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Original Paper

Microarray Expression Profile of Circular RNAs in Peripheral Blood Mononuclear Cells from Active Tuberculosis Patients

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Key Words
Tuberculosis • Circular RNAs • Microarray assay • Biomarkers

Abstract
Background/Aims: Dysregulated expression of circular RNAs (circRNAs) was demonstrated to be implicated in many diseases. Here, we aimed to determine circRNA profile in peripheral blood mononuclear cells (PBMCs) from active tuberculosis (TB) patients to identify novel biomarkers for TB. ***Methods:*** Expression profile of circRNAs in PBMCs from 3 active pulmonary TB patients and 3 healthy controls were analyzed by microarray assay. Six circRNAs were selected for validation using real time-quantitative PCR (qRT-PCR) in 40 TB patients and 40 control subjects. Receiver operating characteristic (ROC) curve was constructed to evaluate their values in TB diagnosis. Hsa_circRNA_001937 was chosen for further evaluation in an independent cohort consisting of 115 TB, 40 pneumonia, 40 COPD, 40 lung cancer patients and 90 control subjects. An eight-month follow up was performed in 20 newly diagnosed TB patients to investigate the expression change of hsa_circRNA_001937 after chemotherapy. ***Results:*** We revealed and confirmed that a number of circRNAs were dysregulated in TB patients. Of the six studied physio circRNAs, the levels of hsa_circRNA_001937, hsa_circRNA_009024 and hsa_circRNA_005086 were significantly elevated and hsa_circRNA_102101, hsa_circRNA_104964 and hsa_circRNA_104296 were significantly reduced in PBMCs from TB patients as compared to healthy controls. ROC curve analysis suggested that hsa_circRNA_001937 has the largest area under the curve (AUC = 0.873, $P<0.001$). Hsa_circRNA_001937 was significantly increased in patients with TB compared with patients with pneumonia, COPD and lung cancer. Hsa_circRNA_001937 was correlated with TB severity (r = 0.4053, P = 0.010) and its expression significantly decreased after treatment. ***Conclusion:*** This study identified a set of deregulated circRNAs in active TB PBMCs, our data also suggest that hsa_circRNA_001937 can be used as a potential diagnostic biomarker of TB.

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Introduction

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (Mtb) infection, remains a leading cause of morbidity and mortality worldwide [1]. In 2015, World Health Organization (WHO) reported that an estimated 10.4 million cases of TB occurred and 1.8 million died of TB [2]. Early diagnosis of TB infection is essential for controlling the spread of the disease [3]. Nevertheless, TB is a stubborn disease hard to be early diagnosed. Classical methods for TB diagnosis in clinical settings include smear microscopy and mycobacterial culture. Acid-fast staining is simple and rapid but has poor sensitivity. As the Lowenstein–Jensen culture takes an average of 4-5 weeks to yield results, this method can hardly meet clinical demand. Immunological tests are time consuming and require confirmation in longitudinal analyses and further functional studies [4]. New automatic molecular methods for the diagnosis of TB are currently available, but the cost per test is too high for resource-limited settings [5]. Therefore, new rapid, sensitive and cost-effective biomarkers or methods for TB diagnosis are urgently needed.

Circular RNAs (circRNAs) are a special type of RNA that formed from the covalent linkage of the 3' and 5' ends to form a closed loop [6]. CircRNAs are generally considerate as non-coding RNA, although Pamudurti et al. found some specific circRNAs can also transcribed into proteins [7]. Increasing evidence reveals that circRNAs have key functions in regulating various physiological and pathological processes [8, 9]. It has been demonstrated that circRNAs could serve as competing endogenous RNAs (ceRNAs) to inhibit the activity of microRNAs (miRNAs) [10]. Many circRNAs are presented with characteristics of abundance, conservatism, and often exhibiting tissue/developmental-specific expression [11]. Unlike linear RNA, circRNAs have a special circular covalently bonded structure, which enable higher stability and resistance against RNA exonuclease [12]. Recent researches showed that circRNAs can function as potential molecular markers for disease diagnosis. For example, Cui et al. showed that hsa_circRNA_103636 in peripheral blood mononuclear cells (PBMCs) can be used as a novel biomarker for the diagnosis and treatment of major depressive disorder [13], Chen et al. found that hsa_circ_0000190 could be used as a new biomarker for gastric cancer [14], and Zhao et al. discovered that hsa_circ_0124644 in peripheral blood can be used as a diagnostic biomarker for coronary artery disease [15]. However, there is no report regarding the use of circRNAs as biomarkers for TB. This study was designed to determine whether circRNAs in PBMCs could be used as novel biomarkers for TB diagnosis.

