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Analysis of Factors Lowering Sensitivity of Interferon- γ Release Assay for Tuberculosis

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Abstract

Background: Imperfect sensitivity of interferon- γ release assay (IGRA) is a potential problem to detect tuberculosis. We made a thorough investigation of the factors that can lead to false negativity of IGRA.

Methods: We recruited 543 patients with new smear-positive pulmonary tuberculosis in Hanoi, Viet Nam. At diagnosis, peripheral blood was collected and IGRA (QuantIFERON-TB Gold In-Tube) was performed. Clinical and epidemiological information of the host and pathogen was collected. The test sensitivity was calculated and factors negatively influencing IGRA results were evaluated using a logistic regression model in 504 patients with culture-confirmed pulmonary tuberculosis.

Results: The overall sensitivity of IGRA was 92.3% (95% CI, 89.6%–94.4%). The proportions of IGRA-negative and indeterminate results were 4.8% (95% CI, 3.1%–7.0%) and 3.0% (95% CI, 1.7%–4.9%). Age increased by year, body mass index <16.0, HIV co-infection and the increased number of HLA-DRB1*0701 allele that patients bear showed significant associations with IGRA negativity (OR = 1.04 [95% CI, 1.01–1.07], 5.42 [1.48–19.79], 6.38 [1.78–22.92] and 5.09 [2.31–11.22], respectively). HIV co-infection and the same HLA allele were also associated with indeterminate results (OR = 99.59 [95% CI, 15.58–625.61] and 4.25 [1.27–14.16]).

Conclusions: Aging, emaciation, HIV co-infection and HLA genotype affected IGRA results. Assessment of these factors might contribute to a better understanding of the assay.

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Introduction

Tuberculosis (TB) remains a disease of serious concern; one third of the global population is infected with *Mycobacterium tuberculosis* (MTB) and eight to ten million people develop the disease every year [1]. The primary step to control TB is detecting infection by a sensitive test.

Recently, an immunoassay that measures interferon (IFN)- γ response to MTB-specific antigens (interferon- γ release assay; IGRA) has been developed. Studies on the use of IGRA in patients with active TB have had two purposes: (1) to evaluate performance of IGRA in latent TB infection (LTBI) using active TB as a surrogate, and (2) to determine whether IGRA plays a supplementary role in the exclusion of active TB disease in optimal setting [2–4].

IGRA use in diagnosis of LTBI has been established and supported by European and American guidelines [5,6], whereas its use has not been recommended to rule out active disease particularly in high-burden countries, because of low sensitivity and low negative predictive values [7,8]. Consequently, so far the sensitivity of IGRA varies from 64% to 92% [3], but the number of reports from high-burden countries is limited.

Imperfect sensitivity is a potential problem when using this assay to exclude LTBI as well as active TB. Due to the lack of a gold standard for LTBI identification, mechanisms by which IGRA gives false-negative results in LTBI are largely unknown [2,3]. Identification and characterization of factors that lower the test sensitivity, by using active TB patients as a surrogate for LTBI suspects, would delineate active TB-disease specific and non-specific mechanisms that underlie false negative results of IGRA.

At present, however, there is no comprehensive report on relevant factors including extent of TB lesions, malnutrition, aging, HIV co-infection, and MTB strains. Inherent genetic variations are also candidate factors affecting IGRA results. Among these, polymorphism of human leukocyte antigen (HLA) is classically known to influence T-cell immune response and determines IFN- γ concentrations after stimulation with MTB antigens [9]. In this study, we thus attempted to investigate host- and pathogen-related factors that may influence IGRA results obtained from more than 500 patients with active TB in Viet Nam.

Methods

Ethics statement

A written informed consent was obtained from each participant. The study was approved by ethical committees of the Ministry of Health, Viet Nam and National Center for Global Health and Medicine, Japan respectively.

Study population

This study is a part of our prospective study on active TB in Hanoi. After signing informed consents, 543 unrelated patients with smear-positive pulmonary TB, equal to or more than 16 years of age, and without history of TB treatment, entered this study from July 2007 to March 2009. Information of no previous TB treatment was based on self-declaration of patients and documents in district TB centers.

All had sputum smear-positive TB. Solid MTB culture on Löwenstein-Jensen media was available in 98.2% and confirmed the diagnosis in 504 patients (92.8%). The sensitivity and risk-factor analysis was made in these culture-confirmed pulmonary TB cases, although clinicians diagnosed all 543 patients as active pulmonary TB and treated them with anti-TB drugs based on the guidelines of the national TB program. Spoligotyping was used to distinguish MTB genotypes including Beijing strains [10]. At diagnosis before anti-TB treatment, the peripheral blood was drawn for testing complete blood count, HIV, IGRA and HLA genotyping. After 2 months of treatment, IGRA was tested again. Chest X-ray films were interpreted by two readers independently of IGRA results.

IGRA

In this study, ELISA-based IGRA, QuantiFERON-TB Gold In-TubeTM (QFT-IT) (Cellestis, Victoria, Australia), was used [11]. The algorithm and software (QuantiFERON-TB Gold Analysis Software, version 2.50, Cellestis) provided by the manufacturer were strictly followed for interpretation of the results [11]. The testing procedure was carefully monitored [12] and quality control of the test was done in each run, following the manufacturer's instructions. For analysis of IFN- γ values higher than 10.00 IU/ml, the truncated value (10.00 IU/ml) was used as indicated in the current software.

HLA typing

Genomic DNA was extracted from the whole blood by using the QIAampTM DNA Blood Midi Kit (QIAGEN Sciences, Germantown, MD, USA). DNA-based HLA typing was performed by Luminex Multi-Analyte Profiling system (xMAP) with WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) as described [13]. Briefly, highly polymorphic exon 2 of HLA-DRB1 and -DQB1 genes were amplified. Each PCR product was hybridized with sequence-specific oligonucleotide probes, complementary to the allele-specific sequences.

Linkage disequilibrium analysis and binding peptide prediction for HLA alleles

Haploview version 4.2 (Broad Institute, Cambridge, MA) was used to calculate indicators of linkage disequilibrium, D' and r^2 , between HLA-DRB1 and -DQB1 alleles [14,15].

To predict peptides in the protein sequence of ESAT-6, CFP10 and TB7.7 capable of binding to a given HLA-DRB1 allele *in silico*, we used the ProPred database [16,17] with a threshold of 3%, a recommended setting.

Statistical analysis

Factors negatively influencing IGRA results were initially screened by univariate analysis and then further investigated by multivariate analysis using a polytomous logistic regression model, with IGRA-negative and -indeterminate results as outcome variables and factors that may be involved in host immunity and disease as independent variables. Another logistic regression model using a dichotomous outcome variable, non-positive (negative and indeterminate) versus positive results, was also tested. Odd ratio (OR) and 95% confidential interval (CI) were thus calculated. HLA candidate alleles were initially screened by comparison of allele frequencies between IGRA-negative and -positive groups, and then further investigated by the logistic regression model mentioned above.

Fisher's exact test was used to detect associations. Bonferroni's correction was applied to correct multiple comparisons of association with HLA alleles. Distribution of IFN- γ values was represented by using median with interquartile range (IQR). When a value was higher than 10.00 IU/ml, truncated values (10.00 IU/ml) were presented and a quantile value based on extrapolation was supplied only as parenthetical. Wilcoxon rank-sum test and Kruskal-Wallis test were used to compare non-parametric distribution of two groups and more than two groups, respectively. P value <0.05 was considered to be statistically significant, unless otherwise specified. Statistical analysis was performed using Stata version 10 (StataCorp, College Station, TX).

Results

Characteristic of the study population

Data including QFT-IT results were analyzed in 504 new patients with culture-confirmed pulmonary TB. The median age was 38.8, the proportion of male patients was 79.2%, and HIV was positive in 8.7% of the cases. Body mass index (BMI) showed that more than 50% of the patients were underweight, following the categorization on the basis of international guidelines [18] (table 1).

