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LncRNA and predictive model to improve the diagnosis of clinically diagnosed pulmonary tuberculosis

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LIST OF ABBREVIATIONS

TB: tuberculosis; PTB: pulmonary tuberculosis; MTB: *Mycobacterium tuberculosis*; TB-IGRA: interferon-gamma release assays for tuberculosis; lncRNA: long noncoding RNA; LTBI: latent TB infection; DE: differentially expressed; EHR: electronic health record; TBM: tuberculous meningitis; non-TB DC: non-TB disease control; PBMC: peripheral blood mononuclear cell; VIF: variance inflation factor; DCA: decision curve analysis; LV: lentivirus vector; BCG: Bacillus Calmette-Guerin; LDH: lactate dehydrogenase.

ABSTRACT

Background Clinically diagnosed pulmonary tuberculosis (PTB) patients lack *Mycobacterium tuberculosis* (MTB) microbiologic evidence, and misdiagnosis or delayed diagnosis often occurs as a consequence. We investigated the potential of lncRNAs and corresponding predictive models to diagnose these patients.

Methods We enrolled 1372 subjects, including clinically diagnosed PTB patients, non-TB disease controls and healthy controls, in three cohorts (Screening, Selection and Validation). Candidate lncRNAs differentially expressed in blood samples of the PTB and healthy control groups were identified by microarray and qRT-PCR in the Screening Cohort. Logistic regression models were developed using lncRNAs and/or electronic health records (EHRs) from clinically diagnosed PTB patients and non-TB disease controls in the Selection Cohort. These models were evaluated by AUC and decision curve analysis, and the optimal model was presented as a Web-based nomogram, which was evaluated in the Validation Cohort. The biological function of lncRNAs was interrogated using ELISA, lactate dehydrogenase release analysis and flow cytometry.

Results Three differentially expressed lncRNAs (*ENST00000497872, n333737, n335265*) were identified. The optimal model (i.e., nomogram) incorporated these three lncRNAs and six EHR variables (age, hemoglobin, weight loss, low-grade fever, CT calcification and TB-IGRA). The nomogram showed an AUC of 0.89, sensitivity of 0.86 and specificity of 0.82 in the Validation Cohort, which demonstrated better discrimination and clinical net benefit than the EHR model. *ENST00000497872* may regulate inflammatory cytokine production, cell death and apoptosis during MTB infection.

Conclusions LncRNAs and the user-friendly nomogram could facilitate the early identification of PTB cases among suspected patients with negative MTB microbiologic evidence.

KEYWORDS lncRNA, electronic health record, clinically diagnosed pulmonary tuberculosis, nomogram

INTRODUCTION

Tuberculosis (TB) is the leading cause of death from an infectious agent [1], but only 56% of plmonary tuberculosis (PTB) cases reported to WHO in 2017 were bacteriologically confirmed. Thus, approximately half of all PTB cases are clinically diagnosed worldwide, and this proportion can reach 68% in China [1]. Clinically diagnosed PTB cases are symptomatic but lack evidence of *Mycobacterium tuberculosis* (MTB) infection by smear microscopy, culture or nucleic acid amplification test [1–3]. The diagnostic procedure for clinically diagnosed PTB is inadequate and time-consuming and often results in misdiagnosis or delayed diagnosis [3], leading to an increased risk of morbidity and mortality [4], or overtreatment [5]. There is thus an urgent need to develop rapid and accurate strategies to diagnose PTB cases without MTB microbiologic evidence [6, 7]. The exploration of effective host immune-response signatures represents an attractive approach for this type of assay.

Long noncoding RNAs (lncRNAs) can function as critical regulators of inflammatory responses to infection, especially for T-cell responses [8, 9]. Increasing evidence indicates that blood lncRNA expression profiles are closely associated with TB disease [10–12], suggesting lncRNAs could function as potential noninvasive biomarkers for TB detection. However, previous studies have suffered from small sample size (ranging from 66 to 510) and lack independent validation.

Recent effort has focused on establishing clinical prediction rules or predictive models for TB diagnosis based on patients' electronic health record (EHR) information [13–16]. Such models can cost-effectively facilitate PTB diagnosis with a limited number of clinical-radiological predictors. For example, a 6-signature model from Griesel et al. (a cough lasting ≥14 days, the inability to walk unaided, a temperature > 39°C, chest radiograph assessment, hemoglobin level and white cell count) attained an AUC of 0.81 [0.80-0.82] in seriously ill HIV-infected PTB patients [13]. However, despite these advances, current EHR models remain

insufficient for precise TB diagnosis. Compelling studies have proposed that models incorporating biomarkers and EHR information attain better performance for prediction of sepsis [17] and abdominal aortic aneurysm [18]. We previously reported that combining exosomal microRNAs and EHRs in the diagnosis of tuberculous meningitis (TBM) achieved AUCs of up to 0.97 versus an AUC of 0.67 obtained using EHR alone [19]. Based on these studies, we hypothesized that combining lncRNAs with well-defined EHR predictors could be used to develop improved predictive models to identify PTB cases that lack microbiologic evidence of MTB infection.

This study was therefore performed to investigate the diagnostic potential of lncRNAs and predictive models incorporating lncRNA and EHR data for the identification of PTB cases without microbiologic MTB evidence. This study also explored the regulatory functions of lncRNA candidates during MTB infection to evaluate the biological basis for their predictive abilities.

MATERIAL AND METHODS

Study design

We performed this study through a four-stage approach. LncRNAs that were differentially expressed (DE) between clinically diagnosed PTB patients and healthy subjects were profiled by microarray in the Screening Step. The expression of top five lncRNAs were then analyzed in a large prospective cohort in the Selection Step of the study, which reduced the number of five lncRNAs to three based on expression difference among groups. In the Model Training Step, lncRNAs and EHRs were used to develop predictive models for clinically diagnosed PTB patients and non-tuberculosis disease control (non-TB DC) patients, and the optimal model was visualized as a nomogram. Finally, we validated lncRNAs and the nomogram in

an independent prospective cohort. Functional analyses were also performed to elucidate the biological significance of lncRNAs. The study strategy is shown in Figure 1.

Subjects enrolment

Screening Cohort We retrospectively collected age- and gender-matched 7 PTB cases and 5 healthy controls as the Screening Cohort. They were 6 males and 6 females from ages 22 to 59 years. PTB cases were clinically confirmed PTB patients with positive TB symptoms, negative MTB pathogenic examinations, and good response to anti-TB therapy. Healthy subjects had a normal physical examination and no history of TB.

Selection Cohort and Validation Cohort Inpatients with clinical-radiological suspicion of PTB but lacking evidence of MTB infection were prospectively enrolled from West China Hospital between Dec 2014 and May 2017. The inclusion criteria for highly suspected patients were: (a) new patients with high clinical-radiological suspicion of PTB, (b) anti-TB therapy < 7 days on admission, (c) patients with negative MTB evidence (i.e., at least two consecutive negative smears, one negative MTB-DNA PCR and one negative culture result), (d) age ≥ 15 years, and (e) patients without severe immunosuppressive disease, HIV infection, or cardiac or renal failure. Two experienced pulmonologists reviewed and diagnosed all presumptive PTB patients, and final diagnoses for all cases were based on the combination of clinical assessment, radiological and laboratory results, response to the treatment [1, 2]. A 12-month follow-up observation was used to confirm the classification of PTB and non-TB patients. The detailed description of patients' symptoms and recruitment, inclusion and exclusion criteria, laboratory examinations, diagnostic criteria and procedure, treatment, and sample size estimate are provided in e-Appendix 1 and 2. In addition, healthy subjects were simultaneously recruited from a pool of healthy donors with a normal physical examination and no history of TB.