Materials and Methods

Patient samples

A total of 155 newly diagnosed patients with active pulmonary TB were recruited from the Department of Tuberculosis, Jiangxi Chest Hospital (Nanchang, China) between January 2015 and August 2016. All of these patients were diagnosed on the basis of typical TB clinical symptoms, imaging examinations and confirmed by positive bacteriological examination results. These patients were further classified into minimal, moderate and advanced disease according to the severity of disease on the basis of chest radiographic examination, as described by Abakay et al [16].. TB patients with any other co-existing disease were excluded in this study. Subsequently, 20 active pulmonary TB inpatients received a 2HRZE/6HE treatment regimen, which starts with a 2-month combined treatment with isoniazid (INH, H), rifampicin (RMP, R), pyrazinamide (PZA, Z) and ethambutol (EMB, E), followed by a 6-month combined treatment with HE. Patients after 2HRZE/6HE treatment were evaluated by clinical physicians on the basis of clinical manifest, bacteriological detection and radiology examination. All these 20 patients were fully recovered. Healthy control individuals (n = 130) with no clinical symptoms of any infectious disease, diabetes, cancer, and had no close contact with TB patient were randomly recruited from individuals undergoing annual health check-up at the clinics of the First Affiliated Hospital of Nanchang University (Nanchang, China). To confirm the specificity of candidate circRNAs which were considered as potential biomarkers for TB, a total of 120 subjects including 40 pneumonia patients, 40 COPD patients and 40 lung cancer patients, were

recruited from the First Affiliated Hospital of Nanchang University and Jiangxi Chest Hospital between July 2015 and August 2016. This study was approved by the ethical committee of the First Affiliated Hospital of Nanchang University and conducted in accordance with the Declaration of Helsinki. All participants provided informed consent before commencement of the study.

Isolation of PBMCs

The peripheral blood samples (5 mL) were collected at indicated time points from all subjects. After sample collection, PBMCs were freshly isolated by density gradient centrifugation on Ficoll-Paque (Sigma, USA) according to the manufacturer's protocol. Then, the PBMCs were lysed with TRIzol reagent (Invitrogen, USA) and stored at -80°C. Three samples of each group were used for microarray expression analysis and all samples were used for quantitative real-time PCR (qRT-PCR).

RNA isolation and quality control

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The integrity of the RNA was assessed by electrophoresis on a denaturing agarose gel. A NanoDrop ND-1000 spectrophotometer was used for the accurate measurement of RNA concentration.

Microarray analysis of circRNA expression

Human CircRNA Array v1.0 is manufactured by Arraystar Technologies (Rockville, MD, USA). Three qualified RNA samples of each group were sent to KangChen Bio-tech (Shanghai, China) for the Arraystar circRNA microarray analysis. In short, isolated total RNA was treated with Rnase R (Epicentre, Inc.) to remove linear RNA, then transcribed into fluorescent cRNA using random primer according to Arraystar Super RNA Labeling protocol. The labeled cRNAs were purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Specific activity (pmol Dye/μg cRNA) and concentration (μg/μl) of cRNA were surveyed to assess the labeling efficiency by Nano Drop ND-1000. Then, 1 μg of each labeled cRNA was fragmented by adding 5 μl 10×Blocking Agent and 1 μl of 25× Fragmentation Buffer. The mixture was heated at 60°C for 30 min, and 25 μl 2× Hybridization buffer was added to dilute the labeled cRNA. Then, 50 μl of the hybridization solution was dispensed into the gasket slide, which was assembled with Human Circular RNA Array slides. The slide was incubated at 65 °C for 17 hours in Agilent Hybridization Oven. After washing, slides were fixed and scanned to generate images using an Agilent G2505C Scanner. Scanned images were imported into Agilent Feature Extraction software (version 11.0.1.1) for raw data extraction. Quantile normalization and subsequent data processing were executed using R software package. Student's t test was performed to analyze the statistical difference. The false discovery rate (FDR) is applied to determine the threshold of *P*-value. An FDR < 0.05 was recommended. Differentially expressed circRNAs with statistical significance (fold changes ≥ 1.5 and $P < 0.05$) between groups were identified using fold change cut-off.

Table 1. Primers used for qRT-PCR analysis of circRNA and mRNA levels

Name	Primer sequence 5'-3'	Product size (bp)
β-actin	F: CATGTACGTTGCTATCCAGGG	250
	R: CTCCTTAATGTCACGCACGAT	
hsa_circRNA_001937	F: TGAAGAACAGCTCTCTGGCTG	67
	R: GCCCACTTAATCAGGGTCAGG	
hsa_circRNA_009024	F: TTCCTGAGCAAGAAGTAGCCC	117
	R: TAACCCACAAAGTCCAGCTTCT	
hsa_circRNA_005086	F: ACCTGCTCCTCTGATTCTGCT	142
	R: GGTGAGTGGCAAGTGAGAGA	
hsa_circRNA_102101	F: TGAGTTTGGTGATTCAGCTTGC	121
	R: GCTTTATATGCCTTTCCTGAGCG	
hsa_circRNA_104964	F: TGGAGAATCAAGTGGCACCC	176
	R: CCCATGGTGTGTCTTTTGCTG	
hsa_circRNA_104296	F: CCCTTGCCAGGAGTGTTCAA	125
	R: TGTCGTCTTTTTCAGCCAGC	

Quantitative real-time PCR analysis

qRT-PCR was performed using a SYBR Master Mix (Applied Biosystems) protocol on an Applied Biosystems 7500 Real-Time PCR system. Divergent primers were designed through Circinteractome Divergent Primers web (https://circinteractome.nia.nih.gov/Divergent_Primers/divergent_primers.html), verified through primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and synthesized by Shanghai Shenggong (Shanghai, China). Primers used in this study were listed in Table 1. All qRT-PCR experiments were performed in triplicate. qRT-PCR data was analyzed by the $2^{-\Delta\Delta Ct}$ method, normalized against internal controls (β-actin) [17]. PCR product was examined by agarose gel electrophoresis using 2% (w/v) LE agarose (Seakem) stained with ethidium bromide.

