or TBM symptoms were recruited at the Department of Tubercular Medicine, West China Hospital, between March 2016 and January 2017. Demographic, clinical, and radiological data of patients were obtained within 48 h of admission. *MTB* tests and other laboratory examinations were conducted by the College of American Pathologists (CAP)-certified central lab of West China Hospital. Details of inclusion and exclusion of participants, the rationale for excluding subjects, and patients' examination are in the Supplemental Material (Section 1).

For each patient, the final diagnosis was determined by a panel of two experienced specialists according to the patient's discharge diagnosis and follow-up confirmation [21,22]. Highly suspected PTB cases were further categorized as the PTB group and non-PTB disease control (PTB-DC, for short) group. Highly suspected TBM cases were categorized as the TBM group and non-TBM disease control (TBM-DC, for short) group. TB cases were treated with a standard anti-TB regimen and adjunctive therapy such as prednisone, and sudden situations and urgent symptoms were treated according to the clinical protocol and guideline [23], see Supplemental Material (Section 2). Patients were evaluated every week initially for the first four weeks, and monthly thereafter through outpatient follow-up or telephone interview; the whole follow-up period lasted 12 months. HS individuals were recruited from relatives or close contacts with no history of TB and a normal physical examination. Detailed diagnostic criteria for participants are in the Supplemental Material (Section 2). The study was approved by the Clinical Trials and Biomedical Ethics Committee of West China Hospital, Sichuan University [No. 2014 (198)]. Informed consent was obtained from the participants.

2.2. Exosome isolation and identification

A 5-ml EDTA-treated blood sample was collected from each participant and centrifuged for plasma isolation within 2h. Exosomes were extracted from plasma using the ExoQuick Kit (System Biosciences, USA) [12,24] and thromboplastin D (Thermo Fisher Scientific, USA) according to manufacturer's instruction (the process is described in Supplemental Fig. 1A). The exosome precipitation was re-suspended before Qiazol buffer was added in for the preparation of RNA extraction. To determine whether the extracted exosome was affected by extra-vesicular RNAs, we treated purified exosome with RNase A and the detergent Triton X-100 at 37 °C for 10 min before RNA extraction as described earlier [25,26]. qRT-PCR analysis of miRNA expression remained unchanged when treated with RNase A alone but decreased when simultaneously treated with RNase A and Triton X-100 (Supplemental Fig. 2), which indicated that the majority of our signal was derived from exosomal miRNAs rather than extra-vesicular miRNA.

The isolated exosomes were verified by Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and Western blotting. Particle size and concentration were measured by NTA with a NanoSight NS300 instrument (Malvern Instruments, UK). The morphology of isolated exosomes was examined by TEM (FEI Tecnai™ G2 Spirit, Czech Republic) with a voltage of 80 kV. The extracellular vesicle protein markers CD63, CD81 and HSP70 were adequately detected in

Western blotting, probed with corresponding antibodies (System Bioscience, USA). GAPDH (Santa Cruz Biotechnology, USA) was used as an internal control.

2.3. RNA isolation and cDNA preparation

Exosomal RNA was extracted by using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany). Cel-miR-39 was added as a "spike-in" normalization control for qRT-PCR quantification, as described in previous studies [27,28]. RNA concentration and purity were measured by using a NanoDrop ND-1000 (Thermo Fisher Scientific, USA). Qualified RNA samples had a 260/280-nm absorbance ratio >1.8 and a 260/230-nm absorbance ratio >2.0. Only RNA samples of high quality were used for subsequent experiments. We then used the miScript II RT Kit (Qiagen, Germany) to prepare cDNA according to the standard reverse transcriptional (RT) reaction protocol. Because the absolute number of exosomes and the exosomal RNA content in plasma samples derived from different individuals are difficult to quantify, it is inappropriate to normalize the measured miRNA expression level by matching against the amount of input RNA. We therefore used a fixed volume of plasma sample and RNA eluate rather than a fixed mass of RNA, as input into the RT reaction, and used cel-mir-39 to normalize potential sample-to-sample variations and technical variations as described earlier [28].

2.4. miRNA profiling

Total RNA was extracted and purified, and targets from blinded RNA samples were prepared and hybridized with an Affymetrix Genechip miRNA 4.0 Array (Affymetrix, USA), measuring 2578 miRNAs according to the GeneChip Expression Analysis Technical Manual. Quality control of the gene expression data was performed using gene-specific probes. Microarray quality assurance was carried out by detecting outlier arrays based on standard post-hybridization quality metrics such as the normalized unscaled standard error (NUSE) plot, the relative log expression (RLE) plot, and the mean signal comparison of perfect match probe and background probe, *E. coli* spike-in quality control, and raw microarray images (Supplemental Fig. 3). Raw data was normalized using robust multi-array average (RMA) algorithm and analyzed using a random variance model (RVM) *t*-test in R packages. Differentially expressed (DE) exosomal miRNAs with statistical significance were selected based on the following criteria: an absolute expression fold change >1.5 and a false discovery rate (FDR) value <0.05. DE exosomal miRNAs were visualized using hierarchical clustering and principal component analysis (PCA). The microarray profile data were submitted to NCBI Gene Expression Omnibus (GSE116542, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE116542).