QFT-IT results

In 504 patients tested, the overall sensitivity of QFT-IT was 92.3% (95% CI, 89.6%–94.4%), but decreased to 61.4% (95% CI, 45.5%–75.6%) in HIV-infected patients (table 2). The proportions of QFT-IT-negative and -indeterminate results were 4.8% (95% CI, 3.1%–7.0%) and 3.0% (95% CI, 1.7%–4.9%) respectively. All of the 15 indeterminate cases had low response to phytohaemagglutinin (PHA, or mitogen) and TB-Ag after subtracting Nil value (TBAg-Nil) (0.20 [IQR, 0.04–0.34] IU/ml and 0.03 [IQR, 0.01–0.06] IU/ml, respectively). Compared with the patients who had test-positive results, those with negative results were significantly older (median age: 48.9 [IQR, 33.2–62.6] vs 39.0 [IQR, 29.1–50.6], $P=0.036$), and had significantly lower BMI (median BMI: 16.6 [IQR, 13.9–17.9] vs 18.3 [16.9–19.7] kg/m², $P=0.0001$) (table not shown).

Table 1. Characteristics of patients with smear-positive/culture-positive pulmonary tuberculosis (n = 504).

		Number	%
Age (years old) (median, IQR)		(38.8,	29.2–50.8)
Sex	Male	399	79.2
	Female	105	20.8
Body mass index	<16.0	77	15.3
	16.0–18.4	206	40.9
	18.5–24.9	218	43.2
	≥25.0	3	0.6
Smoking habit	Smoker	199	39.5
	Ex-smoker	136	27.0
	Non-smoker	168	33.3
	No answer	1	0.2
Underlying disease other than HIV*	None	435	86.3
	One	61	12.1
	More than one	8	1.6
HIV status	Positive	44	8.7
	Negative	459	91.1
	Not available	1	0.2
Lymphocyte count	≥1,000/mm ³	442	87.7
	<1,000/mm ³	60	11.9
	Not available	2	0.4

IQR: inter-quartile range, HIV: human immunodeficiency virus; TB: Tuberculosis; *Includes diabetes mellitus, gastrectomy, gastric ulcer, renal failure and gout (hyperuricemia).

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HLA-DRB1 and HLA-DQB1 alleles and IFN- γ responses

Since QFT-IT is based on T-cell response to MTB-specific antigenic peptides that are presented with MHC class II molecules, we investigated the role of HLA-DRB1 and -DQB1 alleles. Among seven most common HLA-DRB1 and -DQB1 alleles tested in the population [13], the allele frequencies of HLA-DRB1*0701 and DQB1*0201 in the test-negative group were significantly higher than that of the positive group ($P < 0.0001$ and $P = 0.001$, respectively, which remained significant after Bonferroni's correction) (table 3).

Nonrandom association between HLA-DRB1*0701 and DQB1*0202 was tested and HLA-DRB1*0701 was found to be in moderate linkage disequilibrium (LD) with HLA-DQB1*0201 allele ($D' = 0.608$ and $r^2 = 0.235$) (table not shown). The TB_{Ag}-Nil values of IFN- γ in the HLA-DRB1*0701-negative/DQB1*0201-

positive group were not significantly lower than those in the both negative group (6.65 IU/ml [IQR, 2.85–10.00 (16.06)] vs 7.76 IU/ml [IQR, 2.58–10.00 (14.87)], $P = 0.989$), whereas the IFN- γ values in the HLA-DRB1*0701-positive/DQB1*0201-negative group was significantly lower than those in the both negative group (2.30 IU/ml [IQR, 1.22–4.44] vs 7.76 IU/ml [IQR, 2.58–10.00 (14.87)], $P < 0.001$) (table not shown).

TB_{Ag}-Nil values of all four patients with two HLA-DRB1*0701 alleles (homozygous for HLA-DRB1*0701) were below 0.35 IU/ml or the cutoff value; three negative, one indeterminate and none had positive results. In patients with one HLA-DRB1*0701 allele (heterozygous for HLA-DRB1*0701), proportions of negative, indeterminate and positive results were 9.4% (5/53), 7.6% (4/53) and 83.0% (44/53). In patients with no HLA-DRB1*0701 alleles (homozygous for non-HLA-DRB1*0701), the proportions were 3.6% (16/447), 2.2% (10/447) and 94.2% (421/447) respectively. Overall distribution of QFT-IT results was significantly different among HLA-DRB1*0701 genotypes ($P < 0.0001$). The effect of two HLA-DRB1*0701 alleles on QFT-IT negativity was significant (3/4 vs 16/447, $P = 0.0002$), whereas the effect of one HLA-DRB1*0701 allele on QFT-IT negativity was weaker than that of two HLA-DRB1*0701 alleles (5/53 vs 3/4, $P = 0.007$) and did not reach significant levels (5/53 vs 16/447, $P = 0.06$) when "no alleles" was regarded as a category for reference purposes.

Distribution of IFN- γ values may provide information about the mechanism by which false negative results are observed. We reviewed the relationship between IFN- γ values and HLA-DRB1*0701 genotypes (Figure 1). HLA-DRB1*0701 genotype significantly affected TB_{Ag} specific IFN- γ response (TB_{Ag}-Nil) ($P < 0.001$): The IFN- γ values in patients with two HLA-DRB1*0701 alleles (homozygous for HLA-DRB1*0701) were significantly lower than those in patients with one HLA-DRB1*0701 allele (heterozygous for HLA-DRB1*0701) (0.15 IU/ml [IQR, 0.06–0.26] vs 1.91 IU/ml [IQR, 0.65–4.21], $P = 0.008$). As a reference, the median of IFN- γ values in patients who did not bear any HLA-DRB1*0701 alleles was 7.59 IU/ml [IQR, 2.63–10.00 (14.92)].

No association was clinically observed between HLA-DRB1*0701-containing genotype and disease severity assessed by either cavity or infiltrate on CXR respectively (data not shown). QFT-IT test was performed again after two months of anti-TB treatment in 17 out of 19 HIV-negative patients with QFT-IT-negative results. All 7 patients who carried one or two HLA-DRB1*0701 alleles showed negative results again, whereas it remained negative only in 6 out of 10 patients without carrying the HLA allele, though this difference did not reach significant levels ($P = 0.103$).

Analysis of 51 HLA-DR alleles registered on the ProPred database revealed that the average number of epitopes predicted in the overall amino acid sequences of ESAT-6 (95 amino acids),

Table 2. QFT-IT results and HIV status in smear-positive/culture-positive pulmonary TB patients.

	Positive		Negative		Indeterminate		p*
	n	% (95% CI)	n	% (95% CI)	n	% (95% CI)	
All (n = 504)	465	92.3 (89.6–94.4)	24	4.8 (3.1–7.0)	15	3.0 (1.7–4.9)	
HIV positive (n = 44)	27	61.4 (45.5–75.6)	5	11.4 (3.8–24.6)	12	27.3 (15.0–42.8)	<0.001
HIV negative (n = 459)	437	95.2 (92.8–97.0)	19	4.1 (2.5–6.4)	3	0.7 (0.1–1.9)	

QFT-IT: QuantiFERON-TB Gold In-Tube; HIV: human immunodeficiency virus; TB: Tuberculosis; CI: Confidence interval.

*Comparison was made between HIV-positive and HIV-negative groups.

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Table 3. Frequencies of HLA class II alleles and QFT-IT positive/negative results.