Statistical analysis

The statistical significance of microarray data was analyzed in terms of fold change using the Student's t-test and FDR was calculated to correct the *P*-value. Numerical data were shown as the mean ± standard error of the mean (SEM). A one-way ANOVA test, Student t-test or Mann-Whitney test were used for statistical analysis. Receiver operating characteristic (ROC) analysis was used to evaluate the power of candidate circRNAs. The correlation between circRNA levels and stages of pulmonary lesions visualized by radiography were calculated using Spearman rank correlation. All statistical tests were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered statistically significant.

Results

Identification of differentially expressed circRNA profiles

The baseline characteristics of all study participants are presented in Table 2. To identify whether circRNAs are differentially expressed between TB patients and normal subjects, purified PBMCs from 3 active pulmonary TB patients and 3 age- and sex-matched healthy controls were analyzed by circRNA microarray assay. Hierarchical clustering and scatter plot visualization showed that the expression levels of circRNAs were variable between TB patients and healthy controls (Fig. 1). Thirty-seven circRNAs were differentially expressed between the two groups, of which 13 circRNAs were up-regulated and 24 were down-regulated in TB patients (fold changes ≥1.5 and $P<0.05$). The top 20 differentially expressed circRNAs are listed in Table 3 by fold change. Among the up-regulated circRNAs, there are 10 exonic, 2 intronic and 1 sense overlapping. Among the down-regulated circRNAs, there are 15 exonic, 4 intronic, 3 sense overlapping, 1 intergenic and 1 antisense. To identify the most clinically applicable biomarker, the top 3 elevated circRNAs and top 3 decreased circRNAs were chosen for further

Table 2. Baseline characteristics of the cohort. Abbreviations: TB: tuberculosis; COPD: chronic obstructive pulmonary disease; TST: Tuberculosis Skin Test; BCG: Bacille Calmette-Guerin vaccine; NA: not available

Characteristic	Microarray analysis		Validation				
	TB	Control	TB	Control	Lung cancer	Pneumonia	COPD
Total number	3	3	155	130	40	40	40
Gender (male/female)	2/1	2/1	108/47	85/45	30/10	29/11	28/12
Age (years, mean)	39.5±15.2	40.5±16.9	43.7±14.5	40.7±17.7	48.9±10.2	47.1±12.9	50.3±13.8
Smoking (Yes/no)	2/1	2/1	96/59	80/50	29/11	25/15	24/16
BCG vaccination (yes/no)	3/0	3/0	152/3	130/0	38/2	40/0	39/1
TST (Positive/Negative)	3/0	0/3	155/0	NA	NA	NA	NA
Sign and symptoms (n)							
Productive or unproductive cough	3	-	132	-	-	-	-
Weight loss	3	-	124	-	-	-	-
Fever	3	-	103	-	-	-	-
Smear (n)							
+	1	NA	82	NA	NA	NA	NA
++	1	NA	20	NA	NA	NA	NA
+++	1	NA	26	NA	NA	NA	NA
Negative	0	NA	27	NA	NA	NA	NA
Status of chest radiograph (n)							
Minimal	1	NA	73	NA	NA	NA	NA
Moderate	1	NA	47	NA	NA	NA	NA
Advanced	1	NA	35	NA	NA	NA	NA

analysis: hsa_circRNA_001937, hsa_circRNA_009024, hsa_circRNA_005086, hsa_circRNA_102101, hsa_circRNA_104964 and hsa_circRNA_104296.

Validation of circRNAs expression

The differential expression of 6 candidate circRNAs were verified by qRT-PCR in another independent cohort consisting of 40 TB patients and 40 control subjects. Results showed that hsa_circRNA_001937, hsa_circRNA_009024 and hsa_circRNA_005086 were increased, while hsa_circRNA_102101, hsa_circRNA_104964 and hsa_circRNA_104296 were decreased in TB patients versus normal control samples (P<0.001) (Fig. 2). The results were generally consistent with the microarray data.

