Title: A novel whole blood miRNA signature for a rapid diagnosis of pulmonary tuberculosis

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Take home message
A novel, whole blood miRNA signature enables to rapidly diagnose TB with 91.21% sensitivity and 87.95% specificity.

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ABSTRACT (200 words maximum)

**Background:** Deregulation of microRNAs (miRNAs) has been associated with various human pathologies including bacterial infections.

**Aim:** To investigate the role of miRNAs as blood biomarkers for the diagnosis of active pulmonary tuberculosis (TB).

**Materials and methods:** We studied 50 patients, classified into 3 groups: (i) 17 individuals with latent tuberculosis infection (LTBI) (ii) 17 active pulmonary TB patients (iii) 16 healthy individuals. Blood was collected in a PAXgene Blood RNA tube for the later isolation of total RNA. MiRNA quantification was performed with SurePrint G3 8x60K miRNA microarrays (Agilent). Real time-quantitative polymerase chain reaction (RT-qPCR) with the miScript PCR System (Qiagen) was used to validate selected miRNA expression levels.

**Results:** MiRNA expression patterns were compared in whole blood from active TB patients, LTBI individuals and healthy controls. A set of miRNAs differentially deregulated during active pulmonary TB vs. LTBI and healthy controls was identified. We then selected nine miRNAs for validation in a larger patient cohort. The set of miRNAs consisting of miR-150, miR-21, miR-29c and miR-194 enabled to rapidly diagnose pulmonary TB with 91.21% sensitivity and 87.95% specificity.

**Conclusions:** The identified novel miRNA signature opens the possibility of developing a simple, rapid and cheap TB diagnostic test from blood.
INTRODUCTION

*Mycobacterium tuberculosis* (MTB) is a major microbial pathogen that threatens global health. The WHO has estimated about 8.6 million new tuberculosis (TB) cases and around 1.3 millions deaths due to TB in the year 2012 [1]. In MTB-infected individuals, bacteria may exist in a quiescent state for prolonged periods of time or may ultimately start multiplying and escape immune control, resulting in clinically active TB in up to 10% of infected cases. However, factors that promote progression from latent tuberculosis infection (LTBI) to disease are not completely understood [2].

One essential aspect for controlling the spread of TB is the ability to diagnose it in an early stage and to supply the adequate treatment. However, the commonly used test systems are still insufficient. For example, TB diagnosis by microscopy or tuberculin skin-testing (TST) has a variable and low sensitivity ranging from 20% to 60% for microscopy [3], and 67% to 80% for skin-testing [4], respectively. Tests based on MTB cultivation, the current gold standard of TB diagnosis, can take several weeks, requiring incubation periods of up to two months [5]. Moreover, the more recent interferon-gamma release assays (IGRAS) are not able to discriminate between active TB vs. LTBI [6-8]. Host biomarkers are therefore needed to diagnose TB. The ideal TB biomarker should provide prognostic information of pathogenesis and level of treatment success. Today, owing to the limited knowledge of promising TB biomarkers, global “omics” approaches are attractive options to follow [2].

MiRNAs are small non-coding RNAs (17-24 nucleotides in length) that regulate post-transcriptional gene expression by targeting the 3’ untranslated region of messenger RNA (mRNA). They have determinant roles controlling a wide range of biological functions as cell proliferation and differentiation, metabolism and apoptosis [9, 10]. Recent data demonstrate that miRNAs also modulate innate and adaptive immune responses against intracellular pathogens [11]. In addition, miRNAs can circulate in a cell-free stable form associated with microvesicles or protein complexes in body fluids like serum, plasma, saliva, urine, among
others [12]. These findings open the attractive possibility of using miRNAs as TB biomarkers for diagnosis and prognosis, and thereby avoiding time-consuming culture techniques or more invasive procedures like bronchial lavage analysis.

In the present study, we have investigated the role of miRNAs as blood biomarkers for active TB diagnosis. We compared different miRNA expression patterns in whole blood from active TB patients, individuals with LTBI and healthy controls by microarray. A set of miRNAs differentially deregulated during active pulmonary TB vs. LTBI and healthy controls was identified. We then selected most promising deregulated miRNAs and validated them by real time-quantitative polymerase chain reaction (RT-qPCR) in a larger patient cohort. From these we have identified several deregulated miRNAs that in combination could serve as a rapid diagnostic signature for pulmonary TB.