2.5. miRNA qRT-PCR quantification

Exosomal miRNA expression was measured using the miScript SYBR® Green PCR Kit and miRNA-specific primers (Qiagen, Germany). Each 10 μl reaction mixture included 2 μl of RNase-free water, 0.5 μl of miRNA-specific primer, 0.5 μl of 10 X Miscript Universal Primer, 5.0 μl of 2 X QuantiTect SYBR Green PCR Master Mix, and 2 μl of template cDNA. The reactions were performed with the following cycling parameters: 95 °C for 10 min as an initial step followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 10 °C for 30 s. After amplification, a high-resolution melting curve was generated (gradually increasing the temperature from 65 °C to 95 °C at a rate of 0.1 °C/s and acquiring fluorescence data every 0.3 °C) to analyze the purity of the amplification products. qPCR amplification was carried out in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Switzerland). All miRNAs were measured in a blinded fashion. The obtained data were analyzed using Gene Scanning software v1.2 (Roche Diagnostics). Double distilled water (PCR grade) was used as a no-cDNA template control in each qPCR run, and in addition, reverse transcription negative controls and the use of DNA as a qPCR template were tested to ensure the accuracy of the q RT-PCR process. All of the above negative controls showed no detectable cycle of quantification (Cq) values, indicating the lack of any contamination or nonspecific signal. Each sample was run in triplicate, and a CV < 15% cutoff was established to validate the technical replicates. The average Cq was calculated, and $10 < Cq < 33$ was considered acceptable based on our prior qRT-PCR experience. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta Cq}$ method [29], where ΔCq = Cq miRNA – Cq miR-39, $\Delta\Delta Cq = \Delta Cq$ – average ΔCq (healthy states).

2.6. EHR and miRNA data used for modeling

EHR data, including the patients' demographic, clinical, laboratory, and radiological findings, were collected separately by two authors (Lijuan Wu and Minjin Wang), who were blinded to the diagnostic categorization. DE exosomal miRNAs were determined and selected through the Exploratory Step and Selection Step (Fig. 1, Discovery Phase), and the expressions of DE exosomal miRNAs among participants in the selection cohort were used for modeling. The initial EHR and miRNA features before modeling are listed in Supplemental Material (Section 3).

2.7. Data Pre-processing

We applied a 20% missing value and 2% difference between groups as thresholds to remove incomplete and uninformative EHR and miRNA features. A total of 41 out of 62 features in TBM patients *vs* TBM-DC patients, 34 out of 50 features in PTB patients *vs* PTB-DC patients, and all 18 features in TBM or PTB patients *vs* HS controls remained after this filtering step, including the log conversion of miRNA expression, listed in Supplemental Material (Section 3). All variables in both the selection and testing cohorts were normalized to a mean of 0 and variance of 1. We tested the distribution of each feature for verification purposes. We found that most features were normally distributed, and the mean and variance were largely consistent between datasets.

2.8. Diagnostic modeling and further evaluation

In the Training Step, the linear kernel SVM algorithm was used which was implemented using Python Scikit-learn script. SVM is considered a well-suited machine learning method for classification with limited number of samples, high dimensional pattern recognition and reasonable interpretability and has been applied in multiple clinical scenarios [30,31]. We trained SVM model using exosomal miRNAs, EHR data and their combination in the selection cohort for four tasks: PTB *versus* PTB-DC, TBM *versus* TBM-DC, PTB *versus* HS and TBM *versus* HS. We adjusted the parameter Penalty C of the error term for regularization, which aimed to trade off the misclassification of training examples against simplicity of the decision surface and reduce the redundancy between features. SVM hyper-parameter were tuned using k-fold ($k = 3$) cross-validation. The hyper-parameter was selected based on its performance in the internal validation sets, especially through AUC. A final model was constructed through training on the whole selection cohort with the best hyper-parameter. Considering our limited sample size and relatively larger number of features during modeling, we further chose the top 5, top 10, and top 15 features in the "EHR+miRNA" model to establish the diagnostic model, with the purpose of avoiding overfitting; the top 10 features were selected due to their higher performances in the re-established "EHR+miRNA" model. Feature coefficients in the trained models were calculated to represent the contribution of individual feature to the diagnostic model. As the raw data were normalized, feature with a higher absolute coefficient value was considered more important. Unsupervised PCA with the top 10 features was used to ascertain and visualize the classification capacity of the optimal model.

In Testing Step, the final model from the Training Step was further evaluated in the independent testing cohort. Diagnostic classifications for patients using SVM algorithm were made by an author (Shun Liao), who was blinded to the diagnoses made by the clinicians. Code and normalized data are available from: https://github.com/shun1024/tb-exosomal-mirna.

2.9. Bioinformatics analysis

We predicted *in silico* the target genes of the DE exosomal miRNAs by intersecting the predicted targets of the TargetScan and miRanda databases [32]. The predicted target genes were annotated with gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the DAVID software. GOs and enriched pathways with FDR of <0.05 were significant. Networks of DE exosomal miRNAs and related GO terms or pathways were constructed using Cytoscape software.