Allele name	Number of alleles (% [95%CI])			p*
	Total	IGRA positive	IGRA-negative	
	2n‡ = 1008	2n = 930	2n = 48	
HLA-DRB1				
1202	337 (33.4 [30.5–36.4])	318 (34.2 [31.1–37.3])	11 (22.9 [12.0–37.3])	0.119
0901	129 (12.8 [10.8–15.0])	119 (12.8 [10.7–15.1])	6 (12.5 [4.7–25.2])	>0.999
0701	61 (6.1 [4.7–7.7])	44 (4.7 [3.5–6.3])	11 (22.9 [12.0–37.3])	<0.0001
1502	59 (5.9 [4.5–7.5])	53 (5.7 [4.3–7.4])	5 (10.4 [3.5–22.7])	0.198
0301	54 (5.4 [4.0–6.9])	49 (5.3 [3.9–6.9])	4 (8.3 [2.3–20.0])	0.324
0803	53 (5.3 [4.0–6.8])	51 (5.5 [4.1–7.1])	0 (0.0 [0.0–7.4])	0.170
1001	51 (5.1 [3.8–6.6])	49 (5.3 [3.9–6.9])	2 (4.2 [0.5–14.3])	>0.999
others	264 (26.2 [23.5–29.0])	247 (26.6 [23.7–29.5])	9 (18.8 [8.9–32.6])	0.312
HLA-DQB1				
0301	383 (38.0 [35.0–41.1])	361 (38.8 [35.7–42.0])	12 (25.0 [13.6–39.6])	0.067
0303	152 (15.1 [12.9–17.4])	138 (14.8 [12.6–17.3])	9 (18.8 [8.9–32.6])	0.414
0501	94 (9.3 [7.6–11.3])	87 (9.4 [7.6–11.4])	6 (12.5 [4.7–25.2])	0.448
0201	92 (9.1 [7.4–11.1])	74 (8.0 [6.3–9.9])	12 (25.0 [13.6–39.6])	0.001
0502	81 (8.0 [6.4–9.9])	76 (8.2 [6.5–10.1])	3 (6.3 [1.3–17.2])	>0.999
0601	70 (6.9 [5.5–8.7])	68 (7.3 [5.7–9.2])	0 (0.0 [0.0–7.4])	0.072
0401	42 (4.2 [3.0–5.6])	40 (4.3 [3.1–5.8])	1 (2.1 [0.1–11.1])	0.717
others	94 (9.3 [7.6–11.3])	86 (9.2 [7.5–11.3])	5 (10.4 [3.5–22.7])	0.797

*Comparison was made between QFT-IT-positive and -negative groups. After Bonferroni's correction, $P < 0.006$ was statistically significant, considering the number of comparisons.

‡Allele number is shown.

HLA: Human leukocyte antigen; QFT-IT: QuantiFERON-TB Gold In-Tube.

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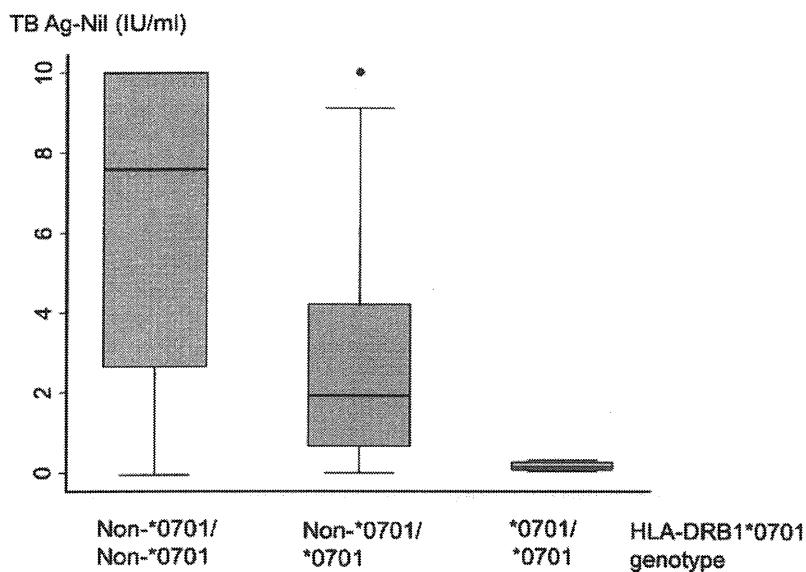


Figure 1. IFN- γ response to TB antigens stratified by HLA-DRB1*0701 genotypes in smear-positive/culture-positive pulmonary TB patients ($n = 504$). HLA-DRB1*0701 genotype significantly affected TB-Ag specific IFN- γ response (TB-Ag-Nil) ($P < 0.001$). IFN: Interferon; TB: Tuberculosis; HLA: Human leukocyte antigen; Ag: Antigen.
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CFP10 (100 amino acids) and TB7.7 p4 (18 amino acids) to bind a given allele was median of 4 with IQR in 3 to 5, but the number of epitopes predicted for HLA-DRB1*0701 was only one (data not shown).

Univariate analysis

Host factors including age, sex, BMI, underlying diseases, disease status, and inherent characteristics of pathogen were analyzed. The number of HLA-DRB1*0701 alleles carried by the patients appeared to be associated with the test-negative results. For this reason, this variable was also included in the statistical model.

In univariate analysis, increased age by year, BMI < 16.0, HIV co-infection and the number of HLA-DRB1*0701 alleles carried by patients showed significant associations with the negative results (OR = 1.04 [95% CI, 1.01–1.06], 7.27 [95% CI, 2.17–24.38], 4.26 [95% CI, 1.48–12.28], and 5.47 [95% CI, 2.58–11.61] respectively) (table 4). Sex, underlying diseases other than HIV infection, hospitalization, presence of infiltrates in more than half of the lung field and cavitary lesions on chest X-ray (CXR) did not show significant associations (data not shown). Beijing MTB strains were less frequently seen in the test-negative group (OR = 0.29 [95% CI, 0.11–0.76]). Multi-drug resistant (MDR)-TB strains showed no association with IGRA-negative results.

With QFT-IT indeterminate results, HIV co-infection, low lymphocyte count, MDR and the number of HLA-DRB1*0701 alleles showed significant associations (OR = 64.74 [95% CI, 17.23–243.20], 26.19 [95% CI, 8.00–85.72], 6.37 [95% CI, 1.64–24.68] and 4.66 [95% CI, 1.83–11.88], respectively).

Multivariate analysis

Age, sex, BMI, HIV status, lymphocyte count, and the number of HLA-DRB1*0701 alleles were put into the initial model for multivariate analysis. MTB strain and MDR-TB were not put together into this model because of the considerable number of missing values but analyzed separately as described later. In the final model, increased age by year, BMI < 16.0, HIV co-infection and the number of HLA-DRB1*0701 alleles showed significant association with QFT-IT negativity (OR = 1.04 [95% CI, 1.01–1.07], 5.42 [95% CI, 1.48–19.79], 6.38 [95% CI, 1.78–22.92] and 5.09 [95% CI, 2.31–11.22] respectively) (table 5).

HIV co-infection and the number of HLA-DRB1*0701 alleles were also significantly associated with QFT-IT indeterminate results (OR = 99.59 [95% CI, 15.85–625.61] and 4.25 [95% CI, 1.27–14.16] respectively).

When non-positive (negative and indeterminate) results of QFT-IT were compared with positive results, increased age by year, BMI < 16.0, HIV co-infection and the number of HLA-DRB1*0701 alleles showed similarly high odds ratios (Table S1).

Bacterial characteristics and IFN- γ responses

Among 488 patients for whom information of QFT-IT and MTB strains were both available, concentrations of IFN- γ responding to MTB-specific antigens were neither different between patient groups with Beijing and non-Beijing MTB strains (6.92 IU/ml [2.19–10.00 (14.42)] vs 6.00 IU/ml [2.12–10.00 (14.54)]) nor between patient groups with MDR-TB and non-MDR-TB strains (4.19 IU/ml [0.62–10.00 (15.72)] vs 6.57 IU/ml [2.19–10.00 (14.47)]) (table not shown).

Discussion

We calculated the test sensitivity of ELISA-based IGRA among active TB patients in Viet Nam and made an extensive analysis of

the factors associated with the false-negative results, which include increased age by year, extremely low BMI, HIV co-infection, and the number of HLA-DRB1*0701 alleles carried by the patients.

Aging is known as a risk factor for false-negative results [19,20]. Kobashi et al. [19] reports that the positive rate for both ESAT-6 and CFP-10 antigens of QuantiFERON TB-2G tested in the patients ≥ 80 years old is significantly lower than that in younger patients. In another study conducted by Liao et al. [20], using ELISPOT assay, increasing age is associated with false-negative results. HIV co-infection was associated with indeterminate results as well as false-negative results, presumably due to strong suppression of mitogenic response [21].