MATERIALS AND METHODS

Patients and inclusion criteria

Blood samples were collected from five different institutions located in Barcelona (Spain). Participating centres were Hospital Universitari Germans Trias i Pujol, Serveis Clítics, Unitat de Prevenció i Control de la Tuberculosis (CAP Drassanes) and Hospital del Mar. Blood was extracted using PAXgene Blood RNA tubes (PreAnalytiX). Written informed consent from all blood donors participating in the study was obtained. The corresponding Ethics Committees provided ethics approval. Demographical and clinical patient data was collected by a detailed questionnaire.

We prospectively enrolled a total of 50 patients, who were classified into three groups:

Group 1: 17 individuals with LTBI coming from contact tracing studies. The inclusion criteria for all patients were a positive TST, at least one positive result for one of the IFN-γ assays (T-SPOT.TB and/or QFN-G-IT) and no more than two weeks of chemoprophylaxis.
Group 2: 17 active pulmonary TB patients. The inclusion criteria were a positive culture for MTB and no more than two weeks of anti-TB therapy.

Group 3: 16 healthy individuals with negative TST and negative IFN-γ assay results (T-SPOT.TB and/or QFN-G-IT) who were identified by routine examinations.

The main demographic characteristics of the patients included in the study are summarized in Table 1.

**Tuberculin skin test**

Two intradermal tuberculin units of PPD RT23 (Statens Serum Institute, Copenhagen, Denmark) were used to perform the TST. The tuberculin was administered according to the Spanish Society of Pneumology guidelines [5]. A chest radiograph was taken from all individuals with positive TST indurations (≥5 mm). Chemoprophylaxis was given to asymptomatic patients with normal chest radiography while TB patients were treated with standard combination therapy.

**Interferon-gamma release assays (IGRAS)**

IGRAs rely on the detection of IFN-γ secreted by effector T cells after specific stimulation with MTB antigens (ESAT-6, CFP-10 and TB7.7). In this study, we used the commercial QuantiFERON-TB Gold In-Tube (Qiagen, Germany) and T-SPOT.TB (Oxford Immunotec, UK) assays. IGRAs were performed and interpreted according to the manufacturer’s instructions.

**RNA isolation and quality control**

A total of 2.5 ml of blood was extracted in PAXgene Blood RNA tubes (PreAnalytiX) that ensure stabilization of RNA and hence stabilization of the miRNA expression profiles. The PAXgene Blood miRNA kit (Qiagen) was used to isolate total RNA (>18 nucleotides including
miRNA). The protocol is from the manual of the PAXgene Blood miRNA kit. Isolated RNA was stored at -80°C until use.

Absorbance measurements at 260 and 280 nm were made on a spectrophotometer (NanoDrop 2000 Spectrophotometer, Thermo Scientific) in order to determine RNA concentration and purity. A ratio between 1.8-2.0 of absorbance at 260 nm and 280 nm corresponds to a sufficient RNA purity for subsequent microarray experiments. RNA integrity was determined with the Agilent RNA 6000 Pico kit (Agilent Technologies). Agilent's 2100 Bioanalyzer software estimated the integrity of total RNA samples (RIN). In general, a RIN value higher than 7-8 was accepted to work well for miRNA expression profiling by microarray.

**Identification of miRNA expression patterns by microarray hybridization**

We performed microarray analysis as previous described [13] using SurePrint G3 8 × 60K miRNA microarrays (Agilent, Cat No. G4870A) that contained 40 replicates of each of the 1,205 miRNAs of miRBase v16 (http://www.mirbase.org/ [14]). Microarray scan data were further processed using Feature Extraction software (Agilent).

**Real time-quantitative polymerase chain reaction (RT-qPCR) validation**

RT-qPCR was performed in nine selected miRNAs (hsa-miR-150, hsa-miR-18a, hsa-miR-194, hsa-miR-21, hsa-miR-223, hsa-miR-29c, hsa-miR-374a, hsa-miR-93* and hsa-miR-96) for validation and confirmation of microarray results. The miScript PCR System (Qiagen) was used. All procedures were performed according manufacture’s instructions and recommendations. First, using a total amount of 200 ng RNA, we selectively transcribed mature miRNAs into cDNA using the miScript HiSpec Buffer and the miScript II RT Kit. Second, RT-qPCR was performed with the miScript SYBR Green PCR Kit. We used the two endogenous controls, i.e., the snoRNA RNU48 (official symbol: SNORD48) and the snRNA RNU6B (official symbol: RNU6-2) for normalization of threshold cycle (Ct) values in samples.
All samples were run in duplicates. Fold-change values were computed by using the $2^{-\Delta\Delta C_{\text{t}}}$ method [15].

