Original Article

Circulating circular RNAs hsa_circ_0001204 and hsa_circ_0001747 act as diagnostic biomarkers for active tuberculosis detection

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Abstract: In recent years, increasing evidence has suggested that circRNAs can serve as novel diagnostic markers for many diseases. However, little is known about the value of circRNAs in the diagnosis of active tuberculosis (TB). In this study, 10 circRNAs which we previously found to be involved in Mycobacterium tuberculosis infection were selected as candidate targets for subsequent circulating circRNA assay. Compared with healthy controls, plasma levels of hsa_circ_0001204 and hsa_circ_0001747 were significantly decreased (P < 0.001). Plasma levels of hsa_circ_0001204 and hsa_circ_0001747 were correlated with TB severity. Hsa_circ_0001204 and hsa_circ_0001747 were selected for further analysis in another 145 TB patients and 120 control individuals. The area under the receiver operating characteristic curve (AUC) for distinguishing TB patients was 0.928 (95% confidence interval: 0.897-0.960; sensitivity = 86.21%, specificity = 89.17%) when hsa_circ_0001204 and hsa_circ_0001747 were used in combination. Further evaluation on potential biomarkers showed that hsa_circ_0001204 and hsa_circ_0001747 may specifically identify patients with TB. Additionally, hsa_circ_0001204 and hsa_circ_0001747 plasma levels after treatment were significantly higher than that pre-treatment (P < 0.001). Our present study indicates that circulating hsa_circ_0001204 and hsa_circ_0001747 may represent novel plasma biomarkers for TB diagnosis.

Keywords: Tuberculosis, circular RNAs, biomarker, diagnosis

Introduction

Tuberculosis (TB) is the second leading cause of death from an infectious disease worldwide. It is estimated that 10.4 million cases of TB occurred in 2015 and that 1.4 million died of TB [1]. Early diagnosis of TB infection is essential for controlling the spread of the disease. Conventional methods for TB diagnosis at present are primarily smears for acid-fast bacilli (AFB) and Mycobacterium tuberculosis culture [2]. AFB smear 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 [3]. Immunological tests are time-consuming and require confirmation in longitudinal analyses and further functional studies. New automatic molecular methods such as GeneXpert MTB/RIF for the diagnosis of TB are currently available, but the cost per test is too high for resource-limited settings without committed long-term, external funding [4, 5]. Therefore, new rapid, sensitive, and cost-effective biomarkers or methods for TB diagnosis are urgently needed.

CircRNAs are a class of endogenous RNAs, which are characterized by covalently closed loop structures without a 5' cap or a 3' Poly A tail [6, 7]. Increasing evidence reveals that circRNAs have important roles in the regulation of gene expression at the post-transcriptional level [8, 9]. Accordingly, it has been demonstrated that many circRNAs serve as competing endogenous RNAs (ceRNAs) to bind with microRNAs (miRNAs) and to inhibit the activity and function of the targeted miRNAs [10, 11]. Many circRNAs are abundant, conserved, and often exhibit tissue/developmental-specific
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We previously found that many circRNAs were differentially expressed in the human monocyte derived macrophage response to M. tuberculosis infection, which indicates that circRNAs have the potential to be novel biomarkers for TB [20]. In the present study, we performed a two-stage study to explore the plasma levels of the ten circRNAs (hsa_circ_0003528, hsa_circ_0001417, hsa_circ_0043497, hsa_circ_0038929, hsa_circ_0000994, hsa_circ_0068784, hsa_circ_0057090, hsa_circ_0056247, hsa_circ_0001204 and hsa_circ_0001747) which we previously found to be involved in M. tuberculosis infection and we investigated their potential as biomarkers in TB.

Materials and methods

Patients and specimens

In this study, consecutive hospitalized patients who were newly diagnosed with active pulmonary TB were selected from Jiangxi Chest Hospital and the First Affiliated Hospital of Nanchang University (Nanchang, China) from June 2015 and May 2017. All of these patients (n = 195) were diagnosed on the basis of typical TB clinical symptoms, imaging examinations, and were 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. [21]. TB patients with any other co-existing disease were excluded in this study. Subsequently, 25 active pulmonary TB inpatients received a 2HRZE/6HE treatment regimen, which started 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.