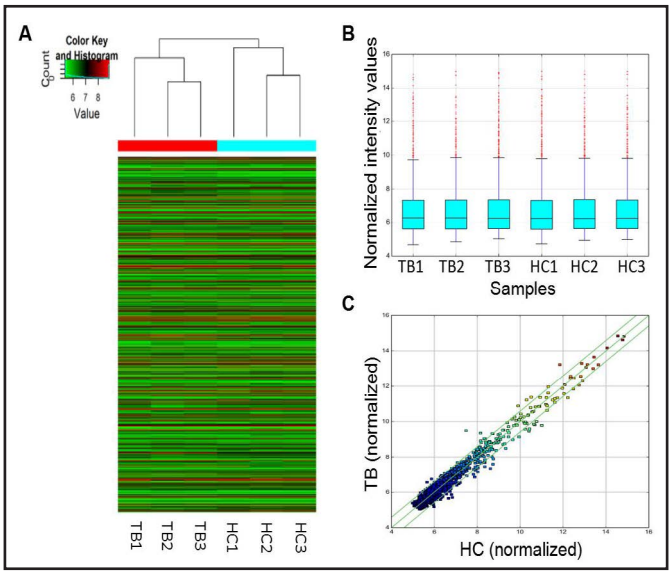


Fig. 1. Analysis of differentially expressed circRNAs in TB patients and healthy controls. (A) Hierarchical clustering results of circRNAs expression profiles among the TB group and control group. "Red" indicated high relative expression and "green" indicated low relative expression. (B) Box plots show the distribution of circRNAs for the two groups. The distributions were nearly the same after normalization. (C) CircRNAs in the Scatter-Plot above the top green line and below the bottom green line indicated more than 1.5 fold change of circRNAs between the two groups. HC: healthy controls; TB: tuberculosis.

Evaluation of the diagnostic potential of circRNAs for active TB

To evaluate the values of these differentially expressed circRNAs in serving as candidate biomarkers for TB diagnosis, ROC curve analysis was performed for each circRNA. As shown in Fig. 3, the AUC was larger than 0.750 for all 6 candidate circRNAs, suggesting their potential diagnostic value. Notably, the AUC of hsa_circRNA_001937 reached 0.873 (95%

Table 3. Top 20 differently expressed circRNAs in TB patients. Abbreviations: FDR: false discover rate; FC: fold change

circRNA	P value	FDR	FC	Regulation	circRNA_type	GeneSymbol
hsa_circRNA_001937	0.00870	0.12103	4.09329	up	intronic	CHD9
hsa_circRNA_009024	0.00002	0.00727	2.65734	up	exonic	TXLNGY
hsa_circRNA_005086	0.00137	0.07060	2.01377	up	exonic	RNF10
hsa_circRNA_103948	0.04069	0.20675	1.86743	up	exonic	SEC24A
hsa_circRNA_003524	0.04779	0.22032	1.70489	up	exonic	FAM168B
hsa_circRNA_015879	0.03556	0.19214	1.67151	up	exonic	PKP1
hsa_circRNA_009377	0.00252	0.08265	1.60347	up	exonic	RER1
hsa_circRNA_103285	0.00157	0.07548	1.57263	up	exonic	ATG7
hsa_circRNA_406505	0.01675	0.16027	1.56877	up	intronic	PPP3CA
hsa_circRNA_005232	0.02816	0.18048	1.56630	up	exonic	SLC8A1
hsa_circRNA_102101	0.00235	0.08164	2.45390	down	exonic	CDC27
hsa_circRNA_104964	0.00199	0.07607	2.39258	down	exonic	DPH7
hsa_circRNA_104296	0.00424	0.10053	2.24028	down	exonic	RNF216
hsa_circRNA_003416	0.02592	0.17719	2.24126	down	sense overlapping	TMSB4X
hsa_circRNA_002971	0.00050	0.04482	2.06249	down	exonic	SNX9
hsa_circRNA_007738	0.00749	0.11494	1.95143	down	sense overlapping	SHC3
hsa_circRNA_000686	0.00206	0.07607	1.87543	down	intronic	QPRT
hsa_circRNA_048148	0.00880	0.12103	1.76536	down	exonic	CNN2
hsa_circRNA_092458	0.04870	0.22032	1.67056	down	intronic	EEF2
hsa_circRNA_002465	0.04677	0.21791	1.64552	down	exonic	CD109

