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Original Article

Biomarkers Can Identify Pulmonary Tuberculosis in HIV-infected Drug Users Months Prior to Clinical Diagnosis

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ABSTRACT

Background: Current diagnostic tests cannot identify which infected individuals are at risk for progression to tuberculosis (TB). Our aim was to identify biomarkers which can predict the development of TB prior to clinical diagnosis.
Method: In a retrospective case–control study, RNA of 14 HIV-infected drug users obtained before TB diagnosis (cases) and of 15 who did not develop TB (controls) was analyzed for the expression of 141 genes by dcRT-MLPA followed by Lasso regression analysis.
Findings: A combined analysis of IL13 and AIRE had the highest discriminatory power to identify cases up to 8 months prior to clinical diagnosis. Cases expressing IL13 had a gene expression pattern strongly enriched for type I IFN related signaling genes, suggesting that these genes represent processes that contribute to TB pathogenesis.
Interpretation: We here demonstrated that biomarkers, such as IL13-AIRE, can identify individuals that progress to TB within a high risk population, months prior to clinical diagnosis.
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1. Introduction

The WHO estimates that there were 8.6 million cases of tuberculosis and 1.3 million deaths due to tuberculosis (TB) in 2012 (World Health Organization (WHO): Global Tuberculosis Report (2013)). Infection with *Mycobacterium tuberculosis* (*Mtb*) has an unpredictable outcome. The proportion of individuals who truly remain infected after tuberculin skin test (TST) or interferon gamma release assay (IGRA) conversion is unknown (Mack et al., 2009). An estimated 5–10% of infected individuals will develop pulmonary TB, mostly within the first 2 years following infection (Comstock et al., 1974; Vynnycky and Fine, 2000). At present it is impossible to predict who will progress to disease and who will remain latently infected.

Biomarkers (characteristics that can be objectively measured as indicators of a pathogenic process) (Biomarkers Definitions Working Group, 2001) have the potential to contribute significantly in the battle against *Mtb*. The vast majority of *Mtb*-infected individuals remain healthy, suggesting that immune responses in individuals that control latent infection with *Mtb* throughout their lifetime differ from responses in those who develop TB within the first years following infection (Weiner and Kaufmann, 2014; Schuetz et al., 2011). If true, correlates of TB progression should be identifiable, and these could be valuable in early identification of individuals at risk of developing – and transmitting – TB (Ottenhoff et al., 2012a). Furthermore, in low incidence countries biomarkers predictive of TB progression would be useful in selecting high-risk individuals for preventive therapy or regular screening. Examples are individuals with known or frequent exposure, such as contacts of infectious pulmonary TB patients or drug users. Likewise, TB-HIV co-infected individuals have an increased risk of progression to TB, reactivation of latent TB infection (LTBI) and TB related mortality (Nunn et al., 2005). Determination of such biomarkers could be embedded in existing programs in the Netherlands, in which HIV-infected individuals are actively screened for TB.

Several studies have identified host gene expression patterns in the blood from patients with pulmonary TB that differ from patterns in latently infected individuals or patients with other inflammatory

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conditions (Berry et al., 2010; Maertzdorf et al., 2011a,b, 2012; Ottenhoff et al., 2012b; Cliff et al., 2013). Individual studies have mostly pointed towards type I interferon (IFN) signaling components as key markers of pulmonary TB, however, analysis of combined data indicated that besides type I IFN signaling also myeloid cell activation, general inflammation and B cell related markers are important players during TB (Joosten et al., 2013). To date no studies have identified biomarkers that can predict disease development well before the onset of clinical symptoms, and thus before the onset of contagious disease. The aim of this study was to explore whether biomarkers exist that may predict clinical TB. We used peripheral blood mononuclear cell (PBMC) samples of HIV-infected drug users, participating in a unique cohort, the Amsterdam Cohort Study (ACS), which undertook regular blood sampling and storage over several decades, independent from TB symptoms or diagnosis and thus allowed retrospective selection of samples prior to TB diagnosis.

2. Materials & Methods

2.1. Study Population

The Amsterdam Cohort Study (ACS) among drug users is an open and ongoing prospective cohort study, initiated in 1985 in Amsterdam, the Netherlands (van den Hoek et al., 1988). The ACS was approved by the medical ethics committee of the Academic Medical Center of Amsterdam. Regular users of hard drugs, such as heroin, cocaine or amphetamines, at least 3 times per week, were included in the ACS. Enrolment is voluntary and written informed consent was obtained from every participant at intake visit. Participants were asked to return for follow-up visits every 4 to 6 months at the Public Health Service (PHS). At every cohort visit, blood was drawn for laboratory testing and storage. Blood specimens have been tested for HIV-1 antibodies using consecutive generations of commercially available screening assays (obtained from Abbott Laboratories, Abbott Park, Illinois, USA; Organon International, The Netherlands, and bioMérieux, France) and confirmed by Western blot analyses (Genelabs Diagnostics, Singapore). Most of the participating drug users were also taking part in the methadone substitution program of the PHS Amsterdam. Methadone clients participating in the ACS were screened for tuberculosis at the TB department of the PHS Amsterdam by chest radiograph at six month intervals. A TB diagnosis was made by specialized TB clinicians, based on symptoms, chest radiograph, sputum smear and culture results, and/or by clinical response to treatment, based on national guidelines (KNCV Tuberculosis Foundation, 2008). In this cohort HIV infection increased the risk of tuberculosis 13-fold (Keizer et al., 2000). Since PBMCs were stored prospectively over decades, samples prior to clinical diagnosis of TB were available, and could be used to study gene expression profiles before the onset of TB.