We finally enrolled a Selection Cohort of 878 participants (141 clinically diagnosed PTB, 159 non-TB DC, and 578 healthy subjects) and an independent Validation Cohort of 482 participants (97 clinically diagnosed PTB, 140 non-TB DC, and 245 healthy subjects). Details of the non-TB DC are listed in e-Table 1. Ethical approval was obtained from the Clinical Trials and Biomedical Ethics Committee of West China [no. 2014 (198)]. Informed consents were obtained from every participant.

LncRNA detection

RNA isolation and cDNA preparation Peripheral blood mononuclear cell (PBMC) samples were isolated from fresh 3 ml blood samples of each participant using a Human Lymphocyte Separation Tube Kit (Dakewe Biotech Company Limited, China). Total RNA was extracted from PBMC isolates using Trizol reagent (Invitrogen, USA). RNA concentration and purity were evaluated spectrophotometrically, and RNA integrity was determined using agarose gel electrophoresis (e-Figure 1A). The PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Takara, Japan) was used to remove contaminating genomic DNA and synthesize cDNA.

LncRNA microarray profiling LncRNA profiles were detected using Affymetrix Human Transcriptome Array 2.0 Chips based on a standard protocol [20]. Raw data were normalized using the Robust Multi-Array Average Expression Measure algorithm. DE lncRNAs with p-values < 0.05 and fold-changes > 2 were identified using the empirical Bayes moderated t-statistics and presented by hierarchical clustering and volcano plot [21]. Microarray data have been deposited in the Gene Expression Omnibus under the accession GSE119143.

qRT-PCR for lncRNAs LncRNA expression was measured using the SYBR® Green PCR Kit (Takara, Japan) in a blinded fashion, normalized to the endogenous control *GAPDH*, and calculated according to the $2^{-\Delta\Delta Cq}$ method where and Cq < 35 was considered acceptable [22]. Specific primers are presented in e-Table 2. PCR curves and the standard curve are shown in

e-Figure 1B-C. Detailed methodology for RNA isolation, reverse transcription, qRT-PCR detection (procedure, quality control, product verification, and stability test) are listed in e-Appendix 3.

Modeling

Data used for modeling A total of 41 EHRs, including demographic, clinical, laboratory, and radiological findings were collected (see e-Appendix 4), and a 20% missing value threshold was applied to remove incomplete features. Features with p-values < 0.05 in univariate analysis or definite clinical significance were included for modeling. A total of 14 of the 44 original variables (41 EHRs and 3 lncRNAs) remained after filtering, including 11 EHRs and 3 lncRNAs (see e-Appendix 4).

Diagnostic modeling Multivariable logistic regression was used to develop predictive models to distinguish clinically diagnosed PTB from patients with suspected PTB cases in the Selection Cohort. Feature subsets were selected and compared using the best subset selection procedure [23] and 10-fold cross-validation. The "EHR+lncRNA", "lncRNA only" and "EHR only" models were developed according to their respective best feature subset in the Selection Cohort. A cutoff of each model was determined by combining the Youden's index and the sensitivity for the samples in the training dataset equal to or greater than 0.85. The models including their cutoff were used for evaluation of the Validation Cohort.

Nomogram presentation and evaluation We further adopted the nomogram to visualize the optimal model with the best AUC [24, 25]. Nomogram calibration was assessed with the calibration curve and Hosmer-Lemeshow test (p-value > 0.05 suggested no departure from perfect fit). The performance of the nomogram was tested in the independent Validation Cohort, with total points for each patient calculated. Decision curve analysis (DCA) [25] was performed by evaluating the clinical net benefit of the nomogram and "EHR only" model

122 across the overall datasets. Assessing clinical value involves comparing the nomogram and
123 "EHR only" model using the 500 bootstrap method. The nomogram was implemented as a
124 Web-based app using R Shiny.

125 **Analysis of *ENST00000497872* (lnc AL) function**

126 The lncRNA with the most significant difference in our analysis, *ENST00000497872* (lnc AL)
127 was analyzed in functional studies. THP-1 cells with stable overexpression and knockdown
128 of lnc AL were constructed using recombinant lentivirus vector (LV). THP-1 cells transfected
129 with these vectors were incubated with Bacillus Calmette-Guerin (BCG) to imitate active
130 MTB-infection [26]. This study examined the effect BCG exposure on THP-1 cells in five
131 groups transfected with vectors to overexpress (LV-lnc AL) or suppress (shRNA-lnc AL) lnc
132 AL expression, their respective empty vector constructs (LV-control and shRNA-control), or
133 with no vector (blank control). Cell culture supernatants were harvested to measure lnc AL
134 and the expression of six cytokines (TNF-α, IL-1β, IL-12 p70, IL-10, IFN-γ, and IL-6). Cell
135 apoptosis and cytotoxicity after 24 h infection were detected by flow cytometry and the
136 lactate dehydrogenase (LDH) release analysis, respectively. Detailed methodology for these
137 experiments is presented in e-Appendix 5.

138 **Statistical analysis**

139 Categorical variables were analyzed by univariate analysis with a Chi-square test and
140 continuous variables were analyzed using Mann-Whitney U tests or Student's t-tests. All tests
141 were 2-sided, and p-values < 0.05 were considered statistically significant. Modeling was
142 constructed and validated by individuals who were blinded to diagnostic categorizations. R
143 code and data for modeling are available from
144 https://github.com/xuejiaohu123/TBdiagnosisModel.
145

RESULTS

Characteristics of prospectively enrolled participants

The demographic and clinical characteristics of participants in the Selection and Validation Cohorts are provided in Table 1. PTB patients were younger and had greater IGRA positivity rates than their non-TB DC (p-value < 0.0001 for both the Selection and Validation Cohorts), but these groups did not differ by gender, BMI, or smoking status. Healthy subjects were age-, gender-, and BMI-matched with PTB patients, who had significantly different blood test results compared with PTB patients (Table 1).

Clinically diagnosed PTB patients were responsible for 29.82% (238/798) of all active PTB patients (see e-Appendix 1). This rate is markedly lower than a nationwide estimate of 68% based on primary public health institutions [1], but represents the clinically diagnosed PTB rate in a referral hospital with experienced specialists.

LncRNAs microarray profiles and candidate selection

In the Screening Step, microarray profiling identified a total of 325 lncRNAs that were differentially expressed (287 upregulated and 38 downregulated) in the clinically diagnosed PTB patients versus healthy subjects. Hierarchical clustering and a volcano plot revealed clearly distinguishable lncRNA expression profiles (e-Figure 2). Top five lncRNA candidates were chosen based on a set of combined criteria: fold-change > 2 between groups, p-value < 0.05, signal intensity > 25 [27], and including unreported lncRNAs in TB literature [28]. Three of these five lncRNAs were upregulated (*n335265*, *ENST00000518552* and *TCONS_00013664*) and two were downregulated (*n333737* and *ENST00000497872*) in PTB versus control subjects (e-Table 3).