Severe wasting disease or malnutrition causes unhealthy emaciation with extremely low BMI, debilitating the patients and also suppressing systemic immune response [22]. In our study, BMI < 16.0 kg/m², was significantly associated with IGRA negativity whereas moderate and mild underweight (BMI from 16.0 to less than 18.5) were not. The proportion of BMI < 18.5 in the general population in Hanoi was only 13.3% [23], indicating that very low BMI in our study population is associated with active TB disease. However, it is not known whether this emaciation is observed mainly as a result of the current wasting disease or partly a risk factor for disease development.

In this study, we newly demonstrated that a particular MHC class II allele, HLA-DRB1*0701, was strongly associated with low TB_{Ag}-Nil values observed in indeterminate and negative results. HLA-DRB1*0701-positive/DQB1*0201-negative group but not HLA-DRB1*0701-negative/DQB1*0201-positive group suppressed the IFN- γ response, which suggests that HLA-DQB1*0701, but not HLA-DQB1*0201 has a primary role. The negative effect of HLA-DRB1*0701 on the IFN- γ values appeared to intensify in proportion to the number of HLA-DRB1*0701 alleles. The association between the increased number of the HLA alleles and QFT-IT negative results was demonstrated by the analysis using a logistic regression model.

After two months of anti-TB treatment, all of our IGRA-negative patients bearing the HLA allele continued to show negative IGRA results. There was no significant association between the extent of disease on CXR and the HLA-DRB1*0701 genotype (data not shown), suggesting that the allele does not seem to affect the assay results through modulation of disease severity. *In vivo* analysis suggested the low affinity of HLA-DRB1*0701 in binding with both ESAT-6 and CFP10 epitopes, and possibly failing to present them to T-cells for initiation of Th1 immune response efficiently [24].

Considering the low frequency of HLA-DRB1*0701 in the population tested, this finding may not have major clinical implications. However, we should bear in mind that negative QFT-IT results might be experienced in TB-infected individuals within a certain genetic background of the host even without apparent cause of immunodeficiency. In addition, it might be necessary to be investigated carefully in Southwestern Europe, North Africa, East Sub-Saharan Africa, West and South Asia among others, where high frequency (>15%) of the allele has been reported [25] and more than 2% of the people are supposed to possess this allele as homozygote. Further clinical investigations about HLA type and IGRA and *in vitro* experiments would contribute to a better understanding of IGRA performance in general and of QFT-IT in particular.

In analogy with negative results of tuberculin skin testing occasionally obtained in severe TB disease [26], IGRA-false-negative results may be caused by inefficient activation of antigen-specific CD4 T-cells [27], based on poorly-defined regulatory mechanism [28,29]. T-cell trafficking to the active TB sites or

Table 4. Univariate analysis using polytomous logistic regression model for factors associated with QFT-IT-negative and -indeterminate results (n = 503).

		QFT-IT-negative results			QFT-IT-indeterminate results		
		Proportion (%)	OR*	95% CI	Proportion (%)	OR*	95% CI
Sex	Male	21/398 (5.3)	1.00		13/398 (3.3)	1.00	
	Female	3/105 (2.9)	0.52	0.15–1.78	2/105 (1.9)	0.56	0.12–2.52
Age (years)			1.04	1.01–1.06		0.98	0.94–1.02
BMI	18.5–24.9	4/217 (1.8)	1.00		3/217 (1.4)	1.00	
	<16.0	9/77 (11.7)	7.27	2.17–24.38	3/77 (3.9)	3.23	0.64–16.40
	16.0–18.5	11/206 (5.3)	3.10	0.97–9.92	9/206 (4.4)	3.39	0.90–12.70
	≥25.0	0/3 (0.0)	NA	NA	0/3 (0.0)	NA	NA
Underlying condition	None	20/434 (4.6)	1.00		14/434 (3.2)	1.00	
	One	4/61 (6.6)	1.43	0.47–4.34	1/61 (1.6)	0.51	0.07–3.96
	More than one	0/8 (0.0)	NA	NA	0/8 (0.0)	NA	NA
HIV status	Negative	19/459 (4.1)	1.00		3/459 (0.7)	1.00	
	Positive	5/44 (11.4)	4.26	1.48–12.28	12/44 (27.3)	64.74	17.23–243.20
Lymphocyte count (cells/mm ³)	≥1,000	19/441 (4.3)	1.00		4/441 (0.9)	1.00	
	<1,000	5/60 (8.3)	2.51	0.89–7.04	11/60 (18.3)	26.19	8.00–85.72
Direct smear result	Scanty	3/65 (4.6)	1.00		3/65 (4.6)	1.00	
	1+ and more	21/438 (4.8)	1.02	0.30–3.52	12/438 (2.7)	0.58	0.16–2.13
Cavity on CXR	No	6/145 (4.1)	1.00		9/145 (6.2)	1.00	
	Yes	16/327 (4.9)	1.13	0.43–2.95	4/327 (1.2)	0.19	0.06–0.62
Infiltrate in >3 lung zones	No	15/391 (3.8)	1.00		12/391 (3.1)	1.00	
	Yes	7/83 (8.4)	2.26	0.89–5.75	1/83 (1.2)	0.40	0.05–3.16
Hospitalization	No	17/375 (4.5)	1.00		9/375 (2.4)	1.00	
	To TB ward	5/104 (4.8)	1.10	0.40–3.07	6/104 (5.8)	2.50	0.87–7.21
	To ER	2/24 (8.3)	1.87	0.41–8.59	0/24 (0.0)	NA	NA
HLA-DRB1*0701 (the number of alleles)			5.47†	2.58–11.61		4.66†	1.83–11.88
MDR	No	21/466 (4.5)	1.00		12/466 (2.6)	1.00	
	Yes	2/22 (9.1)	2.43	0.53–11.19	3/22 (13.6)	6.37	1.64–24.68
MTB strain	Non-Beijing	17/229 (7.4)	1.00		8/229 (3.5)	1.00	
	Beijing	6/259 (2.3)	0.29	0.11–0.76	7/259 (2.7)	0.73	0.26–2.03

QFT-IT: QuantIFERON-TB Gold In-Tube; BMI: Body mass index; CXR: Chest X-ray; MDR: Multi drug resistance; TB: Tuberculosis; ER: Emergency room; MTB: *Mycobacterium tuberculosis*; CI: Confidence interval; NA: Not available.

*OR: Multinomial odds ratio, also known as relative risk ratio, that is obtained by exponentiating the logit coefficient.

†OR per unit change in the number of alleles: Distribution of QFT-IT results and the number of HLA-DRB1*0701 alleles was shown in the text.

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compartmentalization may also be involved in the suppressive response in circulating blood [28]. However, this mechanism may not explain a major part of false-negative results in our study because the extent of infiltrates or presence of cavity on CXR did not show significant effects on the assay results.

Beijing MTB strains have spread rapidly in Asia and previous reports show that these are more adapted to the human body evading immune mechanism than others [30]. Although an inverse association was apparently observed between isolation of Beijing strains and IGRA negativity in our study, it may be attributed to unknown factors we could not access, since we made no demonstration of difference in TB_{Ag}-induced IFN- γ levels between Beijing and non-Beijing strains.

The overall sensitivity of QFT-IT in our population was considerably high among high TB burden countries from Cape Town in South Africa, the Gambia, Zambia, India, and some other countries [3]. This seems to be due to the lower proportion

of false-negative results in our study (4.8%) compared to (9.1% to 29%) in those studies. Several possible reasons for the interpretation of this point derive from our findings and others [19,20]: low proportion of underlying diseases including HIV, very few patients receiving immunosuppressive therapy, and recruitment of only new patients with sputum smear-confirmed pulmonary TB.

Our study had some limitations. Firstly, a clinical laboratory to measure CD4 count was not accessible during the study period, although CD4 count is an important parameter for this assessment [31]. Decrease in total lymphocyte count was used as a surrogate marker. Secondly, only smear-positive patients without previous treatment have been recruited, which may not allow us to generalize our results to all types of TB. Thirdly, further investigation is necessary to know whether all of the factors identified here affect results of ELISPOT-based IGRA as well. Lastly, the number of patients showing negative results was rather small despite the large number of recruited patients in our study.