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence 5'-3'</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: TGTTGCATCAATGACCCCCTT R: TCTACAGAGCTCAGCCG</td>
<td>202</td>
</tr>
<tr>
<td>hsa_circ_0003528</td>
<td>GCTGGAGCTGTTGGTCTT</td>
<td>135</td>
</tr>
<tr>
<td>hsa_circ_0001417</td>
<td>ACTGTGCTAGCCGAAACGTTCC</td>
<td>152</td>
</tr>
<tr>
<td>hsa_circ_0043497</td>
<td>TCAACCGGACATTGCATGTTA</td>
<td>130</td>
</tr>
<tr>
<td>hsa_circ_0038929</td>
<td>TTTCTGACTGAGCAGGGGC</td>
<td>160</td>
</tr>
<tr>
<td>hsa_circ_0000994</td>
<td>AGAGTTAGTTGCGGAGACTGT</td>
<td>120</td>
</tr>
<tr>
<td>hsa_circ_0068784</td>
<td>GGTTGTCTGTATCCTAACCACATCCTG</td>
<td>126</td>
</tr>
<tr>
<td>hsa_circ_0057090</td>
<td>CCACTGACAGCCACACCTT</td>
<td>143</td>
</tr>
<tr>
<td>hsa_circ_0056247</td>
<td>TTCCAAAGGAAGGTGTGTGTT</td>
<td>175</td>
</tr>
<tr>
<td>hsa_circ_0001204</td>
<td>AAGATGACAGGTGGAGACTGT</td>
<td>82</td>
</tr>
<tr>
<td>hsa_circ_0001747</td>
<td>AAGGAGGAGGGAACATGGAAC</td>
<td>130</td>
</tr>
</tbody>
</table>
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Table 2. Characteristics of study subjects in the screening and validation stage

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Screening stage</th>
<th>Validation stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TB Control TB</td>
<td>Lung cancer Pneumonia COPD</td>
</tr>
<tr>
<td>Total number</td>
<td>50 145</td>
<td>120 50 120</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>32/18</td>
<td>95/60 78/42</td>
</tr>
<tr>
<td>Age (years, mean)</td>
<td>38.2±14.3</td>
<td>40.6±13.9 43.8±15.0</td>
</tr>
<tr>
<td>Smoking (Yes/no)</td>
<td>30/20</td>
<td>82/63 69/51</td>
</tr>
<tr>
<td>BCG vaccination (yes/no)</td>
<td>49/1 50/0</td>
<td>135/10 115/5</td>
</tr>
<tr>
<td>TST (Positive/Negative)</td>
<td>50/0 NA</td>
<td>145/0 NA NA NA NA</td>
</tr>
<tr>
<td>Sign and symptoms (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Productive or unproductive cough</td>
<td>35 -</td>
<td>115 - - - - -</td>
</tr>
<tr>
<td>Weight loss</td>
<td>30 -</td>
<td>109 - - - - -</td>
</tr>
<tr>
<td>Fever</td>
<td>29 -</td>
<td>93 - - - - -</td>
</tr>
<tr>
<td>Smear (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>24 NA 72 NA NA NA NA</td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>9 NA 23 NA NA NA NA</td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>5 NA 16 NA NA NA NA</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>12 NA 34 NA NA NA NA</td>
<td></td>
</tr>
<tr>
<td>Status of chest radiograph (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>26 NA 66 NA NA NA NA</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>13 NA 43 NA NA NA NA</td>
<td></td>
</tr>
<tr>
<td>Advanced</td>
<td>11 NA 36 NA NA NA NA</td>
<td></td>
</tr>
</tbody>
</table>

Note: NA: not applicable.

examination. All these 25 patients were fully recovered. Healthy control individuals (n = 170) with no clinical symptoms of any infectious disease, diabetes, cancer, and had no close contact with TB patients 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 150 subjects in the differential diagnosis group (50 pneumonia patients, 50 COPD patients and 50 lung cancer patients), confirmed clinically after eliminating pulmonary TB disease, were recruited from the First Affiliated Hospital of Nanchang University and Jiangxi Chest Hospital between August 2015 and January 2017. This study was approved by the Ethical Committee of the First Affiliated Hospital of Nanchang University and was conducted in accordance with the Declaration of Helsinki. All participants provided informed consent before commencement of the study. Peripheral blood (~5 mL) from all study subjects was collected into EDTA-anticoagulated tubes. The blood samples were centrifuged (2000 g for 10 minutes at 4°C, then 12,000 g for 10 minutes at 4°C) to obtain plasma. After separation, plasma samples were transferred to tubes and stored at -80°C until total RNA extraction.

Total RNA isolation

Total RNA was isolated by using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. The concentrations of RNA were measured using a NanoDrop™1000 spectrophotometer (Thermo Scientific, USA).