Differentially expressed lncRNAs in clinically diagnosed PTB

The expression level of these five candidate lncRNAs was measured by qRT-PCR in the Selection Cohort, which consisted of 141 clinically diagnosed PTB, 159 non-TB DC, and 578 healthy subjects. Two lncRNAs (*ENST00000518552* and *TCONS_00013664*) were excluded from further analysis due to their low abundance expression (Cq > 35) in this cohort. Of the three remaining lncRNAs, *ENST00000497872* and *n333737* were downregulated and *n335265* was upregulated in PTB patients versus healthy subjects (e-Table 4). Comparison between clinically diagnosed PTB cases and non-TB DC patients revealed a decreased expression of *ENST00000497872* and *n333737* in PTB patients (e-Figure 3A), age-adjusted p-values both < 0.0001).

Short-term stability, an essential prerequisite of a potential lncRNA biomarker, was assessed in PBMC samples. This study found that incubation up to 24 h had minimal effect on the expression of *ENST00000497872*, *n333737*, and *n335265* (e-Table 5), in accordance with a previous report of lncRNA stability in blood [29].

Diagnostic modeling and nomogram visualization

Three logistic regression models, "EHR+lncRNA", "EHR only", and "lncRNA only" were evaluated as part of the training step in the Selection Cohort (see e-Appendix 4). The variance inflation factors between the features ranged from 1.02 to 1.29, indicating no collinearity within models. The "EHR+lncRNA" model yielded the highest AUC (0.92) for distinguishing clinically diagnosed PTB from suspected PTB patients, compared to AUCs of 0.87 and 0.82 for the "EHR only" and "lncRNA only" models, respectively (Figure 2A). The "EHR+lncRNA" model also had the best performance in sensitivity, specificity, accuracy, positive predictive value, and negative predictive value (Table 2).

The optimal "EHR+lncRNA" model was displayed as a nomogram (Figure 3A), and the top five features of the nomogram were *ENST00000497872*, age, *n333737*, CT calcification, and TB-IGRA results (e-Table 6). Seneitivity and specificity of the nomogram for prediction

194 of clinically diagnosed PTB was 0.89 (0.82-0.93) and 0.80 (0.73-0.85) at a cutoff of 0.37
195 (Table 2). A calibration curve in the Selection Cohort (Figure 3B) indicated a good
196 agreement between nomogram prediction and actual PTB cases and was confirmed by the
197 nonsignificant Hosmer-Lemeshow test (p-value = 0.957). This nomogram was generated as a
198 free online app (available at https://xuejiao.shinyapps.io/shiny/) to facilitate its access for
199 other studies. This app allows the user to insert the values of specific predictors and provides
200 the risk prediction as a whole number percentage.

201 **Validation for lncRNAs and the nomogram**

202 In the Validation Step, the three candidate lncRNAs were analyzed in an independent
203 Validation Cohort contains 97 clinically diagnosed PTB cases, 140 non-TB DC and 245
204 healthy subjects. This analysis observed an lncRNA expression pattern similar to that
205 observed in the Selection Cohort (e-Table 4, e-Figure 3B). All three models were applied to
206 the Validation Cohort, and as reported in Table 2 and Figure 2 it was found that the
207 nomogram achieved superior discrimination (AUC: 0.89 [0.84-0.93]), good calibration
208 (Figure 3B, and p-value = 0.668 for Hosmer-Lemeshow test) for clinically diagnosed PTB
209 prediction. The sensitivity and specificity of the nomogram at the cutoff of 0.37 in the
210 Validation Cohort was 0.86 (0.77-0.90) and 0.82 (0.75-0.87), respectively. DCA indicated
211 that the nomogram outperformed the conventional "EHR only" model with a higher clinical
212 net benefit within a threshold probability range from 0.2 to 1 (Figure 3C).

213 **LncRNA response to anti-TB treatment**

214 LncRNAs were next analyzed for the ability to predict anti-TB treatment response. Paired
215 samples were collected from 22 clinically diagnosed PTB patients before and after 2-month
216 intensive therapy [30], and the expressions of *ENST00000497872*, *n333737*, and *n335265* were
217 measured by qRT-PCR. All these patients had good response to therapy based on the clinical

and radiological findings, and *ENST00000497872* and *n333737* levels significantly increased post-treatment (p-values = 0.005 and 0.0005, respectively, Figure 4), suggesting that lncRNA expression increased in response to therapy.

Functional studies of *ENST00000497872*

We investigated whether *ENST00000497872* (i.e., lnc AL) could affect the host immune response. At 24 h and 48 h post BCG-infection, lnc AL overexpression (e-Figure 4) led to decreased production of proinflammatory cytokines TNF-α and IL-1β and an increase in INF-γ (Figure 5A). Conversely, knockdown of lnc AL resulted in a significant TNF-α and IL-1β increases and an INF-γ reduction. Lnc AL knockdown was also associated with an increasing trend of cell apoptosis (Figure 5B and 5C) and cell death (Figure 5D). These results implicate an inflammatory regulation of lnc AL during MTB-infection.

DISCUSSION

The present work focused on the challenge of accurately diagnosing PTB patients without microbiological evidence of MTB infection. Our study showed that three lncRNAs (*ENST00000497872*, *n333737*, and *n335265*) were potential biomarkers for clinically diagnosed PTB patients. Addition of three lncRNAs (*ENST00000497872*, *n333737* and *n335265*) to a conventional EHR model improved its ability to identify PTB cases from TB suspects, with the AUCs increasing from 0.83 to 0.89. The lncRNA that was most significantly enriched in the PTB group of this study, *ENST00000497872* (chr14:105703964-105704602), is located close to *IGHA1* (chr14: 105703995-105708665), and functional analyses indicated that expression of this lncRNA was involved in the regulation of inflammatory cytokine production and cell apoptosis in MTB-infected macrophages,

241 although further studies are needed to investigate the mechanisms responsible. Consistent
242 with published lncRNA data [8–12, 31], this data provide new evidence that lncRNAs could
243 participate in TB immunoregulation and serve as promising biomarkers for TB diagnosis.

244 In addition to the three lncRNAs, we identified six EHR predictors (age, CT calcification,
245 positive TB-IGRA, low-grade fever, elevated hemoglobin, and weight loss) that were
246 essential in TB case finding, as proposed by prior findings [15, 16]. Age was an important
247 negative predictor for clinically diagnosed PTB, which appears to conflict with the consensus
248 that advanced age correlates with higher TB susceptibility [32]. This may be explained by
249 differences in the enrollment of the PTB patients and control subjects. Previous studies
250 included healthy and/or vulnerable subjects as controls, while we enrolled inpatients with a
251 wide range of pulmonary diseases and older ages as disease controls.

252 This study serves as a first proof-of-concept study to show that integrating lncRNA
253 signatures and EHR data could be a more promising diagnostic approach for PTB patients
254 with negative MTB pathogenic evidence. The "EHR+lncRNA" model had good
255 discrimination (through AUC and diagnostic parameters), reliable calibration (via calibration
256 curve and Hosmer-Lemeshow test), and potential clinical utility for decision-making (using
257 DCA). The "EHR+lncRNA" model avoided some common problems associated with
258 sputum-based features, such as poor sputum quality or problematic sampling [33], to improve
259 its reliability and clinical utility. Nomogram has been shown to remarkably promote early
260 diagnosis of intestinal tuberculosis [24] and prognosis prediction in PTB [34] and TBM [35].
261 "EHR+lncRNA" model herein was visualized as a nomogram and further implemented in an
262 app. The online nomogram uses readily obtainable predictors and automatically outputs a
263 personalized quantitative risk estimate for PTB. Utilizing this user-friendly tool may facilitate
264 the rapid identification of PTB cases among suspected TB patients without MTB

265 microbiologic evidence to improve TB diagnosis, especially in resource-constrained areas
266 with high TB prevalence.