Table 5. Multivariate analysis using polytomous logistic regression model for factors associated with QFT-IT-negative and indeterminate results (n = 503).

	QFT-IT-negative results			QFT-IT-indeterminate results		
	Proportion (%)	OR*	95% CI	Proportion (%)	OR*	95% CI
Age (years)		1.04	1.01–1.07		1.04	0.97–1.11
BMI	18.5–24.9	4/217 (1.8)	1.00	3/217 (1.4)	1.00	
	<16.0	9/77 (11.7)	5.42	3/77 (3.9)	1.82	0.29–11.18
	16.0–18.5	11/206 (5.3)	2.65	9/206 (4.4)	1.92	0.43–8.48
	≥25.0	0/3 (0.0)	NA	0/3 (0.0)	NA	NA
HIV status	Negative	19/459 (4.1)	1.00	3/459 (0.7)	1.00	
	Positive	5/44 (11.4)	6.38	12/44 (27.3)	99.59	15.85–625.61
HLA-DRB1*0701 (the number of alleles)			5.09†		4.25†	1.27–14.16

QFT-IT: QuantiFERON-TB Gold In-Tube; BMI: Body mass index; HIV: human immunodeficiency virus; CI: Confidence interval; NA: Not available.

*OR: Multinomial odds ratio, also known as relative risk ratio, that is obtained by exponentiating the logit coefficient.

†OR per unit change in the number of alleles: Distribution of QFT-IT results and the number of HLA-DRB1*0701 alleles was shown in the text.

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This is a limitation to analyze statistical significance in general. However, we were able to identify a novel host genetic factor, HLA*DRB1-0701. If well-known factors such as HIV co-infection were predominant in the studied population, individuals bearing the host genetic factor might have a chance of having those extrinsic factors together and it might be difficult to demonstrate that their genetic difference is a primary cause of false negativity.

Although some of the factors associated with IGRA-negative results have been proposed or even studied adopting a piecemeal method [2,3], the strong point of our study is that effects of all factors have been evaluated simultaneously by using appropriate statistical models, which provided a comprehensive insight into this area of interest.

In conclusion, we identified a specific HLA class II allele and characterized a variety of factors that possibly lead to false negativity of IGRA in active pulmonary TB. Detailed investigation of these unfavorable factors is necessary and would help to understand further the performance of the assay.

Supporting Information

Table S1 Univariate and multivariate analysis using logistic regression model for factors associated with

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QFT-IT non-positive (negative and indeterminate) results (n = 503). (DOC)

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Author Contributions

Conceived and designed the experiments: NTLH LTL N. Kobayashi SS PHT N. Keicho. Performed the experiments: NTLH LTL PHT LTH DBT MH IM NVH. Analyzed the data: NTLH TS N. Keicho. Contributed reagents/materials/analysis tools: KH NH N. Keicho. Wrote the paper: NTLH N. Keicho.

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RESEARCH ARTICLE

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Identification of tuberculosis-associated proteins in whole blood supernatant

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Abstract

Background: Biological parameters are useful tools for understanding and monitoring complicated disease processes. In this study, we attempted to identify proteins associated with active pulmonary tuberculosis (TB) using a proteomic approach.

Methods: To assess TB-associated changes in the composition of human proteins, whole blood supernatants were collected from patients with active TB and healthy control subjects. Two-dimensional difference gel electrophoresis (2D-DIGE) was performed to analyze proteins with high molecular weights (approximately >20 kDa). Baseline protein levels were initially compared between patients with active TB and control subjects. Possible changes of protein patterns in active TB were also compared *ex vivo* between whole blood samples incubated with *Mycobacterium tuberculosis* (*Mtb*)-specific antigens (stimulated condition) and under unstimulated conditions. Immunoblot and enzyme-linked immunosorbent assays (ELISA) were performed to confirm differences in identified proteins.

Results: Under the baseline condition, we found that the levels of retinol-binding protein 4 (RBP4), fetuin-A (also called α -HS-glycoprotein), and vitamin D-binding protein differed between patients with active TB and control subjects on 2D gels. Immunoblotting results confirmed differential expression of RBP4 and fetuin-A. ELISA results further confirmed significantly lower levels of these two proteins in samples from patients with active TB than in control subjects ($P < 0.0001$). *Mtb*-specific antigen stimulation *ex vivo* altered clusterin expression in whole blood samples collected from patients with active TB.

Conclusions: We identified TB-associated proteins in whole blood supernatants. The dynamics of protein expression during disease progression may improve our understanding of the pathogenesis of TB.

Background

Tuberculosis (TB) is one of the most important infectious causes of death worldwide [1]. Despite its long historical interaction with humans, our understanding of host response to the TB pathogen remains incomplete. Investigation of the molecular basis of differences in the host immune status and metabolism between patients with active TB and control subjects may provide a clue to understand the disease process, and thus contribute to future strategies for TB prevention and treatment.

Recent advances in comprehensive analytical techniques, such as transcriptomics and proteomics, have enabled us to identify proteins associated with active TB in humans. As a pioneering approach, Jacobsen et al. compared the gene expression profiles of peripheral blood mononuclear cells from patients with TB and *Mtb*-infected healthy donors by microarray analysis [2], and Mistry et al. analyzed gene expression patterns in whole blood in an attempt to find a candidate biomarker for discriminating cured patients from those with a risk of relapse [3].

Agranoff et al. [4] identified amyloid A and transthyretin in human serum as potential indicators for distinguishing patients with TB from those with non-TB

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inflammatory conditions. They also reported that a combination of four protein markers, including amyloid A and transthyretin, achieved a diagnostic accuracy of up to 78%. Chegou et al. [5] reported that EGF, VEGF, TGF- α , and sCD40L in supernatants obtained from interferon-gamma (IFN- γ)-release assays (IGRAs) are informative markers for differentiating active disease from latent infection. Although the above studies are promising, such comprehensive analytical techniques are still in the developmental stages and further investigations are required before they can be applied clinically.

IGRA detects TB infection by measuring the *Mtb*-specific immune response with high specificity [6]. IFN- γ is released by reactivation of *Mtb*-specific effector memory T cells in whole blood. Despite its advantages, IGRA is not a perfect tool for use in most developing countries. In countries with a high TB burden, patients with active TB, and not those with latent TB infection, need to be immediately identified and treated in order to prevent disease transmission. However, IGRA is not capable of distinguishing active TB from latent infection. Also, cytokine measurements to be performed for IGRA are rather expensive in a resource-limited setting and difficult to distribute. Thus, from a practical as well as a research standpoint, development of new markers for TB is desired.

In the present study, by high-resolution two-dimensional difference gel electrophoresis (2D-DIGE) followed by liquid chromatography-mass spectrometry (LC-MS), we analyzed the expression profiles of high molecular weight proteins (approximately >20 kDa) that have not been studied fully among components of residual whole blood supernatants after performing IGRA.

We used two comparative frameworks. One was the direct comparison of plasma supernatants collected from patients with active TB and healthy control subjects. This comparison aimed to identify proteins that are markedly upregulated or downregulated in the disease state; even if such proteins are not disease specific, they might act as useful markers for monitoring the disease before, during, and after treatment. The other comparative framework was more TB specific since whole blood samples from patients were stimulated with *Mtb*-specific antigens or left unstimulated, and the results were compared.

Methods

Patients and control subjects

In this study, whole blood samples collected from Japanese and Vietnamese individuals were used. The study was approved by the ethical review committees of the National Center for Global Health and Medicine (formerly the International Medical Center of Japan), Tokyo, Japan, and the Ministry of Health, Vietnam.

Written informed consent was obtained from each participant. Blood samples were collected from patients with active TB immediately before (Vietnamese patient samples) or within 7 days (Japanese patient samples) of treatment initiation. Patients with potential complications attributable to malignancies, autoimmune diseases, or HIV coinfection were excluded from the study.