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA were reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio Inc., Japan). SYBR®Premix Ex Taq™ II (TaKaRa) was used for fluorescent quantitative real-time PCR (RT-qPCR) assay. The relative expression level of circRNA in plasma was normalized to the GAPDH expression [18, 19]. Divergent primers were designed through Circinteractome Divergent Primers and web verified through primer-BLAST and synthesized by Shanghai Shenggong (Shanghai, China). Primers used in this study were listed in Table 1. All qRT-PCR was performed on a ABI 7500 Real Time PCR System (Applied Biosystems, CA, USA). Expression levels of circRNAs were calculated using 2-ΔΔCt method.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software version (SPSS Inc.).
Figure 1. Screening of ten selected circRNAs in plasma of TB and healthy subjects. The expression levels of ten circRNAs (hsa_circ_0003528, hsa_circ_0001417, hsa_circ_0043497, hsa_circ_0038929, hsa_circ_0000994, hsa_circ_0068784, hsa_circ_0057090, hsa_circ_0056247, hsa_circ_0001204 and hsa_circ_0001747) were validated by qRT-PCR in plasma from 50 TB patients and 50 healthy controls. Statistical analysis was performed using the nonparametric Mann-Whitney U test. The relative expression levels of circRNAs were normalized to levels of the control (GAPDH).
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![Figure 1](image1.png)  ![Figure 2](image2.png)

**Figure 2.** Correlation between the expression levels of candidate circRNAs (hsa_circ_0001204 and hsa_circ_0001747) 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 = 26), moderate (2) (n = 13) and advanced (3) (n = 11) disease. Levels of two circRNAs (hsa_circ_0001204 and hsa_circ_0001747) were correlated with the degree of lung injury in patients with active TB through Spearman’s rank correlation test. The values of ρ and r are specified in each chart.

**Figure 3A.** Association of plasma circRNAs levels with disease severity in patients with active TB

**Figure 3B.** Clinical verification of the biomarker

**Results**

**Patient characteristics**

In this study, we recruited 195 pulmonary TB patients, of which 50 were pneumonia patients, 50 were COPD patients, 50 were lung cancer patients and 170 were healthy controls. The demographic and baseline clinical data of the study subjects in the two stages are summarized in Table 2.

**Screening of ten selected circRNAs in plasma of TB and healthy subjects**

The ten selected circRNAs (hsa_circ_0003528, hsa_circ_0001417, hsa_circ_0043497, hsa_circ_0038929, hsa_circ_0000994, hsa_circ_0068784, hsa_circ_0057090, hsa_circ_0056247, hsa_circ_0001204 and hsa_circ_0001747) were first tested in plasma from 50 newly diagnosed patients with TB and 50 healthy controls using qRT-PCR. Compared with healthy controls, the expression level of hsa_circ_0001204 and hsa_circ_0001747 was significantly downregulated in patients with TB (P < 0.001). No significant difference in the other eight circRNAs was found between TB patients and healthy controls (all P > 0.05, shown in Figure 1).

**Association of plasma 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 of two candidate circRNAs (hsa_circ_0001204 and hsa_circ_0001747) identified in the screening stage were related to TB disease severity, we examined the correlation between circRNA levels and the radiological severity scores by Spearman’s rank correlation test. As shown in Figure 2, hsa_circ_0001204 (r = -0.504, P < 0.001) and hsa_circ_0001747 (r = -0.407, P = 0.003) were moderately correlated with the radiological severity scores.

**Clinical verification of the biomarker**

To evaluate the actual diagnostic value of hsa_circ_0001204 and hsa_circ_0001747 in clinical settings, we tested hsa_circ_0001204 and hsa_circ_0001747 in another independent cohort consisting of 145 TB patients and 120 healthy controls. Compared with healthy controls, plasma levels of hsa_circ_0001204 and hsa_circ_0001747 were significantly decreased in TB patients (Figure 3A). The AUC of the ROC curve was 0.871 (95% CI: 0.827-0.916) for hsa_circ_0001204 and 0.830 (95% CI: 0.780-0.880) for hsa_circ_0001747 (Figure 3B). Further analysis of the diagnostic performance of hsa_circ_0001204 and hsa_circ_0001747 revealed that, the plasma level of hsa_circ_0001204 could distinguish TB from healthy controls with 73.10% sensitivity and 92.50% specificity; hsa_circ_0001747 could distinguish TB from healthy controls with 71.03% sensitivity and
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82.50% specificity. When hsa_circ_0001204 and hsa_circ_0001747 were combined, the AUC increased to 0.928 (95% CI: 0.897-0.960; sensitivity = 86.21%, specificity = 89.17%).

Figure 3. ROC curve analyses of hsa_circ_0001204 combined with hsa_circ_0001747. A. The expression levels of hsa_circ_0001204 and hsa_circ_0001747 in TB patients (n = 145) and controls (n = 120). B. ROC curves of hsa_circ_0001204 and hsa_circ_0001747 between active TB patients and healthy control. The AUC values are given on the graphs.

82.50% specificity. When hsa_circ_0001204 and hsa_circ_0001747 were combined, the AUC increased to 0.928 (95% CI: 0.897-0.960; sensitivity = 86.21%, specificity = 89.17%).