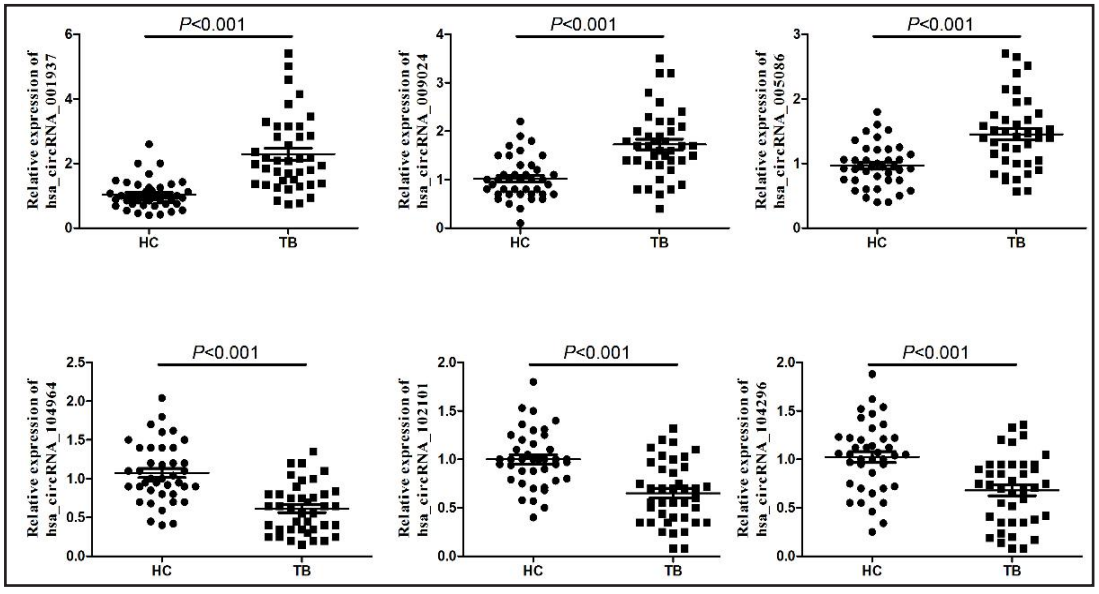


Fig. 2. Confirmation of the differential expression of circRNAs by qRT-PCR. The expression levels of six circRNAs were validated by qRT-PCR in PBMCs from 40 TB patients and 40 healthy controls. Mann-Whitney test was used for hsa_circRNA_001937, hsa_circRNA_009024 and hsa_circRNA_005086; Student's t-test was used for hsa_circRNA_102101, hsa_circRNA_104964 and hsa_circRNA_104296. Data are expressed as the means ± SEM.

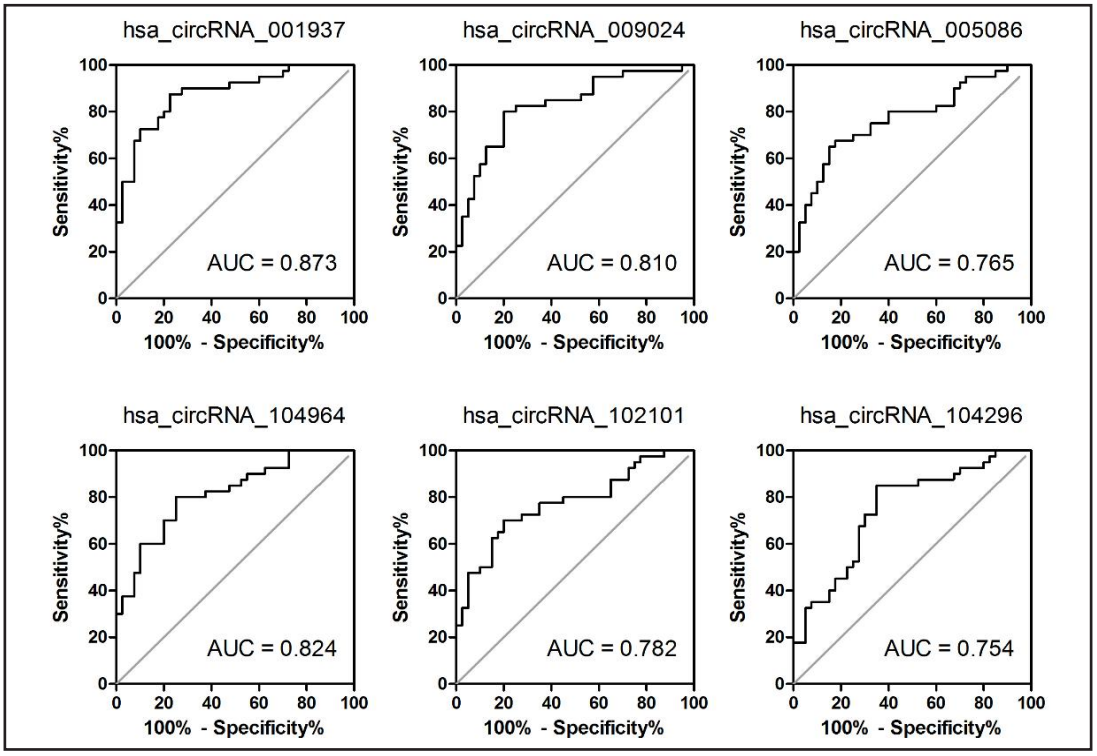


Fig. 3. Receiver operating characteristic (ROC) curve analysis of confirmed circRNAs in PBMCs from TB patients. The AUC values are given on the graphs.

CI 0.796-0.951, *P*<0.0001), which was the largest among these 6 circRNAs. The AUC of the other circRNAs were 0.810 (95% CI 0.714-0.906, *P*<0.0001) for hsa_circRNA_009024, 0.765

Table 4. Sensitivity and specificity of the candidate biomarkers in controls and active pulmonary TB patients. Abbreviations: AUC: area under the curve

Candidate biomarkers	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	P value
hsa_circRNA_001937	0.873 (0.796-0.951)	85.0 (70.2-94.3)	77.5 (61.6-89.2)	<0.0001
hsa_circRNA_009024	0.810 (0.714-0.906)	75.0 (58.8-87.3)	80.0 (64.4-91.0)	<0.0001
hsa_circRNA_005086	0.765 (0.660-0.870)	62.5 (45.8-77.3)	85.0 (70.2-94.3)	<0.0001
hsa_circRNA_102101	0.782 (0.681-0.883)	70.0 (53.5-83.4)	80.0 (64.4-91.0)	<0.0001
hsa_circRNA_104964	0.824 (0.735-0.914)	80.0 (64.4-91.0)	75.0 (58.8-87.3)	<0.0001
hsa_circRNA_104296	0.754 (0.648-0.861)	85.0 (70.2-94.3)	65.0 (48.3-79.4)	<0.0001

