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

To the Editor:

One essential aspect for controlling the spread of tuberculosis (TB) is to diagnose it in an early stage. However, the commonly used test systems are still insufficient. Moreover, current assays perform poorly in discriminating between active TB and latent tuberculosis infection (LTBI). Due to limited knowledge of promising TB biomarkers, global “omics” approaches bear attractive options to follow [1]. MiRNAs are important post-transcriptional regulators shown to be involved in the modulation of immune responses against intracellular pathogens [2]. These findings open the possibility of using miRNAs as TB biomarkers for diagnosis. In the present study, we have investigated their role as blood biomarkers for detecting active TB.

Blood samples were collected from five different participating institutions located in Barcelona (Spain). We enrolled a total of 50 individuals, classified into three groups: 1) 17 LTBI individuals. They had been in close recent contact with a high exposure to a smear positive or negative pulmonary TB (pTB) patient. The inclusion criteria were a positive tuberculin skin test (TST), at least one positive result for one of the interferon (IFN)-γ assays and no more than 2 weeks of chemoprophylaxis. 2) 17 active pTB patients with a positive culture for Mycobacterium tuberculosis and no more than 2 weeks of anti-TB therapy. 3) 16 healthy individuals with negative TST and negative IFN-γ assay results. They were healthcare workers with no risk of TB exposure and recruited in the course of routine examinations that excluded comorbidities (further details on patient’s characteristics can be provided by the authors upon request).

Written informed consent was obtained from all study participants. The corresponding Ethics Committees provided ethics approval. A total of 2.5 mL of blood was extracted in PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland). Total RNA was isolated with a PAXgene Blood miRNA kit (Qiagen, Hilden, Germany). Microarray analysis was performed as described [3] using SurePrint G3 8×60 K miRNA microarrays (Agilent Technologies, Santa Clara, California). Microarray results were validated by RT-qPCR using miScript PCR System (Qiagen).

To identify miRNAs differentially expressed between the three study groups, blood samples were first analysed by microarrays. Differentially deregulated miRNAs in active TB and LTBI individuals with respect to healthy controls (TB versus HC and LTBI versus HC) are detailed in fig. 1a. MiRNA levels in the active TB group compared with controls were 3.27–1.32-fold over-expressed and 2.02–1.31-fold under-expressed, respectively.

Nine selected differentially expressed miRNAs were further analysed by RT-qPCR. Three miRNAs were significantly deregulated in active TB patients with respect to those with LTBI and healthy controls: hsa-miR-150 (down-regulation), hsa-miR-21 (up-regulation) and hsa-miR-29c (up-regulation) (fig. 1b). While most of validated miRNAs showed the same direction of deregulation as in the microarray-based analysis, in some occasions the microarray threshold of detecting expression differences seemed quite low. This enforces the need for RT-qPCR validation of microarray results.

In order to define a miRNA-signature for rapid pTB diagnosis, validated miRNAs with mean fold-change values >1.5, and a significant deregulation in the comparisons TB versus LTBI and/or TB versus HC were selected. These were hsa-miR-150, hsa-miR-21, hsa-miR-29c and hsa-miR-194. We then applied a support vector machine with linear kernel (20 repetitions of standard 10-fold cross validation) for computing the accuracy, sensitivity, 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 pTB (comparison TB versus LTBI+HC).

Several studies aiming to identify deregulated miRNAs in active TB have been performed; however, only some of them addressed the issue of diagnostic performance [4–8]. The data of these studies revealed that the quantification of a single miRNA is not a reliable indicator of active TB [4, 5]. 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 [7, 8]. These latter studies using available samples excluding patients with comorbidities. b) Relative fold-change of miRNA transcripts analysed by RT-qPCR from different patient groups and controls. Nine selected miRNAs were analysed by RT-qPCR with the following selection criteria: i) a strong fold-change deregulation and/or ii) relevance as suggested in the literature. Data represents the mean fold-change of each miRNA validated by RT-qPCR for TB versus HC (white bars), LTBI versus HC (black bars) and TB versus LTBI (grey bars). The fold-change calculated for each miRNA indicates downregulation or upregulation when the value is negative or positive, respectively. We used two endogenous controls for normalisation of threshold cycle (Ct) values in samples (snoRNA RNU48 (official symbol: SNORD48) and snRNA RNU6B (official symbol: RNU6-2)). All samples were run in duplicates. Fold-change values were computed by using the $\Delta\Delta C_{\text{T}}$ method. Validation of differentially-expressed miRNAs by RT-qPCR was assessed with the samples from the complete study group covering 17 pulmonary active TB patients, 17 individuals with LTBI and 16 healthy controls. They were randomly picked from all available samples excluding patients with comorbidities.

It is intriguing to see that different miRNA signatures are being proposed for the diagnosis of active TB. Most studies were composed of two 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 [9]. Furthermore, the choice of starting material for miRNA quantification like serum, individually separated blood cell populations or whole blood is expected to affect miRNA signatures. The observed link between our deregulated miRNA signature and active TB urges to better understand the underlying mechanisms. Ma et al. [10] have described that miR-29 suppresses the immune response to intracellular pathogens by targeting IFN-γ mRNA. The upregulated miR-21 is involved in the reduction of host T-helper (Th) 1 responses [11]. The third overexpressed miRNA was miR-194. This miRNA together with miR-29 targets components of the Wnt signalling pathway that seems to play a role in TB pathogenesis [12, 13]. Finally, miR-150 is under-expressed in active TB. Its primary target is a negative regulator of natural killer (NK) cell maturation. Thus a reduction of miR-150 levels may indicate the development of fewer mature NK cells which are early innate effector cells controlling invading pathogens [14]. Altogether, these findings are in line with the hypothesis that the four deregulated miRNAs can create an immunologically favourable environment for M. tuberculosis expansion.

Limitations of our study need to be addressed. First, the number of patients included is limited and does not allow distinction between ethnic groups. Nonetheless, the newly observed deregulated miRNAs are consistent with previous study results or immunological concepts on TB pathogenesis. 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 directly suggests experiments to move forward in this matter. Third, blood count differences between groups may impact miRNA expression profiles. However, statistical evaluation of such influences reveals that they contribute only partly to a miRNA profile and that they do not significantly affect the feasibility to correlate a miRNA signature with a disease phenotype in humans [15].

Taken together, we here describe a novel, whole blood-derived miRNA signature that enables rapid diagnosis of pTB with 91.21% sensitivity and 87.95% specificity. Larger cohort studies are required to validate this signature in extrapulmonary TB cases and other pulmonary pathologies common in TB differential diagnosis. Finally, if the accurate differentiation between infection and disease is validated, it should be possible to build up a simple, rapid and cheap point-of-care test that is urgently needed even today.

A novel, whole-blood miRNA signature enables to rapidly diagnose TB with 91.21% sensitivity and 87.95% specificity

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