**Bioinformatic analysis**

Microarray data was log-transformed and quantile normalization was applied to normalize expression values using the freely available R software. MiRNA expression values were compared between different groups of patients by employing an unpaired two-tailed t-test. The fold-change calculated for each miRNA indicated down-regulation or up-regulation when the value was negative or positive, respectively. Area under the curve (AUC), fold-change and significance (p-value) were calculated for each miRNA. AUC values suggest how well a miRNA can separate between groups. This parameter indicates good discriminatory capacity when it is close to 1 or 0 values. Differences were considered significant when the p-value was lower than 0.05. In this study, no adjusted p-values were considered. Sensitivity, specificity, accuracy and positive predictive values (PPV) were calculated for active TB diagnosis using a Support Vector machines (SVM) with linear kernel and employing 20 repetitions of standard 10-fold cross validation.

**RESULTS**

**Analysis of blood-derived miRNA levels by microarray**

To identify miRNAs that are differentially expressed between TB patients, LTBI and healthy controls, blood samples from the 3 study groups were analysed by microarrays. For this first analysis, a total of 25 samples were tested representing 9 pulmonary active TB patients, 10 individuals with LTBI and 6 healthy controls. They were randomly picked from all available samples excluding patients with comorbidities (Table 1). Total RNA with length greater than 18 nucleotides was isolated and subjected to microarray-based quantification covering 1,205 different miRNAs. Overall, 188 miRNAs were expressed in all active TB patients, 199 in all LTBI individuals and 216 in all healthy controls. For 827 miRNAs, no expression was observed.
An unpaired two-tailed t-test was applied to evaluate statistical differences in miRNA expression between groups. For further analysis, miRNAs were selected by the following criteria, (i) an unadjusted p-value <0.05 for the comparisons, (ii) detection in all samples of at least one group of each comparison, and (iii) up-regulation or down-regulation with a fold-change >1.3. According to these criteria, a total of 26 miRNAs were deregulated in active TB patients with respect to healthy controls (TB vs. HC): 10 miRNAs were up-regulated and 16 miRNAs were down-regulated. MiRNA levels in the active TB group compared with controls were between 3.27-1.32 fold over-expressed and 2.02-1.31 fold under-expressed, respectively (Table 2). When comparing LTBI individuals with healthy controls (LTBI vs. HC), a total of 35 miRNAs were deregulated: 18 miRNAs were up-regulated and 17 miRNAs down-regulated. Interestingly, the comparison between TB vs. HC and LTBI vs. HC groups only provided a small overlap of 5 deregulated miRNAs: hsa-miR-21, hsa-let-7f-1*, hsa-miR-423-3p, hsa-miR-1275 and hsa-miR-505 (Figures 1 and 2) indicating a good separation between the two comparisons.

Validation of selected, differentially-expressed miRNAs by RT-qPCR

The following nine miRNAs were selected and further analyzed by RT-qPCR: hsa-miR-150, hsa-miR-18a, hsa-miR-194, hsa-miR-21, hsa-miR-223, hsa-miR-29c, hsa-miR-374a, hsa-miR-93* and hsa-miR-96. The selection criteria were (i) a strong fold-change deregulation and/or (ii) relevance as suggested in the literature. They were assessed with the samples from the complete study group covering 17 pulmonary active TB patients, 17 individuals with LTBI and 16 healthy controls. Five patients with active pulmonary TB presented comorbidities. Detailed characteristics are summarized in Table 1.

Fold-change values for the three comparisons TB vs. HC, LTBI vs. HC and TB vs. LTBI were calculated for each miRNA. Three of the nine miRNAs studied were significantly deregulated in active TB patients with respect to those with LTBI and healthy controls. These were hsa-miR-150 (down-regulation), hsa-miR-21 (up-regulation) and hsa-miR-29c (up-regulation;
nearly significant for TB vs. HC) with mean fold-change values higher than 1.5 (Figure 3). Furthermore, hsa-miR-194 was significantly up-regulated with a fold-change >1.5 in active TB patients with respect to LTBI; and hsa-miR-93* showed significant down-regulation in active TB patients in comparison with controls. These RT-qPCR results were not totally consistent with those obtained in the microarray-based analysis. While most of validated miRNAs showed the same direction of deregulation, in some occasions the microarray threshold of detecting expression differences seemed quite low. This enforces the need for RT-qPCR validation.