Hsa_circ_0001204 and hsa_circ_0001747 expression in patients with TB, pneumonia, COPD, and lung cancer

We analyzed expression of plasma hsa_circ_0001204 and hsa_circ_0001747 in 145 pulmonary TB patients versus 50 pneumonia, 50 COPD, or 50 lung cancer patients, respectively. The data showed that expression of hsa_circ_0001204 and hsa_circ_0001747 were decreased dramatically in patients with TB compared with pneumonia patients, COPD patients, and lung cancer patients (all P < 0.001), but no significant difference was observed between pneumonia patients, COPD patients, and lung cancer patients (all P > 0.05) (Figure 4).

Figure 4. Relative expression of circRNAs in the validation set of patients with TB and disease controls. qRT-PCR assay validation of hsa_circ_0001204 and hsa_circ_0001747 expression levels in plasma from 145 TB patients versus 50 pneumonia, 50 COPD, or 50 lung cancer patients. A one-way ANOVA test was used for statistical analysis.

Subsequently, a risk score based on hsa_circ_0001204 combined with hsa_circ_0001747 from the clinical verification set was further assessed in TB patients and all controls (healthy controls in the validation set, pneumonia patients, COPD patients, and lung cancer patients), the AUC for the risk score was 0.913 (95% CI: 0.881-0.945; sensitivity = 91.85%, specificity = 80.00%) (Figure 5). The risk score also significantly discriminated the patients with TB from disease controls (pneumonia patients, COPD patients, and lung cancer patients), and the AUC was 0.900 (95% CI: 0.865-0.936; sensitivity = 90.67%, specificity = 77.24%).

Hsa_circ_0001204 and hsa_circ_0001747 expression are significantly increased in patients with active TB after successful treatment

The levels of hsa_circ_0001204 and hsa_circ_0001747 were compared in 25 TB patients before and after successful treatment. As compared to pre-treatment, the levels of hsa_
circ_0001204 and hsa_circ_0001747 were increased after anti-TB treatment (Figure 6). As compared to controls, the mean levels of hsa_circ_0001204 and hsa_circ_0001747 returned to nearly normal after therapy, with no significant difference between the control and TB treated group ($P > 0.05$).

To determine whether hsa_circ_0001204 and hsa_circ_0001747 could be diagnostic biomarkers for TB, we tested hsa_circ_0001204 and hsa_circ_0001747 in larger cohorts. Based on ROC curves, we selected cut-off values that best differentiated TB patients from healthy individuals. Hsa_circ_0001204 was
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found to have a sensitivity of 73.10% and a specificity of 92.50%, whereas hsa_circ_0001204 had a sensitivity of 71.03% and a specificity of 82.50% for TB. Hsa_circ_0001747 had a sensitivity of 71.03% and a specificity of 82.50% for TB. Hsa_circ_0001204 demonstrated a higher discriminating ability compared to hsa_circ_0001747, while not statistically significant ($P > 0.05$). Combined ROC analyses using these two targets may yield an increased AUC of 0.928, with 86.21% sensitivity and 89.17% specificity in discriminating TB patients from normal controls, indicating the additive effect in the diagnostic value of these two circRNAs. Furthermore, we evaluated its ability to effectively distinguish TB from other lung diseases (pneumonia, COPD and lung cancer). Our study revealed that hsa_circ_0001204 and hsa_circ_0001747 may serve as TB-specific signature circRNAs and could be used as candidate biomarkers of TB. Taken together, we observed that the level of hsa_circ_0001204 and hsa_circ_0001747 which were altered in naive TB patients reverted to normal after successful treatment.

Some studies have revealed that circRNAs could function as miRNA sponges or regulate parent gene expression to affect disease initiation and progression [27, 28]. The association of miRNAs with TB indicated that circRNAs may have a regulatory role in TB infection [29, 30]. To evaluate hsa_circ_0001204 and hsa_circ_0001747 potential function, the circRNAs/miRNAs interaction were predicted using Arraystar’s home-made miRNA target prediction software based on TargetScan and miRanda. We found that the potential miRNAs targets of hsa_circ_0001204 include miR-612, miR-657, miR-362-3p, miR-377-3p, and miR-136-5p. For hsa_circ_0001747, the potential miRNAs targets include miR-616-5p, miR-30d-3p, miR-320b, miR-320a, and miR-302c-5p. However, due to the limited known function of circRNAs and miRNAs, a lot of circRNAs/miRNAs interactions should be analyzed in the future.

In conclusion, our results provide novel empirical evidence that hsa_circ_0001204 and hsa_circ_0001747 may specifically identify patients with TB, and that the combination of hsa_circ_0001204 and hsa_circ_0001747 may provide better diagnostic accuracy. 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.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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