2.10. Statistics

Categorical variables with a Chi-square test and continuous variables with the Mann-Whitney *U* test or *t*-test were used for two-group comparison. One-way Analysis of variance (ANOVA) or Kruskal-Wallis test was used for comparing multiple groups. A Friedman test was used as the non-parametric alternative to the one-way ANOVA with repeated measures. The correlation between exosomal miRNAs and EHR features were performed by Spearman rank correlation. FDR was used for *p*-value adjustment. All statistical tests were 2-sided, and $p < .05$ was considered significant. SPSS v20.0 and GraphPad Prism v6.0 were used to analyze and graph the results.

3. Results

3.1. Characteristics of the prospective enrolled subjects

We prospectively enrolled a final number of 351 individuals (Fig. 1); 246 were included in the selection cohort (62 PTB, 41 PTB-DC, 32 TBM, 20 TBM-DC, and 91 HS) and 105 in the testing cohort (28 PTB, 16 PTB-DC, 15 TBM, 7 TBM-DC, and 39 HS). Table 1 shows the participants' baseline characteristics; the selection cohort and testing cohort were combined together since they were statistically indistinguishable. A total of 58.1% of the participants were male (204/351). The PTB-DC patients had older ages and higher BMIs than the PTB cases (both $p < .0001$), similar to reported in a previous study [33]. Bacteriologic tests for *MTB* (TB-DNA, smear, and/or culture) indicated a remarkably lower sensitivity for TBM patients (17.02%) than PTB patients (65.56%), mainly due to the low bacillary load in TBM samples and limited sample volume [2]. Other statistical differences between TB patients and disease control cohorts included higher hematocrit values and lower platelet counts in PTB patients and increased abnormal radiologic detection in TBM than in disease controls. We noticed that the disease control groups had a similar high level of C-reactive protein and a high percentage of positive TB-IGRA result as PTB or TBM patients, which possibly due to a high rate of active pulmonary infectious diseases and a high LTBI rate respectively in our disease control participants [34]. In addition, there were significantly different baseline indicators such as age, BMI, smoking, hematocrit, platelet counts, and leucocytes between HS controls and suspected patients. Details regarding the PTB-DC and TBM-DC patients are summarized in Supplemental Table 1.

3.2. Exosome isolation

NTA analysis showed that vesicles isolated from plasma had a diameter size range of ~96.8 ± 1.6 nm (Supplemental Fig. 1B), consistent with the known size of exosomes. The number of exosomes per 500 μl of plasma was approximately 1.2×10^9. Round, cup-shaped nanovesicles ranging from 90 to 100 nm in diameter were visualized by TEM (Supplemental Fig. 1C). These vesicles were further confirmed to be exosomes based on the expression of the protein markers CD63, CD81 and HSP70 through Western blotting (Supplemental Fig. 1D).

3.3. Distinct exosomal miRNA profiles in TB

In the Exploratory Step, exosomal miRNA profiling yielded a total of 102 DE exosomal miRNAs (98 with upregulated expression and 4 with downregulated expression) between the TB patients and HS controls.

Table 1
Demographic and clinical features of participants in the prospective selection and testing cohorts.

Clinical features	Suspected PTB patients			Suspected TBM patients			HS controls	p1	p2
	PTB ($n = 90, 62 + 28$)	PTB-DCs ($n = 57, 32 + 16$)	p	TBM ($n = 47, 32 + 15$)	TBM-DCs ($n = 27, 20 + 7$)	p	($n = 130, 91 + 39$)		
Gender, male	57 (63.3%)	36 (63.3%)	0.983	23 (48.9%)	16 (59.3%)	0.392	72 (55.4%)		
Age (years)	42.3 ± 18.6	62.8 ± 16.8	<0.0001	34.4 ± 16.7	33.6 ± 16.3	0.843	36.9 ± 10.5	<0.0001	0.191
BMI (kg/m^2)	20.1 ± 3.2	22.4 ± 3.3	<0.0001	21.3 ± 3.1	21.8 ± 2.9	0.463	20.5 ± 3.8	0.125	0.013
Smoking	32 (35.6%)	22 (38.6%)	0.709	14 (29.8%)	8 (29.6%)	0.989	32 (24.6%)	0.030	0.426
Radiologic pathology detected	64 (71.1%)	37 (64.9%)	0.430	38/43 (88.4%)	10/20 (50.0%)	0.003	–	–	–
MTB tests, positive/detected									
Bacteriologic test	59 (65.56%)	–	–	8 (17.02%)	–	–	–	–	–
MTB-DNA	32/71 (45.1%)	–	–	7 (14.9%)	–	–	–	–	–
Smear	30 (33.3%)	–	–	4 (8.5%)	–	–	–	–	–
Culture	23/45 (51.1%)	–	–	7 (14.9%)	–	–	–	–	–
Other related laboratory tests								–	–
Positive TB-IGRA	41/59 (69.5%)	20/30 (66.7%)	0.786	25/47 (53.2%)	8/12 (66.7%)	0.401	–	–	–
C-reactive protein (mg/L)	15.3 (4.1–36.9)	43.7 (18.1–103.8)	0.072	5.7 (2.7–16.8)	8.3 (1.7–18.3)	0.851	–	–	–
ESR (mm/h)	44.0 (23.0–65.0)	43.5 (19.0–64.8)	0.598	18.0 (8.0–45.0)	35.0 (18.0–48.0)	0.741	–	–	–
Hematocrit	0.4 ± 0.1	0.3 ± 0.1	0.008	0.3 ± 0.1	0.4 ± 0.1	0.110	0.4 ± 0.03	<0.0001	<0.0001
Platelets (×10^9/L)	244.0 (182.0–354.0)	259.5 (147.8–306.8)	0.008	190.0 (141.0–275.0)	177.0 (154.0–283.0)	0.736	193.0 (164.8–220.8)	<0.0001	0.601
Leucocytes (×10^9/L)	5.6 (4.4–7.4)	7.1 (4.5–10.4)	0.069	5.9 (3.7–8.7)	6.5 (5.4–11.0)	0.343	5.4 (4.6–6.4)	0.027	<0.0001