2.2. Sample Selection

Twenty HIV-infected drug users diagnosed with TB in the period 1985–2012 were selected as cases using the ACS cohort database. A requirement was that at least one stored PBMC sample was available in the sample repository in the 365-day period preceding the date of the TB diagnosis. We preferred to select samples taken about 6 months before TB diagnosis, but we were limited in the number of samples available prior to TB diagnosis, as many samples from this cohort were already used for other research purposes. All TB cases with samples collected >180 days before diagnosis were invited for at least 1 ACS cohort follow-up visit after the point of sample collection. Thirty HIV-infected drug users who were not diagnosed with TB in the same cohort period were selected as controls. The controls were frequency-matched for age, gender and CD4 cell count. At time of analysis, all controls were at least 4 years passed the PBMC collection time point and were not diagnosed with TB so far.

We checked the clinical files of the TB department to obtain details on TB diagnosis. In the process it was found that one case was diagnosed with LTBI (and not TB) at a time point shortly after sample collection; he developed TB 8 years later. Therefore this person was excluded from the analysis. Among the remaining 49 samples, from 29 (14 cases and 15 controls) sufficient good quality RNA was isolated for analysis. The 29 participants with sufficient RNA in their samples did not differ significantly from the other 20, in terms of age, gender, CD4 cell count, calendar period of sample, and TB status (case or control). Among the 29 included participants, cases did not differ significantly from controls in these respects (Table 1). The majority of cases were classified as PTB (57%) or PTB + EPTB (29%) and diagnosis was based on culture (86%) or clinical response to treatment (14%) (Table 1). There were no previous episodes of TB reported for any of the participants, cases or controls.

2.3. RNA Extraction

PBMCs were thawed using 50% FCS (Greiner Bio-One, Alphen aan den Rijn, the Netherlands), washed in PBS and the pellet was directly dissolved in 1 ml Trizol (Invitrogen, Thermo Fisher Scientific Inc., Merelbeke, Belgium). Total RNA was extracted according to standard protocols recommended by the manufacturer. RNA was quantified using a Nanodrop ND-1000 spectrophotometer and diluted to 50 ng/μl for use in dcRT-MLPA.

2.4. dcRT-MLPA Assay, Data Analysis and Quality Control

A dual-color reverse transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) assay was performed as described previously (Joosten et al., 2012). Briefly, for each target-specific sequence, a specific RT primer was designed, located immediately downstream of the left and right hand half-probe target sequence. Following reverse transcription of 125 ng RNA using MMLV reverse transcriptase (Promega, Leiden, the Netherlands), left and right hand half-probes were hybridized to the cDNA at 60 °C overnight. Annealed half-probes were ligated and subsequently amplified by PCR (33 cycles of 30 s at 95 °C, 30 s at 58 °C and 60 s at 72 °C, followed by 1 cycle of 20 min at 72 °C). Primers and probes were from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands) (Joosten et al., 2012; Geluk et al., 2014) and MLPA reagents from MRC-Holland (Amsterdam, the Netherlands). PCR amplification products were 1:10 diluted in HiDi formamide containing 400HD ROX size standard and analyzed on an Applied Biosystems 3730 capillary sequencer in GeneScan mode (BaseClear, Leiden, the Netherlands).

Trace data were analyzed using the GeneMapper software package (Applied Biosystems). Signals below the threshold value for noise cut-off in GeneMapper (peak area below $\log^2(200) = 7.64$) were assigned the threshold value for noise cut-off. Subsequently, results from target genes were normalized to the average signal of housekeeping gene GAPDH and assigned the threshold value if below 7.64.

2.5. Statistical Analysis

Gene expression levels among cases and controls were plotted after $\log^2$ normalization and differences between cases and controls were compared using Mann–Whitney U testing; differences in expression were considered significant when $P < 0.05$. P-values for each individual comparison were corrected for multiple testing using the Benjamini–Hochberg adjustment (Benjamini and Hochberg, 1995). All gene expression data obtained using dcRT-MLPA were analyzed using Lasso regression analysis to identify the combined biomarkers with the highest discriminative power between cases and controls (Tibshirani, 1996). Lasso regression analysis is a shrinkage and selection method for linear

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