At the initial screening and confirmation stage, blood samples were collected from 14 Japanese patients with bacteriologically confirmed active pulmonary TB (9 men and 5 women; median age 50 years, range 22-75 years) and 13 age- and gender-matched healthy Japanese patients (8 men and 5 women; median age 48 years, range 24-64 years). We could not completely rule out the possibility of latent TB infection in 2 of the 13 control subjects, according to the results of a commercially available IGRA (QuantIFERON[®]-TB Gold in Tube; Cellestis, Victoria, Australia). However, we analyzed all samples together at the initial stage to identify proteins associated with active TB disease. The tuberculin skin test was not useful for detecting latent TB infection in our study since most individuals in the tested populations had received BCG vaccination after birth. Blood samples from 4 patients with active TB and 4 healthy individuals were chosen for screening by 2D-DIGE and immunoblotting. The stability of proteins measured by the enzyme-linked immunosorbent assay (ELISA) was investigated by comparing a set of plasma samples directly separated from EDTA-containing peripheral blood and another set of plasma supernatants obtained from heparinized blood after 18 h of incubation (under the same conditions as the IGRA negative control).

At the next verification stage, we utilized samples from 25 Vietnamese patients with sputum smear-positive active pulmonary TB (13 men and 12 women; median age 35 years, range 20-55 years) and 50 age- and gender-matched Vietnamese healthy control subjects (26 men and 24 women; median age 36 years, range 21-54 years) of which 25 were IGRA positive and 25 were IGRA negative. None of the IGRA-positive individuals had any signs or symptoms of active TB but at least some were reasonably suspected to have latent TB infections because the prevalence of TB in the population is high. Following IGRA, the remaining unstimulated plasma supernatants were used for ELISA.

Sample collection and preparation

Whole blood was separately collected in heparin-containing tubes precoated with mitogen as a positive control or cocktails of ESAT-6, CFP-10, and TB7.7 (p4) peptides as *Mtb*-specific antigens (QuantIFERON[®]-TB Gold in Tube; Cellestis); the negative control tubes had no precoat. After 18 h of incubation at 37°C, each sample was centrifuged and the plasma supernatants were

harvested and stored at -80°C until use in subsequent assays. For proteomic analysis, four sample sets that were either unstimulated or stimulated with *Mtb*-specific antigens or mitogen from patients with active pulmonary TB and four corresponding sets from control subjects were used to screen for candidate proteins by 2D-DIGE. To increase resolution, 14 human major plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha 2-macroglobulin, alpha 1-acid glycoprotein, IgM, apolipoprotein A1, apolipoprotein AII, complement C3, and transthyretin) were removed prior to electrophoresis using a Multiple Affinity Removal LC Column-Human 14 (Agilent Technologies, Santa Clara, CA, USA). The samples were then concentrated by ultrafiltration (Agilent Technologies, Concentrators Spin 5 kDa MWCO, 4 ml) followed by acetone precipitation in preparation for subsequent electrophoresis.

Quantitative analyses by 2D-DIGE

Protein samples were labeled with Cy3 and Cy5 (DIGE Fluors Minimal Labeling Dyes; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The samples (50 μg of total protein per gel) were applied to Immobiline DryStrips (18 cm long, pH 4-7 linear; Amersham Biosciences, Pittsburgh, PA, USA), and isoelectric focusing (IEF) was performed using an Ettan IPGphor IEF system (Amersham Biosciences) according to the manufacturer's instructions. Next, SDS-PAGE was performed using a 10-18% linear gradient gel from DRC Co., Ltd. (Tokyo, Japan). The fluorescence intensity of each protein spot was digitally recorded using a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA, USA) with Quantity One software (Bio-Rad Laboratories), and differential protein expression was quantitatively analyzed using the PDQuest software (Bio-Rad Laboratories). The same gel included a reference sample that had been labeled with Cy2 and was used for spot matching, image analysis, and volume normalization. Initially, all spots were roughly matched using an automated tool in the PDQuest software suite. This estimate was followed by a more detailed manual curation to correct any inappropriately matched pairs of protein spots.

Sample preparation for mass spectrometry

A mixture of all samples (400 μg of total protein per gel) was subjected to 2D-DIGE under the same conditions as described above to isolate selected spots. To visualize individual protein spots, the gels were stained with SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR, USA) for 3 h. The fluorescence intensity of each protein spot was digitally measured using the

Molecular Imager FX system with Quantity One software. Mass spectrometric analysis was performed according to the method reported by Toda *et al.* [7], with slight modification. Briefly, each protein spot on SYPRO Ruby stained gels was picked using a spot picker (Amersham Biosciences). In-gel digestion of proteins was performed according to the method reported by Saeki *et al.* [8].

Mass spectrometric analysis

An ESI ion-trap mass spectrometer (LCQ Deca XP Plus, Thermo Electron) was used for peptide detection. Mass spectrometric analysis was performed as described previously [8]. Protein identification was performed using the Mascot server (Matrix Science, Boston, MA, USA) and Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA, USA). We selected the SWISS-PROT *Homo sapiens* database and used the following parameters: peptide tolerance 1.0 Da and one missed cleavage. Carbamidomethyl modification of cysteine, acetylation of the NH₂-terminal ends of lysine, and phosphorylation of serine, threonine, or tyrosine were considered in this analysis.

Immunoblotting

Immunoblotting to detect the proteins identified as described above was performed using anti-human retinol-binding protein 4 (RBP4) rabbit polyclonal IgG (A-0040; Dako; Glostrup, Denmark), anti-human fetuin-A (AHSG) goat polyclonal IgG (G-20; Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-human vitamin D-binding protein (VDBP) (Gc-Globulin) rabbit polyclonal IgG (Dako), anti-human clusterin- α mouse monoclonal IgG1 (B-5; Santa Cruz Biotechnology), or anti-human clusterin- β rabbit polyclonal IgG (N-18; Santa Cruz Biotechnology).

Total protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). To detect clusterin- α and - β , mixed protein samples (20 μg) were applied to 2D PAGE with 1D IEF using the Immobiline DryStrip (pH 3-5.6 nonlinear). Proteins were then transferred to PVDF membranes. The membranes were probed with polyclonal antibodies, anti-clusterin- α , and anti-clusterin- β . To detect other proteins, each sample (10 μg) was subjected to conventional SDS-PAGE. Membranes were probed with anti-VDBP, anti-fetuin-A, or anti-RBP4 polyclonal antibodies. Anti-mouse and anti-rabbit (GE Healthcare) as well as anti-goat (Santa Cruz Biotechnology) HRP-conjugated secondary antibodies were prepared. Protein bands were detected using the ECL plus detection reagent (GE Healthcare). Band intensities were calculated using the Quantity One software.

ELISA

A competitive ELISA for quantitative determination of RBP4 in human plasma was performed according to the manufacturer's instructions (AdipoGen Inc.; Seoul, Korea). The detection limit was 1 ng/ml. An AHSG ELISA kit was used to detect fetuin-A in plasma (Bio-Vender Laboratory Medicine Inc.; Modrice, Czech Republic). The detection limit was 0.35 ng/ml. A Quantikine[®] Human Vitamin D-Binding Protein Immunoassay kit was used to detect VDBP in plasma (R&D Systems, Inc.; Minneapolis, MN, USA). The mean minimum detectable VDBP level was 0.65 ng/ml. Distribution of levels was represented using the median and interquartile range (IQR).

Statistical analysis

Proteins showing differential expression between two conditions were first determined with *P* values using the Student's *t*-test preinstalled in the PDQuest software suite. To select candidate proteins with expression levels that differed between unstimulated samples from patients with active TB and healthy control subjects, a significance level of *P* < 0.05 was selected. To select candidate proteins showing differential expression in *Mtb*-specific antigen-stimulated and unstimulated plasma samples, a less stringent cut-off value of *P* < 0.10 was applied. Assuming an alpha error of 0.1 and a standardized effect size of 2.0, the power to detect a difference was calculated as 0.8 given our sample size. When a normal distribution of measurements was not predicted, the Wilcoxon rank sum test (Mann-Whitney U test) was applied for confirmation using the JMP software (version 7.0.1; SAS Institute, Cary, NC, USA).