267 Our study has several limitations. Modeling in this study was conducted based on data
268 from a single large hospital, and multi-center validation studies are needed. Further, because
269 Xpert MTB/RIF is still not routinely available in most clinical laboratories of China, and
270 since previous Xpert studies reported moderate sensitivities ranged from 28% to 73% [36–38] in
271 smear-negative PTB patients, we did not consider Xpert in our research, which may limit the
272 generalization of our findings.

273 In summary, a novel nomogram we developed and validated in this study that incorporated
274 three lncRNAs and six EHR fields may be a useful predictive tool in identifying PTB patients
275 with negative MTB pathogenic evidence, and merits further investigation.

276

277 **AUTHOR'S CONTRIBUTIONS**

278 XH, HC, ZZ, and BY developed the concept and experimental design; XH, HB, YZ, JZ, LJ,
279 LW, and MW enrolled patients and performed the experiments; XH, SL, and SG developed
280 and validated the diagnostic models; XC, YZ, XL, and TYH provided expert advice and
281 support; all authors contributed to write or revise the manuscript, provided intellectual input
282 and gave final approval.

283

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TABLES

Table 1. Demographic and clinical features of participants in the Selection and Validation Cohorts.

Clinical features	Suspected clinically diagnosed PTB patients $_{Selection}$			HS $_{Selection}$ (n=578)	p2	Suspected clinically diagnosed PTB patients $_{Validation}$			HS $_{Validation}$ (n=245)	p4
	Clinically diagnosed PTB (n=141)	Non-TB DC (n=159)	p1			Clinically diagnosed PTB (n=97)	Non-TB DC (n=140)	p3		
Gender, male	84 (59.57%)	95 (59.75%)	0.976	284 (49.13%)	0.026	58 (59.79%)	87 (62.14%)	0.715	126 (51.43%)	0.162
Age (years)	37.81 ± 17.93	56.68 ± 14.52	< 0.0001	40.59 ± 13.11	0.084	38.29 ± 17.57	57.96 ± 16.66	< 0.0001	36.82 ± 9.28	0.436
BMI (kg/m^2)	20.81 ± 2.99	20.43 ± 4.03	0.359	20.65 ± 3.19	0.57	21.59 ± 3.43	21.29 ± 3.62	0.52	21.51 ± 3.52	0.843
Smoking	61 (43.26%)	72 (45.28%)	0.725	161 (27.85%)	< 0.0001	41 (42.27%)	66 (47.14%)	0.458	84 (34.29%)	0.167
Radiologic pathology	116(82.26%)	140 (88.05%)	0.158	-		86 (88.66%)	130 (92.86%)	0.264	-	
Laboratory tests										
Positive TB-IGRA	97 (68.88%)	56 (35.22%)	< 0.0001	-		64 (66.00%)	42 (30.00%)	< 0.0001	-	
C-reactive protein (mg/L)	16.30 (5.32-54.05)	17.80 (6.47-60.20)	0.427	-		13.60 (3.34-43.55)	18.60 (6.56-70.63)	0.037	-	
Hematocrit	0.37 ± 0.06	0.36 ± 0.07	0.162	0.44 ± 0.04	< 0.0001	0.38 ± 0.07	0.35 ± 0.07	0.002	0.43 ± 0.04	< 0.0001
Erythrocytes (×10^{12}/L)	4.33 ± 0.72	4.03 ± 0.81	0.001	4.78 ± 0.46	< 0.0001	4.46 ± 0.80	3.89 ± 0.91	< 0.0001	4.80 ± 0.46	< 0.0001
Hemoglobin (g/L)	122.57 ± 23.22	115.82 ± 25.20	0.017	144.46 ± 13.88	< 0.0001	125.08 ± 24.25	113.11 ± 25.52	< 0.0001	145.82 ± 13.73	< 0.0001
Platelets (×10^9/L)	238.00 (177.00-305.00)	233.00 (149.00-299.00)	0.171	193.00 (158.00-223.00)	< 0.0001	220.00 (160.50-313.00)	199.50 (137.00-290.75)	0.059	190.00 (165.00-230.00)	0.001
Leukocytes (×10^9/L)	6.03 (4.76-8.25)	6.36 (4.71-9.07)	0.488	5.92 (5.18-6.67)	0.184	6.96 (5.06-9.14)	5.93 (4.34-8.33)	0.009	5.70 (4.91-6.55)	0.217
Lymphocytes (×10^9/L)	1.15 (0.80-1.52)	1.28 (0.87-1.87)	0.056	1.86 (1.55-2.19)	< 0.0001	1.29 (0.91-1.80)	1.22 (0.86-1.62)	0.343	1.85 (1.57-2.55)	< 0.0001
Neutrophils (×10^9/L)	4.02 (3.23-5.93)	4.08 (2.71-6.32)	0.956	3.47 (2.87-4.09)	< 0.0001	4.03 (2.51-5.69)	4.85 (3.21-6.70)	0.023	3.36 (2.75-3.92)	0.006
Monocytes (×10^9/L)	0.47 (0.35-0.65)	0.42 (0.26-0.61)	0.015	0.36 (0.29-0.44)	< 0.0001	0.43 (0.30-0.64)	0.46 (0.34-0.71)	0.211	0.31 (0.25-0.39)	< 0.0001
Alb (g/L)	36.66 ± 6.78	36.60 ± 6.66	0.973	48.24 ± 2.67	< 0.0001	37.33 ± 7.47	36.69 ± 7.22	0.509	47.06 ± 2.25	< 0.0001
Globin (g/L)	31.69 ± 7.66	30.68 ± 8.10	0.269	28.92 ± 3.29	0.041	30.41 ± 7.89	29.73 ± 7.91	0.514	27.41 ± 3.19	< 0.0001

Subscripted "Selection" or "Validation" refers to the Selection or Validation Cohort, respectively. Radiologic pathology refers to abnormal chest imaging, including at least one of the signs: polymorphic abnormality, calcification, cavity, bronchus sign, and pleural effusion. Abbreviations: non-TB DC, non-tuberculosis disease control patients; HS, healthy subjects. p1, p-value for the comparison of clinically diagnosed PTB patients and non-TB DCs in the Selection Cohort; p2, p-value for the comparison of clinically diagnosed PTB patients and healthy subjects in the Selection Cohort; p3, p-value for the comparison of clinically diagnosed PTB patients and non-TB DCs in the Validation Cohort; p4, p-value for the comparison of clinically diagnosed PTB patients and healthy subjects in the Validation Cohort.

Table 2. Performances of the comparative diagnostic models.