The selection and testing cohorts are combined together. PTB: pulmonary tuberculosis; TBM: tuberculous meningitis; PTB-DCs: non-PTB disease controls; TBM-DCs: non-TBM disease controls; HS: healthy states; ESR: erythrocyte sedimentation rate. Among 47 TBM patients, 22 had accompanied with PTB. Bacteriologic test included *MTB*-DNA, smear, and culture. TB-IGRA: tuberculosis interferon gamma release assay. p1: p-value for the comparison of suspected PTB patients *versus* HS controls. p2: *p*-value for the comparison of suspected TBM patients *versus* HS controls.

Clear discrimination between TB cases and controls was observed in the hierarchical clustering and PCA results (Supplemental Fig. 4).

The top 10 out of the 102 DE exosomal miRNAs that met the criteria for fold change and statistical significance were selected, which included 6 upregulated miRNAs (miR-191, 20b, 26a, 106a, let-7c, and 20a) and 4 downregulated miRNAs (miR-3128, 1468, 3201, and 8084). We also included another DE miRNA, miR-486, together with these top 10 miRNAs. Although not in the top 10 (ranked as no. 22), miR-486 still had a high fold change and an FDR-value of 0.003. Furthermore, miR-486 is known to have elevated expression from earlier blood cell studies (GSE29190, GSE39163, and GSE34608) [35–37] and an exosome study as well [15]. Details regarding the 11 candidate miRNAs are provided in Fig. 2.

3.4. Identification of 6 differentially expressed miRNAs

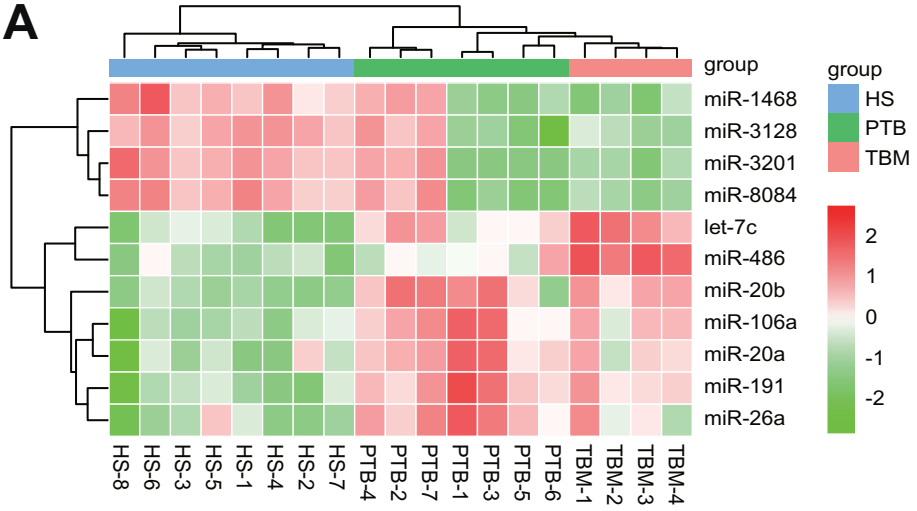
In the Selection Step, we quantified the expression of these 11 exosomal miRNAs by using qRT-PCR in the prospective selection cohort (62 PTB cases, 41 PTB-DC, 32 TBM cases, 20 TBM-DC, and 91 HS). Group comparison was conducted for PTB/TBM (*i.e.*, PTB *vs* PTB-DC, PTB *vs* HS, TBM *vs* TBM-DC, and TBM *vs* HS). The DE exosomal miRNAs were further selected based on the following criteria: median fold change >1.5 in any group comparison, age-adjusted $p < .05$, and $10 < Cq < 33$. Five miRNAs with very low or undetectable expression ($Cq \geq 33$) or fold change <1.5 were excluded from the subsequent study. The six remaining miRNAs (miR-20a, 20b, 26a, 106a, 191, and 486) were upregulated in PTB or TBM patients than in their disease controls or HS counterparts (Supplemental Fig. 5). The increased expression of these miRNAs was also reported in previous studies [15,19].

We next tested these 6 miRNAs in a prospective testing cohort, consisted of 28 PTB cases, 16 PTB-DC, 15 TBM, 7 TBM-DC, and 39 HS controls. When compared between PTB *vs* PTB-DC, PTB *vs* HS, and TBM *vs* HS, all of these 6 miRNAs showed consistent upregulation in diseases cases than in control cases, except for a nonsignificant change of miR-486 in PTB *vs* HS. When compared TBM with TBM-DC, 3 miRNAs (miR-20b, 191, and 486) remained a higher expression in TBM cases (Supplemental Fig. 6).