Results

Quantitative analyses by 2D-DIGE

In a preliminary experiment, we used an immobilized linear pH gel strip with a broad pH range (pH 3-10 linear) for 1D IEF. Although more than 500 protein spots were visualized in fresh plasma with SYPRO Ruby staining, the number of spots after incubation of whole blood with stimuli decreased, and detectable spots were primarily located in the pH range 4-7 (data not shown). Therefore, we performed subsequent analyses using an immobilized linear pH gel strip with a narrower range (pH 4-7 linear) to obtain a finer resolution. We used two comparative frameworks in our analyses, and the corresponding spot patterns are schematically depicted in Figure 1.

Differential gel images were acquired and displayed using the PDQuest 2D gel analysis software (Figure 2A, B). In our comparison of the protein expression profiles of patients with active TB and control subjects, red indicates proteins increased in the supernatants collected

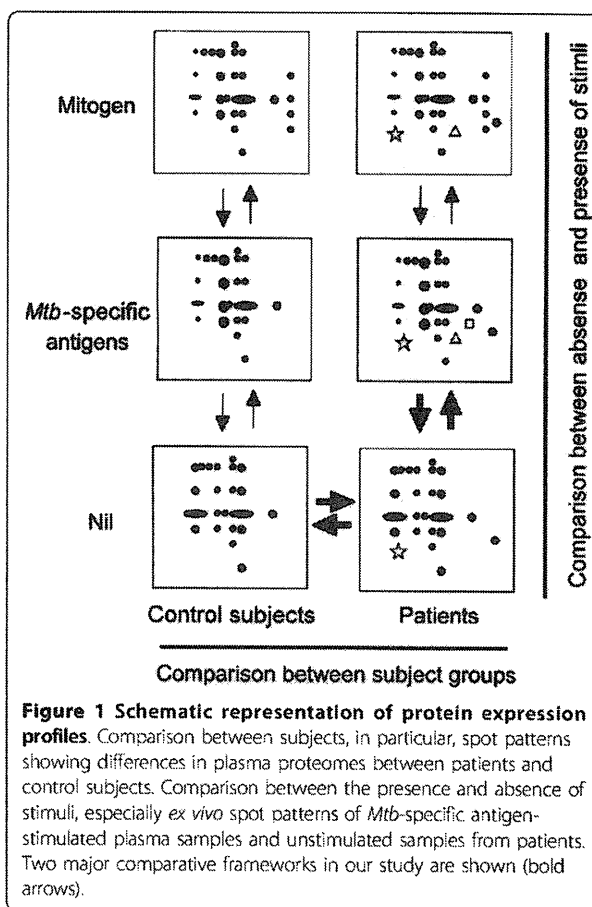
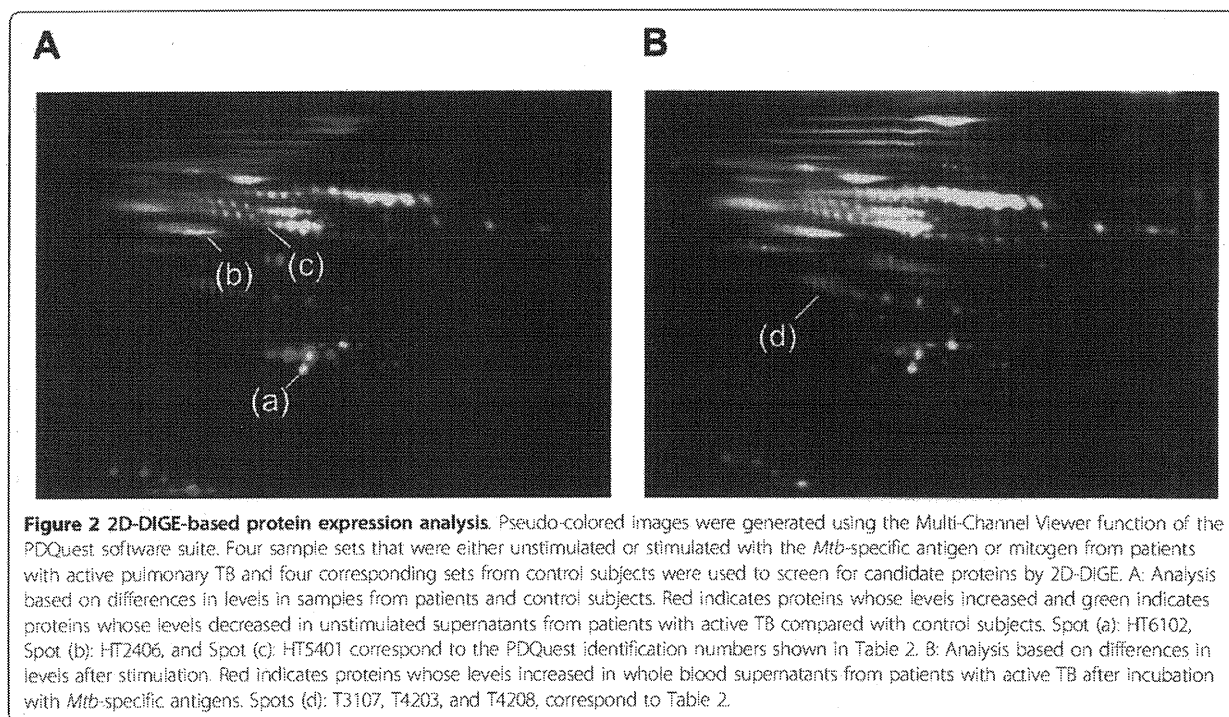


Figure 1 Schematic representation of protein expression profiles. Comparison between subjects, in particular, spot patterns showing differences in plasma proteomes between patients and control subjects. Comparison between the presence and absence of stimuli, especially *ex vivo* spot patterns of *Mtb*-specific antigen-stimulated plasma samples and unstimulated samples from patients. Two major comparative frameworks in our study are shown (bold arrows).

from the patients and green indicates proteins decreased in the patients compared with the control subjects. Yellow indicates no significant differences (Figure 2A). In 2D gel profiles comparing the antigen-stimulated and unstimulated samples collected from patients with active TB, red indicates proteins increased in the supernatants after *Mtb*-specific antigen stimulation, and green indicates proteins decreased after stimulation. Yellow indicates no significant changes (Figure 2B). From 367 spots compared between patients with active TB and control subjects, and 293 spots generated with samples collected from patients with active TB that were either stimulated with *Mtb*-specific antigens or left unstimulated, we selected several candidates for subsequent mass spectrometric analysis (Table 1) according to the criteria described in the Materials and Methods section.

Mass spectrometric analysis

Following the above criteria for selecting candidates of differentially expressed proteins between two conditions, a total of 41 spots were isolated from the corresponding 2D gels on the basis that they showed sufficiently strong



signals. Trypsin digestion of each isolated spot was followed by LC-MS analysis. The proteins corresponding to 14 of these spots were successfully identified (Figure 3).

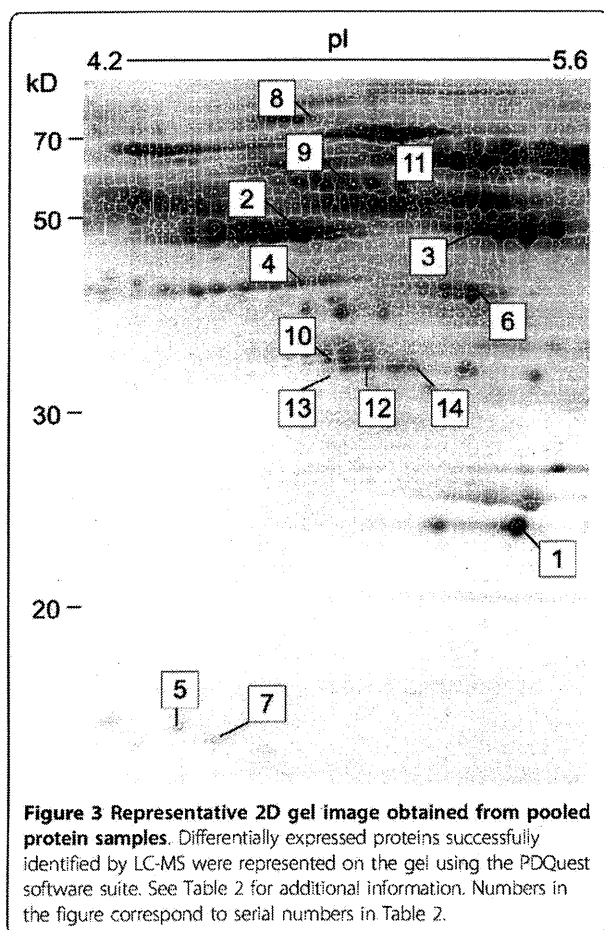
Of the 14 proteins in Table 2, 7 (serial numbers 1 to 7) were obtained as a result of comparisons between patients with active TB and control subjects; number 1 (spot HT6102) was identified as RBP4, number 2 (HT2406) as fetuin-A, and number 3 (HT5401) as VDBP. Four (numbers 8 to 11) were obtained as a result of comparisons between nonspecific mitogen-stimulated and unstimulated samples collected from patients with active TB (not analyzed in this study). The last 3 proteins (numbers 12 to 14) were obtained as a result of comparisons between *Mtb*-specific antigen-stimulated and unstimulated samples collected from patients with