Model performance	Selection Cohort			Validation Cohort		
	EHR+lncRNA (Nomogram)	EHR only	lncRNA only	EHR+lncRNA (Nomogram)	EHR only	lncRNA only
Sensitivity	0.89 (0.82-0.93)	0.89 (0.83-0.93)	0.85 (0.76-0.88)	0.86 (0.77-0.90)	0.89 (0.82-0.94)	0.85 (0.76-0.90)
Specificity	0.80 (0.73-0.85)	0.62(0.54-0.68)	0.55 (0.46-0.61)	0.82 (0.75-0.87)	0.65 (0.56-0.72)	0.54 (0.47-0.62)
Accuracy	0.84 (0.80-0.88)	0.75 (0.69-0.79)	0.69 (0.63-0.74)	0.84 (0.78-0.88)	0.75 (0.69-0.81)	0.67 (0.60-0.73)
Positive predictive value	0.80 (0.73-0.85)	0.67 (0.60-0.74)	0.62 (0.55-0.69)	0.77 (0.68-0.83)	0.64 (0.56-0.72)	0.56 (0.48-0.63)
Negative predictive value	0.89 (0.83-0.93)	0.87 (0.79-0.91)	0.80 (0.72-0.86)	0.89 (083-0.93)	0.90 (0.83-0.94)	0.83 (0.75-0.89)

Note: The cutoff probability in the Selection Cohort was 0.37 for "EHR+lncRNA" model, 0.26 for "EHR only" model, and 0.32 for "lncRNA" model, respectively. Features in each model are provided in e-Appendix 4, 4.4. The "EHR+lncRNA" formula that was developed to classify patients as PTB cases or non-TB disease controls was: -3.32 - 0.053×[age] - 0.94×log(ENST00000497872) - 0.39×log(n333737) + 1.51×[CT calcification] + 1.16×[TB-IGRA] + 1.09×[low-grade fever] + 0.014×[hemoglobin] + 0.23×log(n335265) + 0.43×[weight loss].

FIGURES CAPTIONS

Figure 1. Overview of the strategy for investigating lncRNA and prediction model for clinically diagnosed PTB differential diagnosis.

Abbreviations: PTB, pulmonary tuberculosis; PBMC, peripheral blood mononuclear cell; non-TB DC, non-tuberculosis disease control; DE, differentially expressed; EHR, electronic health record; DCA, decision curve analysis. LDH, lactate dehydrogenase.

Figure 2. Receiver operator curves of different models in the Selection and Validation Cohort.

(A), ROC of the Selction Cohort. The 10-fold cross-validation ROC of "EHR+lncRNA" model is provided in the e-Figure 5. P-values for model AUC comparisons in the Selection Cohort: 0.00012 ("EHR+lncRNA" vs "EHR only"), 1.402×10^{-7} ("EHR+lncRNA" vs "lncRNA only"), and 0.103 ("EHR only" vs "lncRNA only"), respectively. P-values < 0.016 (0.05/3) were considered statistically significant.

(B), ROC of the Validation Cohort. P-values for model AUC comparisons in the Validation Cohort: 0.004 ("EHR+lncRNA" vs "EHR only"), 0.0003 ("EHR+lncRNA" vs "lncRNA only"), and 0.361 ("EHR only" vs "lncRNA only"), respectively.

Figure 3. Nomogram for the prediction of clinically diagnosed PTB based on the optimal models.

(A), Nomogram to predict the risk of clinically diagnosed PTB patients, in which points were assigned based on the feature rank order of the effect estimates. A vertical line is drawn between the "Point" axis and the corresponding point for each feature to generate a total point score and PTB probability.

(B), Calibration plot in the Selection Cohort (left in B) and Validation Cohort (right in B), with lines indicating the ideal (dashed), apparent (dotted) and bias-corrected (unbroken) predictions of the nomogram.

(C), Decision curve analysis for the nomogram and "EHR only" model with lines indicating the nomogram (blue), "EHR only" model (red dash), and assumptions that no patients or all patients have PTB (black and grey, respectively).

Figure 4. Alteration of lncRNAs before and after 2-month intensive therapy.

LncRNA expressions before (blue) and after (red) a 2-month intensive anti-TB treatment regimen. Altered lncRNA expressions were calculated using $\log_2$ lncRNA (post-treatment expression / pre-treatment expression) and the Wilcoxon matched-paired rank test was used for comparisons among 22 paired samples.

The median and interquartile range of $\log_2$ lncRNA were as follows: *ENST00000497872* (before: -1.91 [-2.74, -1.11]; after: -1.55 [-2.61, -0.79]), *n333737*: (before: -3.88 [-4.81, -3.33] ; after: -2.30 [-2.99, -0.50]), *n335265* (before: 2.12 [1.05, 2.34]; after: 1.29 [0.85, 1.69]), respectively.

Figure 5. Regulation of lncRNA on inflammatory cytokine, cell apoptosis and cytotoxicity in BCG-infected THP-1 cells.

(A), Cytokine expression. (B), Flow cytometry analysis of cell apoptosis. (C), Graph of apoptosis data. (D), LDH release analysis of cell cytotoxicity for BCG-infected THP-1 cells. LV-control and shRNA-control mean values considered negative control values, and the blank control is not shown. Three cytokines (IL-12 p70, IL-10 and IL-6) did not significantly differ and are not shown. Difference between groups were analyzed by one-way ANOVA and Bonferroni's post-test comparison among groups (*p-value < 0.05, **p-value < 0.01, and ***p-value < 0.001).

SUPPLEMENRARY FIGURE CAPTIONS

e-Figure 1. RNA electrophoresis, amplification curve of qRT-PCR and standard curve of control cDNA

e-Figure 2. Hierarchical clustering and volcano plot for differentially expressed lncRNA profiles in the Screening Cohort

e-Figure 3. LncRNA expression between clinically diagnosed PTB patients and non-TB disease controls in the Selection and Validation Cohorts

e-Figure 4. qPCR analysis of *ENST00000497872* expression in BCG-infected THP-1 cells

e-Figure 5. Ten-fold cross-validation ROC of "EHR+lncRNA" model developed using the data from the Selection Cohort