3.5. Stability of exosomal miRNAs in plasma

Reasoning that an ideal miRNA biomarker should remain stable in plasma, we evaluated the freeze-thaw stability and short-term (bench-top) stability of samples, which were commonly encountered in clinical practice [38]. We randomly selected plasma samples from 6 healthy individuals, and their plasma samples were subjected to different conditions such as prolonged incubation at 4 °C (0 h, 1 h, 2 h, 6 h,

A



B

miRNA	Fold change	FDR
miR-191	5.13	0.003
miR-20b	4.92	0.003
miR-26a	4.26	0.003
miR-106a	4.21	0.003
let-7c	4.02	0.003
miR-20a	3.95	0.003
miR-486	3.31	0.003
miR-3128	-3.17	0.004
miR-1468	-2.35	0.004
miR-3201	-2.53	0.003
miR-8084	-2.75	0.004

Fig. 2. Eleven candidate differentially expressed miRNAs were selected from the exploratory cohort (A) Hierarchical clustering of the differentiation between TB patients and controls based on the expression of 11 DE exosomal miRNAs. The exploratory set included 11 TB patients (4 TBM patients and 7 PTB patients) and 8 HS controls. A red diamond indicates increased miRNA expression, a green diamond represents decreased miRNA expression, and deeper colour means a larger fold change. (B) The expression of 11 miRNAs in the exploratory cohort. FDR: false discovery rate.

12 h, and 24 h, respectively) and repeated freeze-thaw cycles (0, 1, 2, and 4 times, respectively), as described in Supplemental Material (-Section 6). Time point and freeze-thaw cycle were chosen according to the prevailing storage and analysis conditions in many clinical labs in China. We concluded that such treatment had minimal effect on the levels of exosomal miRNAs (miR-20a, 20b, 26a, 106a, 191, and 486) as measured by the qRT-PCR method (Supplemental Table 2–3, Friedman test, p-values all >0.05), which was consistent with previous literature [28,39].

3.6. Correlation between DE exosomal miRNAs and clinical features

We next evaluated whether the PTB or TBM patients' clinical features ascertained upon admission were correlated with exosomal miRNA abundance. No significant correlations were observed between miRNA expression and age, gender, BMI, laboratory data, and radiological data using Spearman's rank correlation (p-values all >0.05, data not shown, available upon request).

3.7. Response to anti-TB treatment

We randomly selected 15 TB patients with typical and similar symptoms from the selection cohort and the testing cohort, and analyzed their paired plasma samples before and after 2 months of intensive anti-tuberculosis (anti-TB) therapy [23]. Good response to therapy was defined as significant clinical and radiological relief as determined independently by two experienced physicians. All the patients had a good response to therapy except for 4 patients, who suffered from drug-resistance or adverse drug reaction (see Supplemental Material. Section 7). Using a 2-fold change as the cutoff, among a total of 90 points (15 patients × 6 miRNAs), the expression levels of the 6 DE exosomal miRNAs significantly declined in 55 points, statistically unchanged in 29 points, and increased in only 6 points ($p = 4.80 \times 10^{-5}$, Fig. 3). These results suggested that the majority of the dysregulated miRNA expression in these patients was restored after intensive anti-TB therapy, albeit we only profiled 6 miRNAs in a small number of patients. It is worth noting that, among the 4 patients who did not have a good response, miR-191 was increased (4.10 fold) in a patient with anti-TB drug-induced leucopoenia, and miR-286 was increased (3.54 fold) in another patient with multiple drug resistance TB (see Supplemental Material. Section 7).

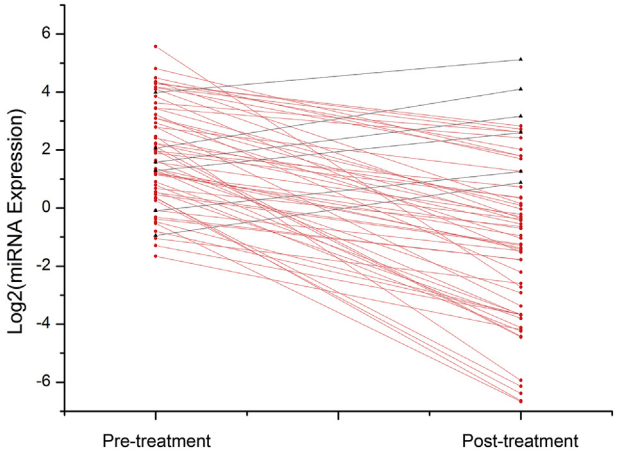


Fig. 3. The alteration of exosomal miRNAs before and after 2-month intensive anti-TB therapy The altered miRNA expression of 6 DE exosomal miRNAs was calculated using $\log_2$ miRNA (post-treatment expression / pre-treatment expression ratio). The fold-change threshold was set at 2. A total of 90 points were detected in 15 TB patients, among which the expression of 55 points significantly declined (red lines), 29 points did not obviously change (not shown in the figure for the sake of brevity), and only 6 points increased (black lines). The Wilcoxon matched-paired rank test was used for comparisons between paired samples ($p = 4.80 \times 10^{-5}$).

