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Diagnostic accuracy of a selected signature gene set that discriminates active pulmonary tuberculosis and other pulmonary diseases

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Summary *Objective:* We validated the accuracy of host selected signature gene set using unstimulated whole blood (WB), and peripheral blood mononuclear cells (PBMC) in the diagnosis of tuberculosis (TB).
Methods: The unstimulated WB and PBMC from 1417 individuals with active pulmonary TB patients, other lung diseases and healthy participants were analyzed using real time polymerase chain reaction (RT-PCR).
Results: The WB cohort test demonstrates that the combination of *GBP5* and *KLF2* can differentiate active TB versus HC with sensitivity and specificity of 77.8% and 87.1%, respectively; but most importantly active TB versus OD with sensitivity and specificity of 96.1% and 85.2%, respectively. Again during treatment course, the TB score of *GBP5* and *KLF2*, analytes secretion and clinical parameters were found to be associated in disease progression. In the PBMC cohort test, we found that the only and best discriminatory combination was *GBP5*, *DUSP3* and *KLF2* in

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the active TB versus HC with a sensitivity and specificity of 76.4% and 85.9%, respectively. *Conclusions:* Our study reveals that *GBP5* and *KLF2* may be useful as a diagnostic tool for active TB, also the two-gene set may serve as surrogate biomarkers for monitoring TB therapy.
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Introduction

Globally, tuberculosis (TB) now ranks as the leading cause of death among infectious diseases.[1] Since the discovery of *M. tuberculosis* by Robert Koch using light microscopy, development and implementation of TB diagnostics have not kept pace with either medical technology or the disastrous explosion of *M. tuberculosis* infection, including drug-resistant *M. tuberculosis* strains, especially in the wake of the human immunodeficiency virus (HIV) pandemic.[2] To date, the only rapid test for diagnosis of TB currently recommended by WHO is the Xpert® MTB/RIF assay, but, some concerns have been raised about the Xpert® MTB/RIF, including operational issues and cost. This leads most resource-limited and high-burden countries, to rely their TB diagnosis principally on sputum smear microscopy, which has limited sensitivity, especially among HIV-infected patients.[3–5] The sputum smear microscopy is usually followed by a culture-based diagnosis, which is relatively costly and takes weeks due to slow mycobacterial growth.[6,7] It is therefore important to note that, these many resource-limited settings lack also microbiology laboratories capability to perform culture-based diagnosis at high volume.[8,9] However, lack of rapid and cost-effective TB diagnosis increases the risks of transmission which may lead to increased TB case fatality.[10–12] Thus, a rapid, cost-effective and sensitive diagnostic test is the first step towards effective treatment of TB. The aim of this study was to expend and validate the ability of selected host genes to discriminate between active TB versus other diseases (OD) and healthy controls (HC) in whole blood (WB), and peripheral blood mononuclear cell (PBMC) using a real time-PCR (RT-PCR) assay. Transcriptional blood signatures for the diagnosis of TB have been described in several studies.[13–16] The gene set we chose for validation was previously identified by Timothy E Sweeney and colleagues[17] using meta-analysis study, where they analyzed published data that utilized whole blood transcriptional, and found that the three-gene set was robust for diagnosis of active TB. The set included genes for Guanylate Binding Protein 5 (*GBP5*), Dual Specificity Phosphatase 3 (*DUSP3*), and Krüppel-like Factor 2 (*KLF2*). Moreover, the performance of this three-gene set in unstimulated samples using RT-PCR has not yet been investigated in a larger sample size. Here, we show at least two-gene set (*GBP5* and *KLF2*) that allows high sensitivity and specificity in discriminating active TB from OD and HC.

Methods

Subject enrolment and inclusion criteria

In total, we recruited 1652 participants, among whom 235 were excluded for not meeting the study inclusion criteria, and the remaining 1417 were eligible for the WB cohort test (active TB n = 144; OD n = 209; HC n = 209), and PBMC cohort test (ATB n = 275; OD n = 290; HC n = 290) (Fig. 1). The study was approved by the Sun Yat-sen University research ethics committee prior to the commencement of the experiment. All subjects agreed and signed the consent form, and were eligible for the study if they were 18 years or older, willing to give written informed consent, including for HIV testing using a rapid test (Roche, USA); and met the following inclusion criteria for active TB: smear and culture confirmed *M. tuberculosis* in either sputum or broncho-alveolar lavage, with a specific positive for ELISPOT, tuberculin skin test (TST), purified protein derivative (PPD) or interferon-gamma release assays (IGRAs), and confirmed chest radiological TB findings (within 3 months of recruitment) according to the WASOG guidelines.[18] The OD participants were not positive to any of the above tests but showed clinical signs of pulmonary disease other than TB. In the OD group, the majority of subjects had pneumonia (58%); other subjects also included in this group were those with pulmonary sarcoidosis (31%), and lung cancer (11%) (Fig. 1). In order to meet the final criteria for study inclusion, the HC subjects had to be negative to all the above mentioned tests with no clinical evidence of TB or other diseases. The exclusion criteria for all recruited patients and healthy participants are listed in Fig. 1. All patients were recruited before initiation of anti-TB treatment unless otherwise stated. Moreover, 45 active pulmonary TB patients were followed up, where, clinical parameters and blood transcriptional profiles of untreated active TB patients were analyzed before (referred to as pre-treatment), during 4, 6, and 7 (also referred to as >6 months treatment) months treatment, n = 45/45 and were compared to healthy individuals (Fig. 1).

RNA isolation

Total RNA from WB and PBMC of active TB, OD patients, and HC was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's recommendation. The RNA concentration was detected at an optical density of 260/230 nm ratio ≥1.5 on a spectrophotometer (Nano Drop 2000C, Thermo scientific, USA). All extracted RNA samples were reverse transcribed into cDNA using the RevertAid™ First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Real Time PCR assay and gene validation

Each cDNA sample was amplified using the Bio-Rad CFX96 real-time detection system with SYBR Green reagent Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on the Step One Plus™ Real-Time PCR system (Applied Biosystem, Foster City, CA, USA). Briefly, the reaction consisted of 10 ng of cDNA and 5 μM of primers in a final reaction volume of 10 μl in SYBR Green mixture. Each reaction was initiated

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