SUPPLEMENRARY TABLE CAPTIONS

e-Table 1. Disease controls in the present study

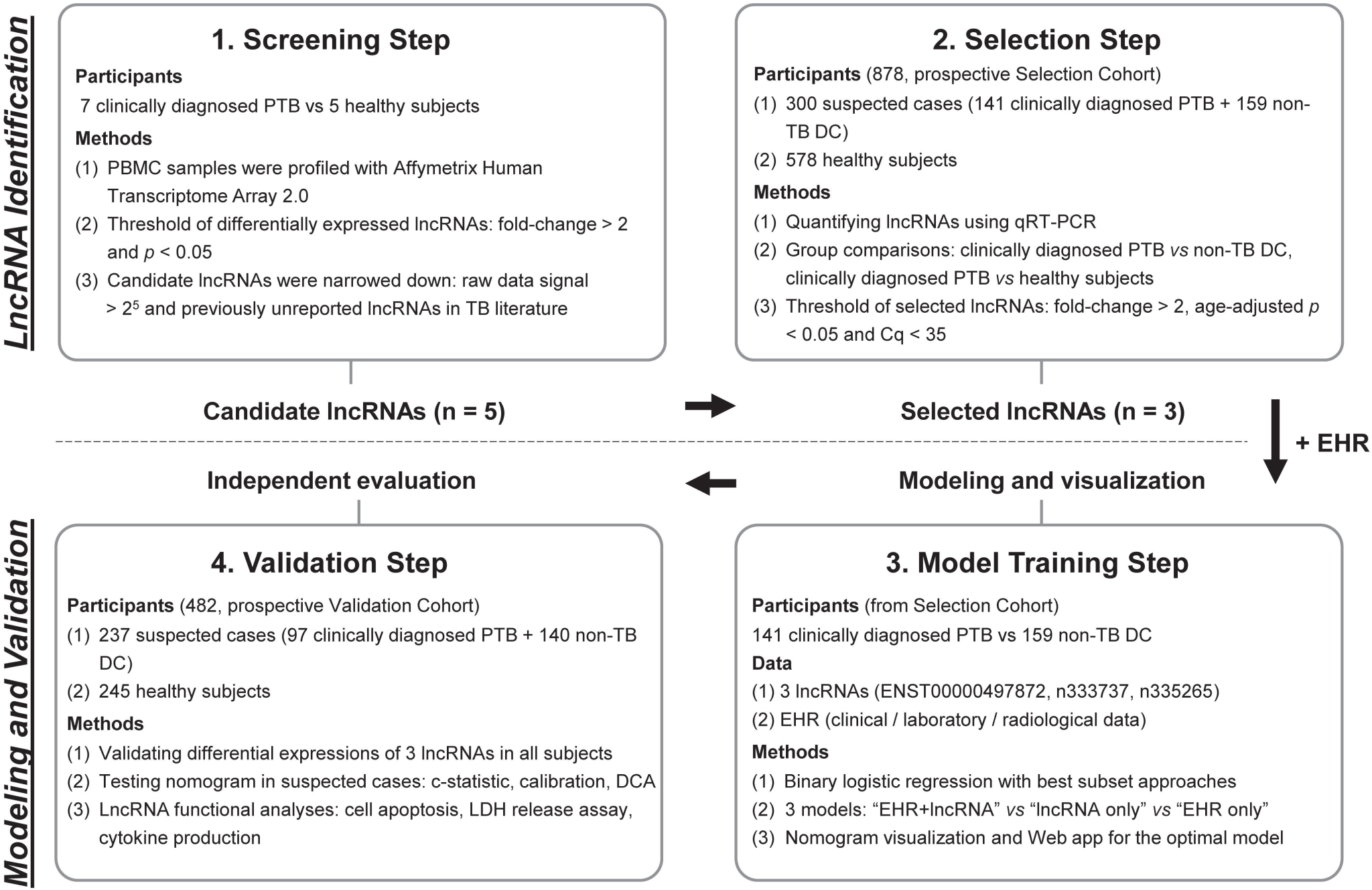
e-Table 2. Specific qRT-PCR primers for lncRNAs

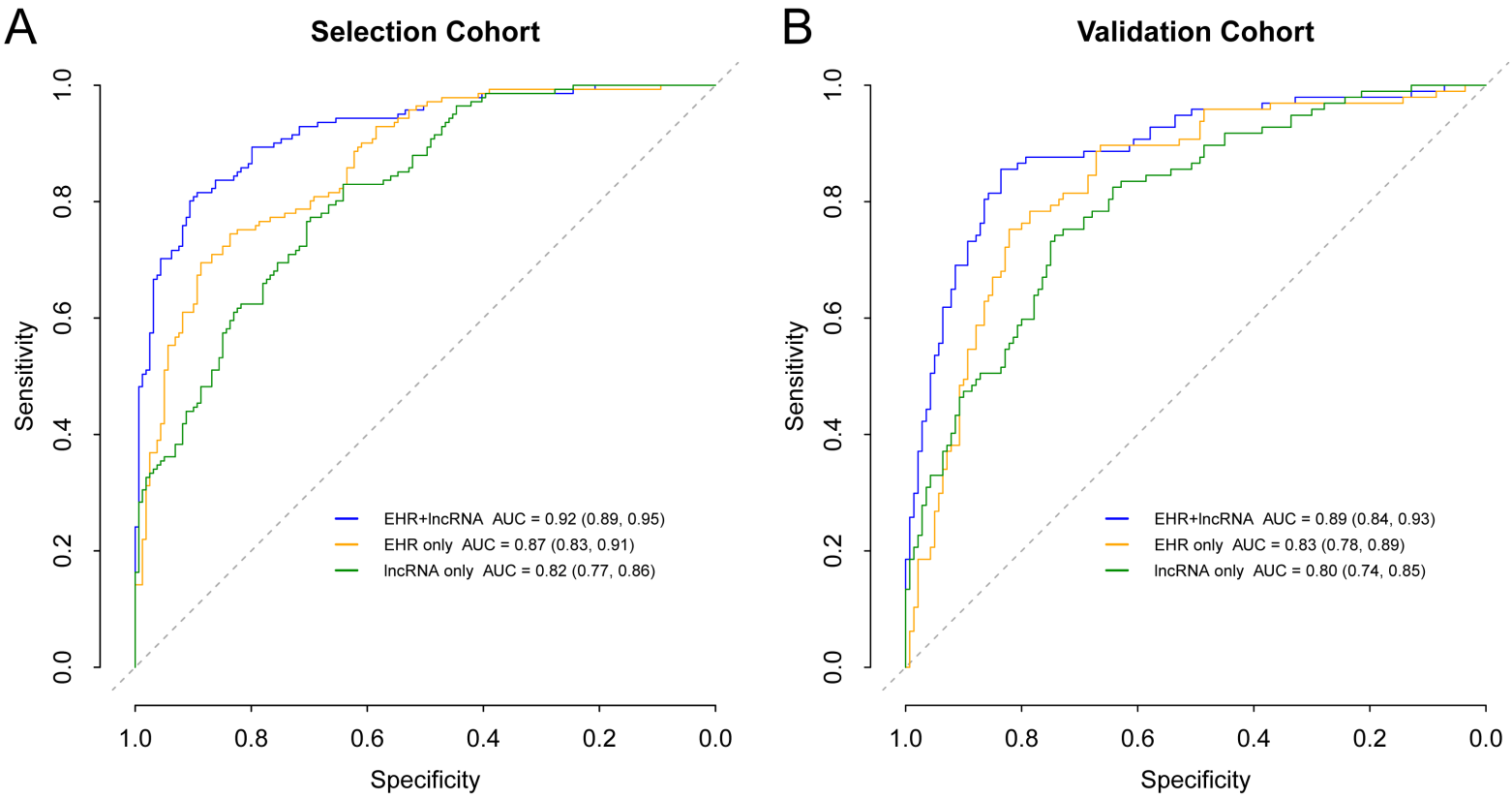
e-Table 3. Expression of five candidate lncRNAs in the Screening Cohort