3.8. Diagnostic models and features analysis

In the training and Testing Step, We developed analytical models to investigate the feasibility of using exosomal miRNAs and EHRs as biomarkers for TB diagnosis. The details on SVM construction can be found in Materials and Methods. Overall, the "EHR+miRNA" model achieved the best AUC in most cases (see Table 2), for both the selection and the testing cohorts.

3.8.1. TBM discriminative model and feature analysis

Fig. 4A shows the performance of the three models in the Training Step, as evaluated by 3-fold cross-validation in the training cohort (see Materials and Methods). We observed that miRNAs remarkably increased the model performance in distinguishing between TBM patients and TBM-DC patients. The "EHR+miRNA" model and "miRNA only" models both achieved a maximum AUC of 0.97 (95% CI 0.88–0.99) in the training cohort regarding Penalty C inside SVM, while the “EHR only” model only obtained a value of 0.60 (0.46–0.72) (Fig. 4A, Table 2). Fig. 4B shows that these three models had consistent performances in the Testing Step on another independent testing cohort. The "EHR+miRNA" model had an AUC of 0.97 (95% CI 0.80–0.99); sensitivity of 0.94 (95% CI 0.84–1.00) and specificity of 0.95 (95% CI 0.86–1.00). This was slightly better than that of the "miRNA only" model, and significantly better than that of "EHR only" model (Fig. 4B and Table 2). Fig. 4C indicates the top 10 features of the "EHR + miRNA" and the "EHR only" model on TBM, which had the highest contribution to the model (see Materials and Methods). Four features of the "EHR + miRNA" model were miRNAs (miR-486, 20b, 191, and 106a), and the remaining 6 were EHR data. The most important feature was miR-486, followed by miR-20b and the CSF/blood glucose ratio. Fig. 4D shows an unsupervised PCA of the 32 TBM cases and 20 TBM-DC cases of the selection cohort, calculated based on the aforementioned 10 features in the "EHR + miRNA" model. The TBM cases and TBM-DC cases were clearly separated.

3.8.2. Comparison with existing TBM diagnostic models

We applied published EHR diagnostic scoring schemes [2] to analyze the TBM testing cohort used in our study (Supplemental Table 4). The Vietnam model had an excellent sensitivity (0.98) but lower specificity (0.11), which was similar to the values reported in the literature [2,20]. The Turkey and Morocco models achieved moderate performances (sensitivity: 0.62 and 0.64; specificity: 0.74 and 0.70, respectively). The Chinese model had a sensitivity of 0.32 and specificity of 1.00 [20].

3.8.3. PTB discriminatory model and feature analysis

Among the three different models, the "EHR+miRNA" model showed superior performance in PTB suspected cases, with an AUC ≥ 0.94 in both the selection and testing cohorts (Supplemental Fig. 7A–B). The sensitivity and specificity of the ‘"EHR+ miRNA" model both increased to 0.89 (0.80–0.98), shown in Table 2. The features of the "EHR + miRNA" model involved 3 miRNAs (miR-20b, 486, and 191) and 7 EHR factors, which completely overlapped the those of the "EHR only" model. The most important factor was TB-smear, followed by miR-20b and hematocrit (Supplemental Fig. 7C). Clear delineation between PTB and PTB-DCs in the PCA also demonstrated the feasibility of modeling (Supplemental Fig. 7D).

3.8.4. Discriminating TB from healthy states

Both "EHR+miRNA" and "EHR only" models had satisfactory discriminative performance in distinguishing PTB or TBM from HS controls, indicating that current EHR information was sufficient for identifying TB patients from the general population. The performances and features of models are shown in Table 2 and Supplemental Figs. 8–9.

Table 2
Performances of the comparative diagnostic models.

Models	Cross-validation in the selection cohort			Testing cohort		
(EHR + miRNA /miRNA / EHR)	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
TBM *vs* TBM-DCs	0.97/0.97/0.60	0.94/0.97/1	1/0.95/0.43	0.97/0.95/0.67	0.94/0.93/0.71	0.95/0.97/1
PTB *vs* PTB-DCs	0.94/0.80/0.89	1/0.62/0.97	0.84/1/0.70	0.97/0.87/0.93	0.89/0.70/0.93	0.89/1/0.82
TBM *vs* HS	0.94/0.86/0.88	1/1/1	0.91/0.77/0.91	0.98/0.85/0.96	1/1/0.93	0.86/0.81/0.95
PTB *vs* HS	0.99/0.78/0.98	1/0.96/0.91	0.98/0.70/0.98	0.99/0.81/0.99	0.95/0.95/1	0.98/0.76/0.98

Notes: The labeling '(EHR + miRNA / miRNA / EHR)' indicates the model with EHR and miRNA data, the model with miRNAs only, and the model with EHR only, respectively. The 95% confidence intervals are presented in the Supplemental Material. Section 8.

