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Research paper

The circular RNA of peripheral blood mononuclear cells: Hsa_circ_0005836 as a new diagnostic biomarker and therapeutic target of active pulmonary tuberculosis

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ABSTRACT

It has been reported that circular RNA (circRNA) is associated with human cancer. However, few studies have been reported in active pulmonary tuberculosis (APTB). The global circRNA expression was detected in the peripheral blood mononuclear cells (PBMCs) of APTB patients (n = 5) and health controls (HC) (n = 5) by using high-throughput sequencing. According to the systematical bioinformatics analysis, the basic content of circRNAs and their fold changes in the two groups were calculated. We selected 6 significant differentially expressed circRNAs, hsa_circ_0005836, hsa_circ_0009128, hsa_circ_0003519, hsa_circ_0023956, hsa_circ_0078768, and hsa_circ_0088452 and validated the expression in PBMCs from APTB (n = 10) and HC (n = 10) by real-time quantitative reverse transcription-polymerase chain reactions (qRT-PCRs). Further, the verification of these specific circRNAs (hsa_circ_0005836 and hsa_circ_0009128) between APTB (n = 34) and HC (n = 30) in PBMCs was also conducted by qRT-PCRs. The RNA-seq data showed the significant differential expression of the 523 circRNAs between the APTB and HC groups (199 circRNAs were significantly up-regulated and 324 circRNAs were down-regulated). Hsa_circ_0005836 and hsa_circ_0009128 expression was significantly down-regulated in the PBMCs of APTB ($P < 0.05$) in the samples of APTB compared to HC in our study. The gene ontology based enrichment analysis of the circRNA-miRNA-mRNAs network showed that cellular catabolic process ($P = 7.10E-08$), regulation of metabolic process ($P = 2.10E-06$), catalytic activity ($P = 3.67E-08$), protein binding ($P = 1.71E-07$), cell part ($P = 3.46E-06$), intracellular part ($P = 1.71E-07$), and intracellular ($P = 3.67E-08$) were recognized in the comparisons between APTB and HC. Based on KEGG analysis, HTLV-I infection, regulation of actin cytoskeleton, neurotrophin signaling pathway and mTOR signaling pathway were relevant during tuberculosis bacillus infection. We found for the first time that hsa_circ_0005836 and hsa_circ_0009128 were significantly down-regulated in the PBMCs of APTB compared with HC. Our findings indicate hsa_circ_0005836 might serve as a novel potential biomarker for TB infection.

1. Introduction

Tuberculosis (TB) is a major global health problem that causes illness among millions of people each year. Now it remains as a leading cause of death worldwide (WHO, 2016). The latest data from WHO showed that, in 2016, TB killed 1.5 million people, of which

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approximately 890,000 were men, 480,000 were women and 140,000 were children (WHO, 2016). In recent years, despite the fact that nearly all cases can be cured, TB remains one of the world's biggest threats in the public health field. The future targets should focus on the falling of TB incidence, not merely new drug development. Early accurate diagnosis and immediate curative treatment are key to tuberculosis control (Uplekar et al., 2015). At present, smear microscopy and bacteriological culture are still the most widely used tools for TB clinical diagnosis, despite of their time-consuming and inaccuracy (Siddiqi et al., 2003). Therefore, we urgently need new progress in finding the specific biological markers of tuberculosis, and the establishment of a rapid, sensitive and efficient method of diagnosis and identification of pulmonary tuberculosis, with the goal of preventing the rapid spread of tuberculosis.

Circular RNA (circRNA) is a novel noncoding RNA molecule with 3′- and 5′- ends covalently linked in a closed loop structure, in contrast to the typical linear RNAs with 5'- caps and 3'- poly-(A) tails. It has been discovered in all domains of life with distinct sizes and sources. While in eukaryotes circRNAs were often considered as products of mis-splicing events (Cocquerelle et al., 1993), recent studies using high-throughput RNA sequencing (RNA-seq) analysis and corresponding experimental validation have proven that they actually represent a class of abundant and stable RNAs which possess important biological functions in animals (Chen et al., 2016; Memczak et al., 2015; Sand et al., 2016). Recent studies have shown that circRNA molecules are enriched with microRNA (miRNA) recognition elements (MREs) and play a role in the miRNA sponge, such as circRNAs CDR1as (ciRS-7) and SRY (Hansen et al., 2013). They could relieve the inhibition of the corresponding miRNA on its targets and increase their expressions. By interacting with miRNAs, circRNA plays a crucial role in the occurrence and development of diseases. Being resistant to exonucleolytic activities, circRNAs are more stable and have longer half-life than linear RNAs (Enuka et al., 2016). Due to their high abundance, stable and accessible characteristics, circRNAs could serve as putative biomarker molecules in clinically relevant samples. It has been found that the expression of circular ANRIL(cANRIL) may be associated with transcription of INK4/ARF and the risk of cardiovascular sclerosis (Burd et al., 2010). The researchers found that the expression level of hsa_circ_002059 was significantly down-regulated in gastric cancer tissues, and it has been used as a potential biomarker for gastric cancer diagnosis (Li et al., 2015a). Qin and Liu et al. discovered that hsa_circ_0005075 is a potential target for the diagnosis and treatment of hepatocellular carcinoma (Qin et al., 2016). The study of Li and Zhang et al. found that circ-ITCH could be used as a biomarker for clinical diagnosis of esophageal cancer (Li et al., 2015b).