(95% CI 0.660-0.870, *P*<0.0001) for hsa_circRNA_005086, 0.782 (95% CI 0.681-0.883, *P*<0.0001) for hsa_circRNA_102101, 0.824 (95% CI 0.735-0.914, *P*<0.0001) for hsa_circRNA_104964 and 0.754 (95% CI 0.648-0.861, *P*<0.0001) for hsa_circRNA_104296. The sensitivity and specificity of each circRNA were determined based on the cut-off value (Table 4), and hsa_circRNA_001937 showed the highest sensitivity and specificity (85.0% and 77.5%). To evaluate the cumulative performances of the circRNAs in discriminating active TB from healthy controls, a logistic regression was performed. The logistic regression model showed that combination of hsa_circRNA_001937 and hsa_circRNA_009024 could provide better diagnostic accuracy, with the AUC of 0.926 (95% CI 0.865-0.987, *P*<0.001) (Fig. 4). The sensitivity and specificity of the combination of hsa_circRNA_001937 and hsa_circRNA_009024 were 95.0% and 80.0%, respectively.

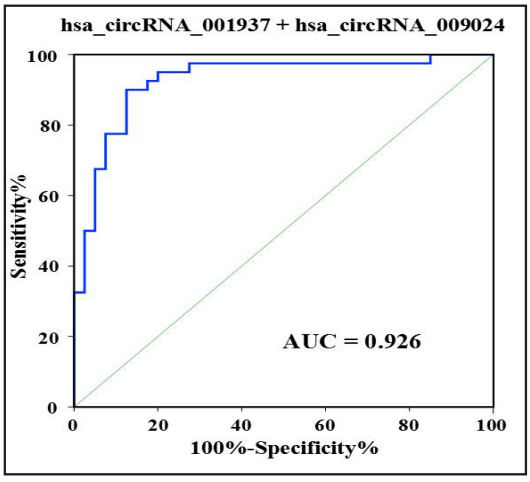


Fig. 4. Receiver operating characteristic (ROC) curve analysis of hsa_circRNA_001937 combined with hsa_circRNA_009024 in PBMCs from TB patients. The AUC values are given on the graphs.

Association of circRNAs levels with disease severity in patients with active TB

Active TB patients were classified regarding the severity of disease according to pulmonary radiographic images using a double blind test and classified as minimal, moderate and advanced disease. To investigate whether the expression levels of these differentially expressed circRNAs are related to the TB disease severity, the correlation between circRNA levels and the radiological severity scores (RSS) was analyzed by Spearman's rank correlation test. We found that 3 of the 6 circRNAs were correlated with the RSS (Fig. 5). Among these circRNAs, hsa_circRNA_001937, hsa_circRNA_009024 and hsa_circRNA_102101 were correlated with the RSS, hsa_circRNA_005086, hsa_circRNA_104964 and hsa_circRNA_104296 were not correlated with the RSS. Based on the AUCs of the 6 candidates and their correlations with the RSS, hsa_circRNA_001937 was chosen for further evaluation as a potential biomarker for TB diagnosis.

Clinical verification of the biomarker

To evaluate the actual diagnostic value of hsa_circRNA_001937 in clinical settings, we tested this circRNA in another independent cohort consisting of 115 TB patients and 90 healthy controls. As shown in Fig. 6, hsa_circRNA_001937 was significantly higher in TB pa-