3.9. miRNA functional analysis

A total of 186 genes were predicted to be targeted by the 6 DE miRNAs (Supplemental Fig. 10). A total of 109 "biological processes" are targeted by targeted by these 6 miRNAs, which included including apoptosis, immune response, transcription, and neurotrophin receptor signaling (Supplemental Fig. 10B). A total of 52 unique KEGG pathway were targeted; the top 10 ranked pathways included pathways related to cancer occurrence, inflammatory response, and migration of immune cells (Supplemental Fig. 10C). We speculated that these 6 DE miRNAs may be involved in immunoregulatory functions in the context of TB, although this hypothesis needs further confirmation.

4. Discussion

The discovery of biomarkers and diagnostic algorithms holds great value for TB diagnostics, in which an increasing clinical concern is how to distinguish active TB patients from those with highly similar diseases. In this study, we identified 6 DE exosomal miRNAs in TB patients, 3 out of which showed a significant discriminatory value for TBM and PTB. We also integrated miRNAs with EHR data into a more comprehensive model and achieved a superior performance in the differential diagnosis of TBM and PTB.

Small biosignatures with robust discriminatory capacities could transform omics data for field use and greatly facilitate translation into clinical tests, as has been highly recommended in recent TB studies

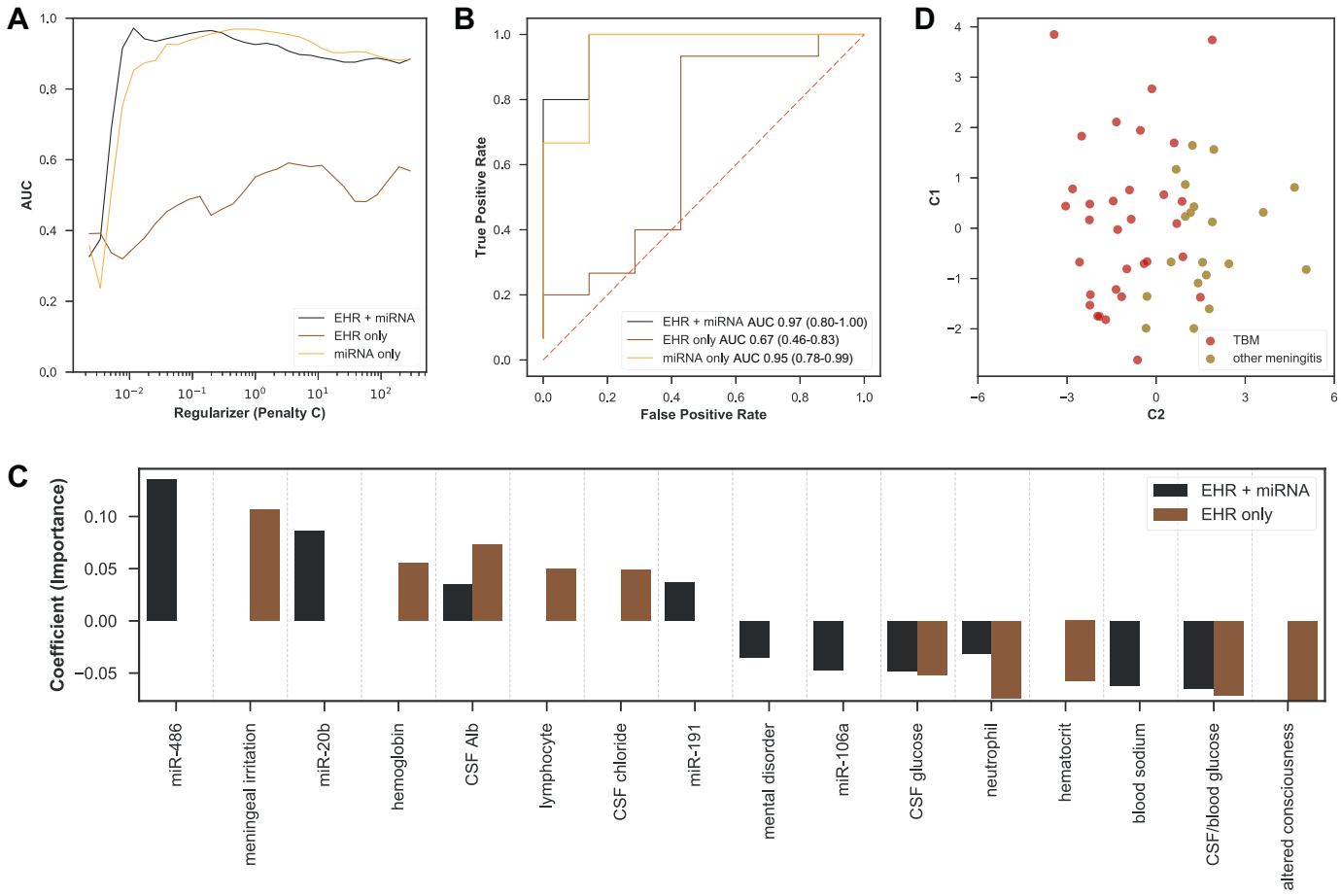


Fig. 4. Modeling result for TBM *versus* TBM-DC (A) AUC values in the selection cohort with tuning parameter Penalty C. Penalty C is a regularization parameter, which aims to trade off the misclassification of training examples against the simplicity of the decision surface and reduce the redundancy between features. The hyper-parameter set with a maximum AUC in the concatenated validation sets was finally used to establish the models. In detail, the model predictions in each validation set (3-fold) are concatenated. (B) AUC values in the testing cohort with models built with the best hyper-parameter set and trained on the whole selection cohort. (C) The top 10 important features of the models and their corresponding ranks. As raw data were normalized, features with higher absolute coefficient values were considered more important. A coefficient >0 and <0 means a positive and a negative correlation with TBM prediction, respectively. (D) Unsupervised PCA visualization for TBM differentiation based on the top 10 features of the trained "EHR+miRNA" model.