TB is a chronic infectious disease caused by the human pathogen, *Mycobacterium tuberculosis* (*Mtb*) infection, which primarily infects innate immune cells patrolling the lung (Cooper, 2009). The mononuclear cells which played an vital role in the innate immune responses are key to the control of tuberculosis infection (Flynn, 2004). Activated mononuclear cells have the ability to prevent *Mtb* infection through secreting pro-inflammatory cytokines, such as IFN-γ and TNF-α (Su et al., 2010; Yu et al., 2015), forming phagolysosome which kills tuberculosis (Armstrong and Hart, 1971), presenting antigen to T lymphocytes and triggering cell-mediated immune reaction (Iwasaki and Medzhitov, 2015). Mononuclear cells serve as barometers of the immune response against *Mtb* infection. Thus in our study, we analyzed circRNAs from peripheral blood mononuclear cells (PBMCs) in the patients with active pulmonary tuberculosis (APTB) and healthy volunteers (HV) by combining RNA-seqence technique with bioinformatics analysis. For the first time, we investigated the possibilities of circRNA as a potential biomarker for early diagnosis of APTB through comparing the circRNA expression levels of the PBMCs of HC and APTB patients. We found hsa_circ_0005836 was significantly down-regulated in PBMCs from APTB patients. Further, we evaluated putative interactions among the differentially expressed circRNA, the miRNA targets and the targets of this miRNAsto identify the circRNA-miRNA-mRNA network. This research suggests that differentially expressed circRNAs might be involved in the process of anti-*Mtb* infection through miRNA interaction and presents a novel molecular target for clinical diagnosis and therapy.

2. Material and methods

2.1. Patients and cell samples collection

In this study, a total of 49 patients with active pulmonary tuberculosis (APTB), from 16 to 65 years old, were diagnosed as described previously (Wang et al., 2016) in the Affiliated Houjie Hospital of Guangdong Medical College, Guangdong Medical College, and the Dongguan Hospital for Prophylaxis and Treatment of Chronic Disease. All subjects were tested for sputum Ziehl-Neelsen acid fast staining and Lowenstein-Jensen slant culture according to the standard methods. Forty five healthy volunteers (HV) individuals aged from 20 to 68 years old who were absent of bacteriological and clinical evidence of tuberculosis were served as controls.

Peripheral blood mononuclear cells (PBMCs) were prepared as previously reported (Zeng et al., 2014). Approximately 5 ml blood was collected from each subject in acid citrate dextrose (ACD)-containing blood collection tubes. PBMCs were freshly isolated from blood by standard Ficoll (GE Healthcare, Little Chalfont, United Kingdom) density gradient centrifugation. Cell viability was determined by trypan blue exclusion (> 95% in all experiments). PBMCs were then stored in liquid nitrogen for further experiments.

2.2. RNA sample preparation for high-throughput sequencing

Total RNA was extracted from each sample of PBMCs by Trizol reagent (Invitrogen, USA) according to the protocol of manufacturer, and treated with RNase-free DNase to remove traces of genomic DNA. Five RNA samples of the same group were pooled and aliquoted into two for sequencing. The quantity of the total RNA was determined by Nano Drop ND-2000C spectrophotometer (Thermo, USA). The integrity of the RNA was assessed by agarose gel electrophoresis.

For high-throughput sequencing, we synthesized the cDNA libraries of the circRNA from each sample based on the Illumina standard protocols. Briefly, total RNA was treated with the Epicentre Ribo-Zero kit (Epicentre, USA) to remove all the ribosomal RNAs, and digested with Rnase R (Epicentre, USA) to remove all the linear RNAs, thus got the pure circular RNAs. Then, the enriched circular RNAs were processed by the NEBNext® Ultra™ Directional RNA Library preparation kit (Illumina, USA)according to the Illumina protocol. The main steps involved: the fragmentation of circRNA, reverse transcription and second-strand cDNA synthesis, end repair, dA-tailing and adaptor ligation. The products of these reactions were purified and amplified by PCR to create the final circular RNA cDNA library. The libraries were paired-end sequenced on the Illumina HiSeq2500 platform (Illumina, USA).

2.3. Circ-seq workflow and bioinformatics data analysis

The Circ-seq workflow and bioinformatics data analysis was consist of five steps: RNA-seq data acquisition and quality control, transcriptome assembly and circRNA identification, differential expression analysis of circRNAs, target miRNA prediction of circRNAs, and functional enrichment analysis of differentially expressed circRNAs.

2.4. RNA-seq data acquisition and quality control

In this study, high-throughput sequencing of the samples was performed using the paired-end sequencing mode on the Illumina HiSeq2500 platform. We got the enormous circRNA sequence raw data of the HV control group and APTB patients group PBMCs by RNA-seq. Based on the distribution of low-quality fraction of illumina system