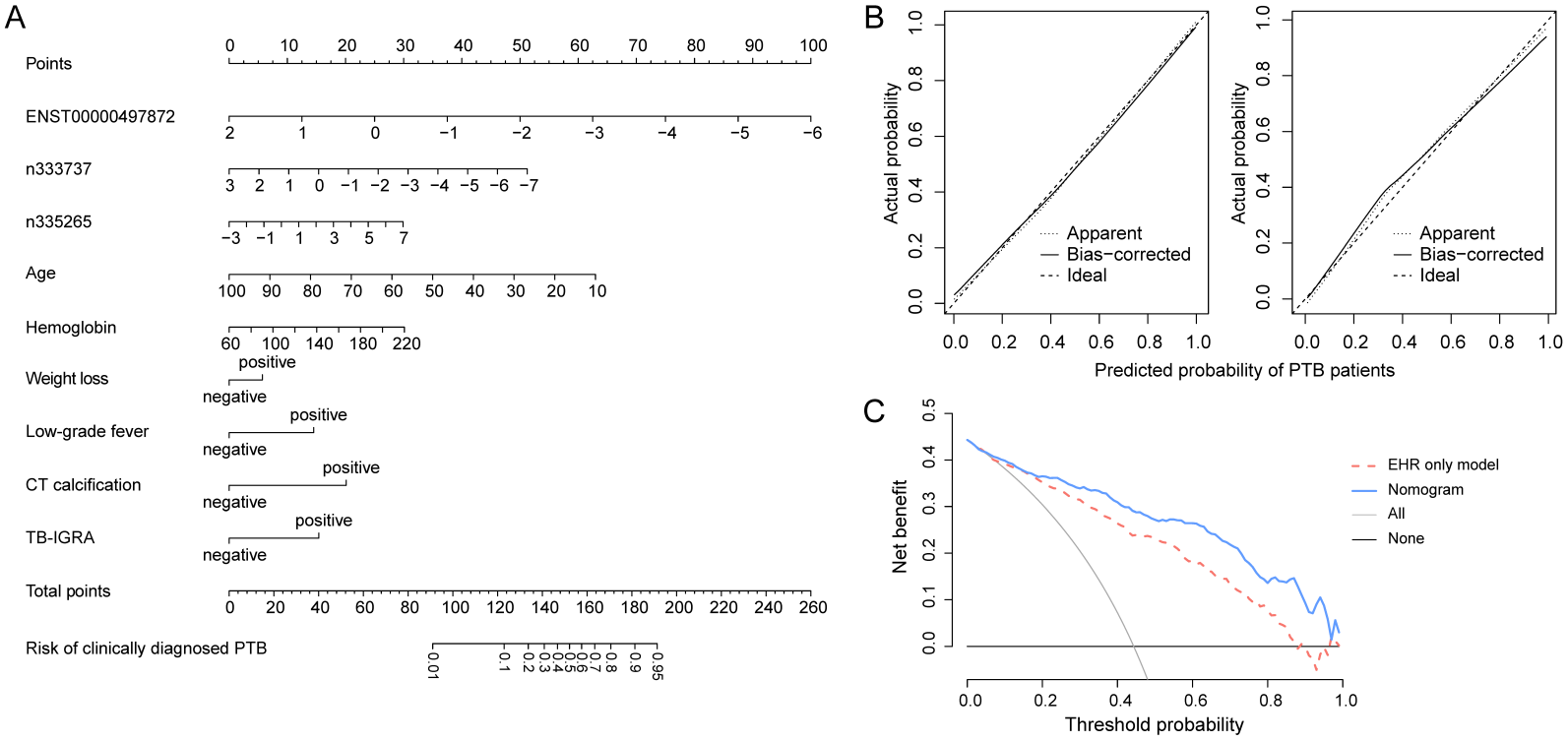
e-Table 4. Comparison of lncRNA expression between clinically diagnosed PTB patients and healthy subjects in the Selection and Validation Cohorts

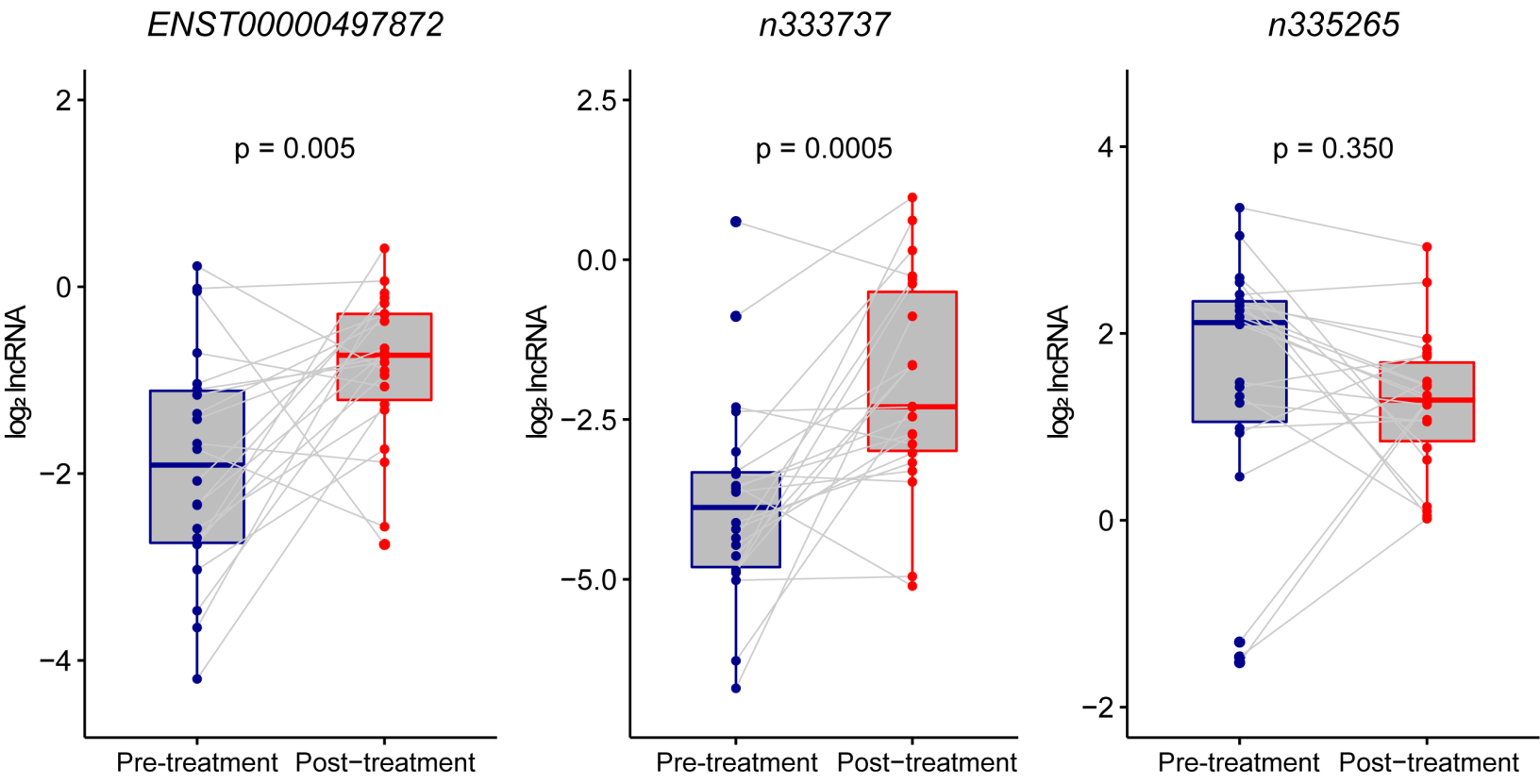
e-Table 5. Short-term stability evaluation of lncRNAs in PBMC samples