[8,9]. Blood-based biomarkers have the potential to rapidly identify delayed responsiveness or no responsiveness to anti-TB treatment [40]. We also found that DE exosomal miRNA expression decreased as the patient attained remission following anti-TB therapy, in accordance with reported evidence that changes in miRNA levels may be potential markers of treatment response [41,42]. Following validation in larger studies, qRT-PCR-based 3 DE exosomal miRNA signatures represent a diagnostic approach that is simple to perform, cost-effective and could complement clinical settings for disease [28], especially for TBM diagnosis.

We also investigated the utility of EHR data for TBM diagnosis. Previous TBM models did not perform well with our EHR data, most likely because our dataset involved a wider range of conditions that was highly similar to TBM and was more representative of the population as a whole for comparison. This finding contrasts with previous models that considered one specific meningitis patient as the control. Similarly, our "EHR only" model had a poor performance for TBM differentiation. The disappointing performance of these models indicates that an EHR-based TBM diagnosis is largely unsatisfactory to discern TBM patients from suspected cases.

Notably, the "EHR+miRNA" model performed significantly better than the "EHR only" and "miRNA only" models in both TBM and PTB discriminatory diagnoses. Although further large-scale validation is needed, we consider our study the first proof of concept that signatures combining gene data with EHR data could represent a more promising approach to TB diagnosis. Apart from its satisfactory performance, the dominant features of the "EHR + miRNA" model were also convincing from a clinical perspective. For instance, CSF/blood glucose and blood sodium were treated as traditional indicators for TBM prediction [2]. However, some well-known determinants, such as TB-DNA and *MTB* culture, failed to rank as important features. This finding may be due to their low yields for TBM patients. Paucibacillary cerebrospinal fluid (CSF) volume is rarely adequate in the clinic and is used for multiple laboratory tests [43]. Therefore, the low diagnostic yield obtained using traditional gold standards is hardly surprising.

Proper modeling is crucial to evaluate diagnostic accuracy because erroneous results can mislead researchers and clinicians. For example, modeling with small sample sizes usually triggers the overfitting problem; we alleviated this issue by using a series of standard strategies and discrete model interpretation, including regularization and an independent testing cohort. Unsupervised PCA illustrations supported the discriminative power of our model. The inclusion of features in the optimal model also made sense, including consistently elevated exosomal miRNAs and reliable EHR indicators. Therefore, despite our small sample size, our study provided sufficient robustness for our models.

Additionally, the functional annotations of these 6 DE exosomal miRNAs highlighted a possible regulatory role for genes involved in immunological processes and neurotrophin-related signaling, and these annotations corroborated with recent TB studies reporting that the innate immune response against *MTB* is significantly regulated by miRNAs [15,19,44–46]. Although the underlying mechanisms are not well-defined, these results demonstrate the consistency of our results with previous findings.

This work represents one exploratory setup: a proof of concept that signatures combining EHR information with exosomal miRNA data *via* a machine learning algorithm can improve TBM clinical diagnosis. If validated, such a pattern may be a more promising approach for disease diagnosis in clinical practice. Some limitations must be acknowledged. Further evaluation using larger populations or multiple settings is warranted due to the small sample size. A high percentage of patients were clinically diagnosed rather than microbiological reference standards, we attempted to confirm the existence of *MTB* with CSF and sputum cultures, but these were almost all negative because of limited sample volume and immediate antibiotic use on admission. Therefore, all patients in our study were followed up for 12 months and evaluated by a panel of experts to avoid the misclassification of cases and disease controls. In addition, miRNA functional analysis remains hypothetical and will be the focus of future experimental work.

5. Conclusion

In conclusion, our results suggest that exosomal miRNAs (miR-20b, miR-191 and miR-486) are potential miRNA biomarkers for TB diagnosis. The combination of miRNA and EHR data through a machine learning algorithm could serve as a feasible approach to promote the differential diagnosis of TBM and PTB and requires validation in larger cohorts.

Conflicts of interests

The authors have declared that they have no competing interests.

Authors' contributions

X-JH and HB performed the experiments, analyzed the data and wrote the initial manuscript. SL was responsible for the data modeling and manuscript writing. X-JH, HB, and SL contributed equally to this work. L-JW and M-JW oversaw participant enrolment and acquisition of clinical and laboratory data. QW, JZ, and LJ searched literature and collected patients' follow-up data. Y-HZ and X-RC provided expert advice and engaged in the data interpretation. X-JL provided administrative, technical, and material support. B-WY, *Z*-LZ, and W-ML supervised the whole project, conceptualized and designed the study, and revised the article. All authors have reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.01.023.

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