**Diagnostic test performance for active TB**

In order to define and evaluate a miRNA-signature for rapid pulmonary TB diagnosis, validated miRNAs with mean fold-change values >1.5, and a significant deregulation in the comparisons TB vs. LTBI and/or TB vs. HC were selected. These were hsa-miR-150, hsa-miR-21, hsa-miR-29c and hsa-miR-194 (Figure 3). We then applied a Support Vector Machine with linear kernel (20 repetitions of standard 10-fold cross validation) for computing the accuracy, the sensitivity, the specificity and positive predictive values (PPV) with the corresponding 95% confidence intervals. Interestingly, the selected miRNA-signature had a 90.1% accuracy (89.75 - 90.45%), a 91.21% sensitivity (90.8 - 91.62%), a 87.95% specificity (87.37 - 88.52%) and a 93.63% PPV (93.35 - 93.91%) for diagnosing active pulmonary TB (for comparison TB vs. LTBI+HC). The AUC value obtained was 0.068 indicating that this miRNA combination has a very good discriminatory capacity.

**DISCUSSION**

The development of a rapid assay for active TB diagnosis with increased sensitivity and specificity continues to be an important goal in clinical microbiology. Given the great success of recent miRNA-based diagnostics in various human pathologies [16] including bacterial infections [17], we have assessed in the present study the potential of miRNA patterns as biomarker to distinguish between active TB and LTBI. Whole blood from active TB patients,
LTBI individuals and healthy controls was first screened for differentially expressed miRNAs with a chip microarray. Promising deregulated miRNAs were then selected and validated by RT-qPCR. A signature of four miRNAs was identified and enabled to diagnose active pulmonary TB with 91.21% sensitivity and 87.95% specificity within a single working day.

Several studies aiming to identify deregulated miRNAs in active TB have been performed [18-30], however, only some of them addressed the issue of diagnostic performance [18, 21, 22, 27, 28]. The data of these studies revealed that the quantification of a single miRNA is not a reliable indicator of active TB as it was associated with a low sensitivity [18, 21]. Amongst the reasons is the neglect of miRNA expression differences between individuals. When miRNAs were evaluated in combination, then reasonable sensitivities and specificities were reached. For example, serum-derived miR-378, miR-483-5p, miR-22, miR-29c, miR-101 and miR-320b together discriminated active TB from healthy controls with 95% sensitivity and 91.8% specificity [28]. With a different, non-overlapping set of 15 miRNAs from serum, a comparable discriminatory capacity was observed [27]. Both study results are in the order of the test performance described here using just 4 miRNAs quantified from whole blood sampling.

It is intriguing to see that so different miRNA signatures are being proposed for the diagnosis of active TB. Clearly they must be related to one or several differences in study design and experimental details. Most studies were composed of 2 separate experimental steps, a primary miRNA screen and a subsequent result validation by RT-qPCR. Therefore, the resolution of the primary screen, for example a microarray platform or RNA sequencing, as well as the patient grouping influences the choice of miRNAs to be evaluated further and thus the final miRNA signature [31]. Furthermore, even when neglecting differences in reference gene selection or measures of RNA quality control, the choice of starting material for miRNA quantification like serum, individually separated blood cell populations or whole blood is expected to affect miRNA signatures. To take miR-29a as an example, Yi and co-
workers found a significant overexpression in serum and sputum of TB patients compared with healthy controls [23], while Kleinsteuber et al. observed it lower expressed in CD4+ T cells from active TB adults compared to LTBI individuals, as well as lower expressed in whole blood from children with active TB [24]. Likewise, in the present study we have observed an overexpression of miR-21 in TB vs. LTBI and healthy controls while Kleinsteuber et al. found it lower expressed [24]. This is suggestive of an important impact of the patient materials on the miRNA quantification results and calls for a need of assay standardization [12].

The observed link between a deregulated miRNA signature and active TB urges to better understand the underlying mechanisms. Previous studies from others already gave a functional hint for our 4 miRNAs. Ma and co-workers have described that miR-29 suppresses the immune response to intracellular pathogens by targeting IFN-γ mRNA [32]. Moreover, miR-29 also targets the mRNAs for Bcl-2 and Mcl-1. As these transcripts code for two central anti-apoptotic proteins, an upregulation of miR-29 may inactivate Bcl-2 and Mcl-1 mRNAs leading to an increased apoptosis of cells involved in the anti-TB response [33, 34]. Another upregulated miRNA of our study was miR-21. It is involved in promoting the production of the anti-inflammatory cytokine IL-10 [35] while inhibiting IL-12, a key cytokine involved in Th1 cell polarization. A consequence may be the reduction of host Th1 responses [36, 37] that are an important component of MTB control. The third overexpressed miRNA in the blood of active TB patients was miR-194 for which the possible relation to TB is less obvious. However, together with miR-29 it targets components of the Wnt signaling pathway that seems to play a role in TB pathogenesis [38, 39]. Finally, miR-150 is under-expressed in active TB. A primary target of this miRNA is c-Myb, a negative regulator of natural killer (NK) and invariant NK T (iNKT) cell maturation. Thus a reduction of mR-150 levels may indicate the development of fewer mature NK and iNKT cells which in turn represent early innate effector cells controlling invading pathogens [40]. Altogether, these findings are in line with the
hypothesis that the four deregulated miRNAs can create an immunologically favorable environment for MTB expansion and active TB development.