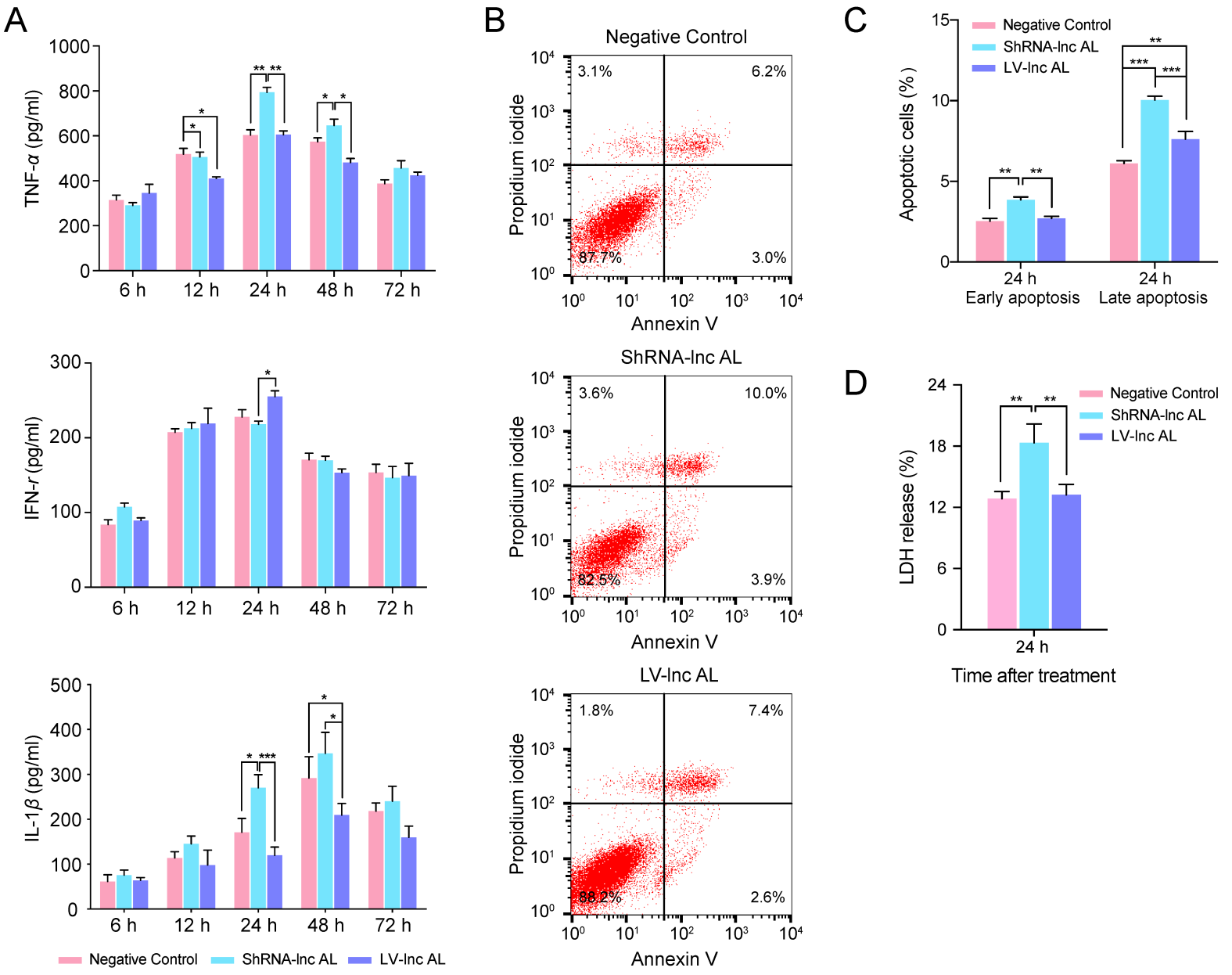
e-Table 6. Details of "EHR+lnRNA" logistic regression model to differentiate clinically diagnosed PTB among 300 highly suspected patients


LncRNA Identification
1. Screening Step
Participants
7 clinically diagnosed PTB vs 5 healthy subjects
Methods
(1) PBMC samples were profiled with Affymetrix Human Transcriptome Array 2.0
(2) Threshold of differentially expressed lncRNAs: fold-change > 2 and p < 0.05
(3) Candidate lncRNAs were narrowed down: raw data signal > 2^5 and previously unreported lncRNAs in TB literature
2. Selection Step
Participants (878, prospective Selection Cohort)
(1) 300 suspected cases (141 clinically diagnosed PTB + 159 non-TB DC)
(2) 578 healthy subjects
Methods
(1) Quantifying lncRNAs using qRT-PCR
(2) Group comparisons: clinically diagnosed PTB vs non-TB DC, clinically diagnosed PTB vs healthy subjects
(3) Threshold of selected lncRNAs: fold-change > 2, age-adjusted p < 0.05 and Cq < 35
Candidate lncRNAs (n = 5)
Selected lncRNAs (n = 3)
+ EHR
Independent evaluation
Modeling and visualization
Modeling and Validation
4. Validation Step
Participants (482, prospective Validation Cohort)
(1) 237 suspected cases (97 clinically diagnosed PTB + 140 non-TB DC)
(2) 245 healthy subjects
Methods
(1) Validating differential expressions of 3 lncRNAs in all subjects
(2) Testing nomogram in suspected cases: c-statistic, calibration, DCA
(3) LncRNA functional analyses: cell apoptosis, LDH release assay, cytokine production
3. Model Training Step
Participants (from Selection Cohort)
141 clinically diagnosed PTB vs 159 non-TB DC
Data
(1) 3 lncRNAs (ENST00000497872, n333737, n335265)
(2) EHR (clinical / laboratory / radiological data)
Methods
(1) Binary logistic regression with best subset approaches
(2) 3 models: "EHR+lncRNA" vs "lncRNA only" vs "EHR only"
(3) Nomogram visualization and Web app for the optimal model


A
Selection Cohort
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
1.0
0.8
0.6
0.4
0.2
0.0
Specificity
EHR+lncRNA AUC = 0.92 (0.89, 0.95)
EHR only AUC = 0.87 (0.83, 0.91)
lncRNA only AUC = 0.82 (0.77, 0.86)
B
Validation Cohort
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
1.0
0.8
0.6
0.4
0.2
0.0
Specificity
EHR+lncRNA AUC = 0.89 (0.84, 0.93)
EHR only AUC = 0.83 (0.78, 0.89)
lncRNA only AUC = 0.80 (0.74, 0.85)


A
Points
0 10 20 30 40 50 60 70 80 90 100
ENST00000497872
2 1 0 −1 −2 −3 −4 −5 −6
n333737
3 2 1 0 −1 −2 −3 −4 −5 −6 −7
n335265
−3 −1 1 3 5 7
Age
100 90 80 70 60 50 40 30 20 10
Hemoglobin
60 100 140 180 220
Weight loss
positive
negative
Low-grade fever
positive
negative
CT calcification
positive
negative
TB-IGRA
positive
negative
Total points
0 20 40 60 80 100 120 140 160 180 200 220 240 260
Risk of clinically diagnosed PTB
0.01 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 0.95
B
Actual probability
Apparent
Bias−corrected
Ideal
Predicted probability of PTB patients
C
Net benefit
EHR only model
Nomogram
All
None
Threshold probability


ENST00000497872
p = 0.005
log2 lncRNA
2
0
−2
−4
Pre-treatment
Post−treatment
n333737
p = 0.0005
log2 lncRNA
2.5
0.0
−2.5
−5.0
Pre-treatment
Post−treatment
n335265
p = 0.350
log2 lncRNA
4
2
0
−2
Pre-treatment
Post−treatment


A
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-1β (pg/ml)
6 h
12 h
24 h
48 h
72 h
Negative Control
ShRNA-lnc AL
LV-lnc AL
B
Negative Control
3.1%
6.2%
87.7%
3.0%
ShRNA-lnc AL
3.6%
10.0%
82.5%
3.9%
LV-lnc AL
1.8%
7.4%
88.2%
2.6%
Propidium iodide
Annexin V
C
Apoptotic cells (%)
Early apoptosis
Late apoptosis
D
LDH release (%)
Time after treatment