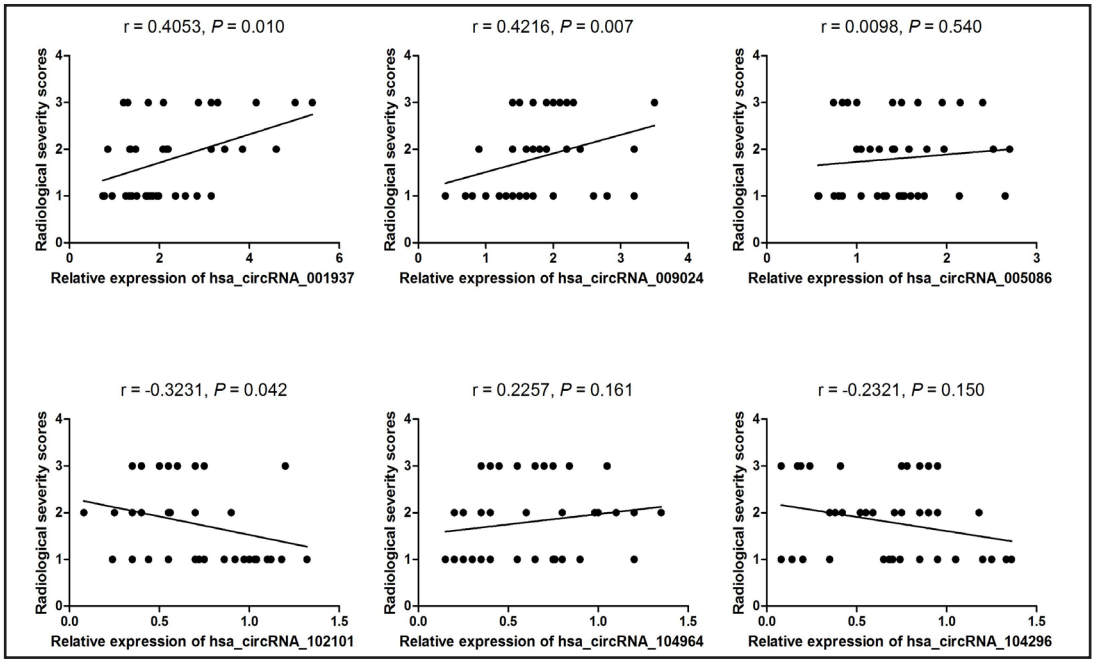


Fig. 5. Correlation between the expression levels of confirmed circRNAs and lung injury in TB patients. Lung injury of TB patients was classified in a double-blind test and then divided into three grades. Images are representative of each grade – minimal (1) (n = 18), moderate (2) (n = 12) and advanced (3) (n =10) disease. Levels of six circRNAs were correlated with the degree of lung injury in patients with active TB through Spearman's rank correlation test. The values of P and r are specified in each chart.

Fig. 6. The expression levels of hsa_circRNA_001937 in TB patients and controls. Control group, n = 90; TB group, n = 115. ROC curve analyses of hsa_circRNA_001937 in TB patients and controls.

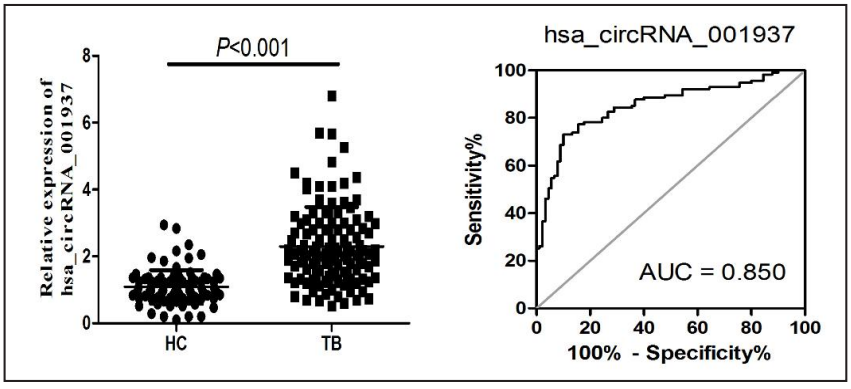
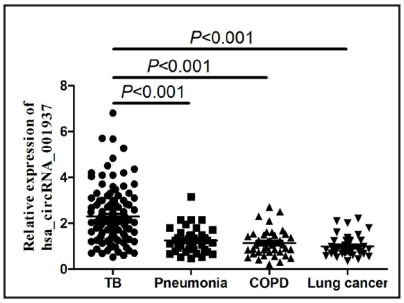


Fig. 7. The qRT-PCR assay validation of hsa_circRNA_001937 expression levels in PBMCs from 115 TB patients versus 40 pneumonia, 40 COPD or 40 lung cancer patients. A one-way ANOVA test was used for statistical analysis. COPD: chronic obstructive pulmonary disease.



tients than controls, with a 2.11-fold change. ROC curve analysis demonstrated an AUC of 0.850 (95% CI 0.796-0.903, *P*<0.001), and its sensitivity and specificity were 72.2% (95% CI 63.1%-80.1%) and 90.0% (95% CI 81.9%-95.3%), respectively.

Hsa_circRNA_001937 expressions in patients with TB, pneumonia, COPD and lung cancer

The expression levels of hsa_circRNA_001937 in 115 pulmonary TB patients were compared to that in 40 pneumonia, 40 COPD or 40 lung cancer patients, respectively. The data showed that the expression of hsa_circRNA_001937 was significantly higher in TB patients (both $P<0.001$) (Fig. 7). No significant difference was observed between pneumonia patients, COPD patients and lung cancer patients ($P>0.05$).

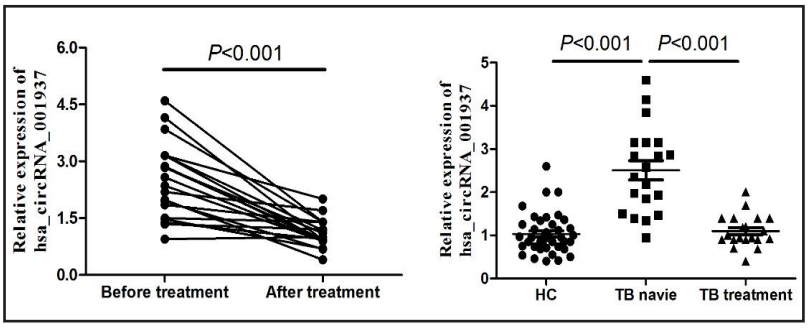


Fig. 8. Change in the levels of hsa_circRNA_001937 in response to therapy. The levels of hsa_circRNA_001937 were measured by qRT-PCR. Dot plot shows relative levels of hsa_circRNA_001937 in the same patients before and after completion of therapy (n = 20). Relative level of hsa_circRNA_001937 in healthy controls (n = 40), pulmonary TB naive patients (n = 20) and TB treated group (n = 20). The value between the healthy control and TB treated group is not significantly different.