Limitations of our study need to be addressed. First, the number of patients included into our first miRNA analysis by microarray and subsequent RT-qPCR validation is limited and does not allow distinction between ethnic groups that may have an impact on the diagnostic performance of the determined miRNA signature. Nonetheless, the observed deregulated miRNAs are consistent with previous study results or immunological concepts on TB pathogenesis and thus strengthen the possible application of miRNAs as biomarkers for active TB diagnosis. Second, the mechanistic link between the observed miRNA signature and active TB remains hypothetical. However, the identified signature together with the functional role of the respective miRNAs studied by others directly suggests experiments to move forward in this matter.

Taken together, we here describe a novel, whole blood-derived miRNA signature consisting of miR-150, miR-21, miR-29c and miR-194 that enables to rapidly diagnose pulmonary TB with 91.21% sensitivity and 87.95% specificity. Despite these promising results, larger cohort studies are required to validate this specific signature against other pulmonary pathologies common in TB differential diagnosis. In addition, further work is required to evaluate whether the miRNA expression pattern can predict anti-TB therapy success in order to assess an adequate treatment. Finally, if an accurate differentiation between infection and disease could be obtained, it should be possible to build up a simple, rapid and cheap point-of-care test that is urgently needed even today.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

Irene Latorre, Jose Dominguez, Malú de Souza-Galvao, Jose Maldonado, Cristina Prat and Andreas Meyerhans are members of the European Tuberculosis Network (TB-NET) Group. The rest of investigators have no relevant financial interest in or a financial conflict with the subject matter or materials discussed in this manuscript.

REFERENCES


Table 1. Demographic characteristics of patients included in the study analyzed by microarray and/or RT-qPCR

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>Microarray</th>
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<th>RT-qPCR</th>
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<tbody>
<tr>
<td></td>
<td>LTBI n=10 (%)</td>
<td>Active TB n=9 (%)</td>
<td>Healthy controls n=6 (%)</td>
<td>LTBI n=17 (%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>6 (60)</td>
<td>5 (55.6)</td>
<td>2 (33.3)</td>
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<tr>
<td>Female</td>
<td>4 (40)</td>
<td>4 (44.4)</td>
<td>4 (66.7)</td>
<td>7 (41.2)</td>
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<tr>
<td>Age, mean±SD</td>
<td>37.7 ± 9.56</td>
<td>32.8 ± 17.33</td>
<td>30.17 ± 3.89</td>
<td>36.82 ± 8.77</td>
</tr>
<tr>
<td>Birth country</td>
<td></td>
<td></td>
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<tr>
<td>Immigrants from countries with high prevalence of TB infection</td>
<td>4 (40)</td>
<td>5 (55.6)</td>
<td>0 (0)</td>
<td>8 (47.1)</td>
</tr>
<tr>
<td>Residents in a non-epidemic TB country</td>
<td>6 (60)</td>
<td>4 (44.4)</td>
<td>6 (100)</td>
<td>9 (52.9)</td>
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<tr>
<td>Comorbidities</td>
<td>0 (0)</td>
<td>0 (0)</td>
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</tr>
</tbody>
</table>

SD: Standard deviation; RT-qPCR: Real time-quantitative polymerase chain reaction; TB: tuberculosis; LTBI: latent tuberculosis infection

* Five patients with active TB presented the following comorbidities:
  Chronic obstructive pulmonary disease (COPD) and lung tumor
  Hepatitis C virus (HCV) and hepatitis B virus (HBV)
  Follicular cyst
  Urothelial carcinoma
  Schizophrenia
Table 2. Up-regulated and down-regulated miRNAs in TB blood with an unadjusted p-value <0.05 for the comparisons of TB versus HC with a deregulated fold-change >1.3.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold-change TB vs. HC</th>
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<tr>
<td>hsa-miR-194</td>
<td>3.27</td>
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TB: tuberculosis; HC: Healthy controls