Hsa_circRNA_001937 expression is significantly reduced in patients with active TB after successful treatment

The levels of hsa_circRNA_001937 were compared in 20 TB patients before and after anti-TB therapy. As compared to their pre-treatment, the levels of hsa_circRNA_001937 decreased after anti-TB treatment (Fig. 8). As compared to controls, the mean levels of hsa_circRNA_001937 came to nearly normal after therapy, with no significant difference between control and TB treated group ($P=0.592$), while that were significantly elevated in TB patient before treatment.

Discussion

CircRNAs are a large class of endogenous RNAs, formed by exon skipping or back-splicing events as covalently closed loops, which are expressed abundantly in mammalian cells [18]. With the increasing studies about circRNAs, researchers have reported that circRNAs are involved in the development of several types of diseases, such as cancer [19], cardiovascular disease [20], and neurological disorders [21]. Recently, several studies have revealed circRNAs can be used as diagnostic or predictive biomarkers for many human diseases [22, 23]. However, to date, research regarding circRNAs dysregulation in TB has not been reported. Serum/plasma is the most commonly used biological specimen for in-vitro diagnosis because of its nature of easy to obtain. However, because the abundance of circRNAs is significant higher in PBMCs than in serum/plasma and it's relatively easy to obtain, PBMCs are the most commonly used sample for circRNAs detection [13, 17].

In this study, circRNA expression profiles in PBMCs from individuals with active TB and healthy controls were determined by circRNA microarray. The microarray expression profiles exhibited that 13 up-regulated circRNAs and 24 down-regulated circRNAs were significantly differentially expressed in TB patients. The distinct expression patterns of circRNAs may be related to their involvement in the pathogenesis of TB and thus serve as biomarkers for TB diagnosis. To identify the clinically applicable biomarkers, we detected six significant differentially expressed circRNAs and investigated the association between their expression levels and disease severity of TB. Compared with healthy controls, hsa_circRNA_102101, hsa_circRNA_104964 and hsa_circRNA_104296 were significantly decreased, while hsa_circRNA_001937, hsa_circRNA_009024 and hsa_circRNA_005086 were overexpressed in TB patients. ROC curve analysis suggested that hsa_circRNA_001937

has signifcant value for TB diagnosis (AUC = 0.873), followed by hsa_circRNA_104964 (AUC = 0.824), hsa_circRNA_009024 (AUC = 0.810), hsa_circRNA_102101(AUC = 0.782), hsa_circRNA_005086 (AUC = 0.765), and hsa_circRNA_104296 (AUC = 0.754). Logistic regression model demonstrated that combination of hsa_circRNA_001937 and hsa_circRNA_009024 had higher AUC (0.926) when compared with each of them. Moreover, the Spearman' s rank correlation test demonstrated that the expression level of hsa_circRNA_001937 was correlated with the radiological severity scores, implying that hsa_circRNA_001937 might be involved in the pathologies of TB.

To determine whether hsa_circRNA_001937 can be a diagnostic biomarker for TB, we tested hsa_circRNA_001937 in larger cohorts. Hsa_circRNA_001937 provided the high diagnostic power for detection of TB (AUC= 0.850; sensitivity, 72.2%; and specificity, 90.0%). Furthermore, we evaluated its ability to effectively distinguish TB from other lung diseases (pneumonia, COPD and lung cancer). Our study revealed that hsa_circRNA_001937 may serve as TB-specific signature circRNA and could be used as candidate biomarker of TB. Taken together, we found that the levels of hsa_circRNA_001937 came to nearly normal after completion of anti-TB therapy, although hat were significantly elevated in TB patient before treatment.

Some studies have revealed that circRNAs could function as miRNA sponges or regulate parent gene expression to affect disease initiation and progression [24]. The association of miRNAs with TB indicated that circRNAs may have a regulatory role in TB infection. To evaluate hsa_circRNA_001937 potential function, the hsa_circRNA_001937/miRNA interaction was predicted using Arraystar's home-made miRNA target prediction software based on TargetScan and miRanda. We found that the potential miRNAs targets of hsa_circRNA_001937 include miR-22-5p, miR-26b-3p, miR-10b-3p, miR-376a-5p and miR-597-3p. MiR-26b participates in the inflammatory response by modulating the NF-κB pathway through targeting PTEN [25]. However, due to the limited known function of circRNAs and miRNAs, a lot of circRNAs/miRNAs interactions should be analyzed in the future.

Several limitations in this study should be acknowledged. First is the relatively small sample size. The conclusions of this study require further verification in larger and more diverse cohorts. Second, PBMCs compose mainly lymphocytes and monocytes. We compared the numbers of monocytes and lymphocytes between the TB and healthy control groups and found no significant difference (data not shown). And, we did not investigated the expression of hsa_circRNA_001937 released in the local target tissues and in specific cell subsets in PBMCs.

Conclusion

We identified differentially expressed circRNAs in PBMCs from subjects with active TB and normal controls. Furthermore, hsa_circRNA_001937 was identified as potential molecular marker for diagnosis of TB. To our knowledge, this is the first research addressing circRNAs expression profiles in TB diseases. Further studies should focus on the function of circRNAs involved in TB infection, which may lead to new theories for TB pathogenesis and give new potentially therapeutic targets in active TB.

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Disclosure Statement

The authors declare no conflicts of interest.

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