active TB; numbers 12 to 14 (T4203, T3107, and T4208) were all identified as clusterin. In Table 2, *P* values indicating a significant difference between the means of the two conditions examined, the SWISS-PROT accession numbers of the identified proteins as well as their molecular weights and theoretical pI values are indicated. We also used the *Homo sapiens* database of expressed sequence tags (ESTs) to identify clusterin in spot T4208.

Mascot search scores (indices of protein matches) were 47, 50, 98, 75, and 72 for spots T4203 (clusterin), T3107(clusterin), HT5401 (VDBP), HT2406 (fetuin-A), and HT6102 (RBP4), respectively, (Table 2), suggesting that identification of these proteins using peak lists of MS/MS spectra obtained from the LC-MS/MS system are fairly reliable since all these scores were significant

Table 1 The number of spots that may show differential expression

A: Comparison between patients with active TB and control subjects		<i>P</i> < 0.02	0.02 ≤ <i>P</i> < 0.05	0.05 ≤ <i>P</i> < 0.10
Patients versus control subjects		18	12	24
B: Comparison between stimulated and unstimulated conditions		<i>P</i> < 0.02	0.02 ≤ <i>P</i> < 0.05	0.05 ≤ <i>P</i> < 0.10
Patients	<i>Mtb</i> antigens versus no stimuli	0	2	2
	Mitogen versus no stimuli	3	5	11
Control subjects	<i>Mtb</i> antigens versus no stimuli	0	1	13
	Mitogen versus no stimuli	2	83	8



above the 5% confidence threshold and no other proteins with comparable scores were detected for each gel spot (See Additional file 1: for supporting information). These proteins were interesting because of their potential biological significance, and we therefore analyzed them further.

Confirmation of differentially expressed proteins by immunoblotting

Immunoblot analysis was used to confirm differential expression of three proteins identified in patients with active TB compared with control subjects (Figure 4A). We measured band densities using the same samples prepared for protein confirmation (Figure 4B). The band density of RBP4 in patients with active TB ($64,283$ arbitrary units $\pm 3,861$) was lower than that in control subjects ($445,894 \pm 16,590$), and fetuin-A expression in the patients was also lower ($42,710 \pm 7,580$) than that in control subjects ($343,617 \pm 58,923$). These results are consistent with those of 2D gel analysis. Moreover, the band density of VDBP tended to be higher in samples from patients with active TB than from control subjects,

which is similar to that observed above; however, the protein levels were widely distributed and the differences in these levels did not reach significance in the control subjects compared with patients with active TB ($33,251 \pm 2,572$ versus $38,971 \pm 11,001$). Because the three clusterin spots altered after *Mtb*-specific antigen stimulation were not clearly distinguished by immunoblotting, we did not attempt any further demonstration of changes in these signals in our study. Instead, pooled samples were run on a 2D gel and followed by immunoblotting with anti-clusterin- α and anti-clusterin- β antibodies (because clusterin consists of clusterin- α and - β subunits) (Figure 4C). Based on immunoreactivity and pI values, the spots detected were confirmed to be clusterin- α . More specifically, the three spots comprised a subset of possible modified forms of clusterin- α that may be detected.

Detection of differentially expressed proteins by ELISA

Because RBP4 and fetuin-A levels determined by immunoblotting were significantly different between samples from patients with active TB and control subjects, we performed further quantitative ELISA to extend the measurements to plasma samples from 14 Japanese patients with active TB and 13 age-, gender-, and ethnicity-matched control subjects. Plasma RBP4 levels in patients with active TB (median = 23.6 $\mu\text{g/ml}$; IQR = 18.4 - 37.9) were significantly lower than those from control subjects (median = 44.6 $\mu\text{g/ml}$; IQR = 34.6 - 53.8 ; $P = 0.0033$; Figure 5A). Plasma fetuin-A levels in patients (median = 147.9 $\mu\text{g/ml}$; IQR = 115.8 - 159.6) were also significantly lower than those in control subjects (median = 211.0 $\mu\text{g/ml}$; IQR = 186.7 - 264.6 ; $P = 0.0002$; Figure 5B). No significant difference were observed in plasma VDBP levels between patients (median = 110.0 $\mu\text{g/ml}$; IQR = 85.2 - 151.3) and control subjects (median = 105.0 $\mu\text{g/ml}$; IQR = 88.1 - 215.6 ; $P = 0.5441$; Figure not shown).

We simultaneously compared the protein levels in plasma immediately separated from EDTA-containing blood with those in plasma supernatants obtained from heparinized blood as a negative control for IGRA after 18 h of incubation without stimulants. We found that the differences between the two types of plasma samples were small (coefficient of variance (CV) = 10.5% for RBP4; CV = 5.0% for fetuin-A; CV = 6.6% for VDBP) and was in a range of variation generally accepted in ELISA (CV < 15%), indicating that the measurements obtained under the latter condition can be substituted for those obtained under the former condition. Indeed, plasma RBP4 and fetuin-A levels in samples from Japanese patients with active TB were significantly lower than those from control subjects, irrespective of plasma conditions (data not shown).

Table 2 Characteristics of proteins identified in this study

A: Comparison between patients with active TB and control subjects									
Condition	Serial Number	2D-DIGE		LC-MS/MS		Mascot search score ^e	Protein name	Da	pI
		PDQ SSP# ^a	P value	+/- ^b	Swiss-Plot				
Patients versus control subjects	1	HT6102	0.0064	-	RET4_HUMAN	72	Retinol binding protein 4	23010	5.76
	2	HT2406	0.0097	-	FETUA_HUMAN	75	α -2-HS-glycoprotein	39325	5.43
	3	HT5401	0.0331	+	VTD8_HUMAN	98	Vitamin D binding protein	52964	5.40
	4	HT2303	0.0419	+	CO4A_HUMAN	86	Complement C4A	192771	6.66
	5	HT1012	0.0271	-	APOC3_HUMAN	105	Apolipoprotein C-III	10852	5.23
	6	HT5303	< 0.001	-	APOA4_HUMAN	190	Apolipoprotein A-IV	45399	5.28
	7	HT1016	0.0024	-	APOC2_HUMAN	61	Apolipoprotein C-II	11284	4.72
B: Comparison between stimulated and unstimulated conditions in active TB									
Condition	Serial Number	2D-DIGE		LC-MS/MS		Mascot search score	Protein name	Da	pI
		PDQ SSP#	P value	+/- ^c	Swiss-Plot				
Mitogen (versus no stimuli)	8	T3601	0.0917	-	C1S_HUMAN	169	Complement-C1S	76684	4.86
	9	T3403	0.0156	+	KNG1_HUMAN	139	Kininogen-1	71957	6.34
	10	T3105	0.0866	-	ZA2G_HUMAN	45	Zinc- α -2-glycoprotein	33872	5.57
	11	T4512	0.0061	-	A1BG_HUMAN	76	α -1B-glycoprotein	54273	5.58
<i>Mtb</i> antigens (versus no stimuli)	12	T4203	0.0640 ^d	+	CLUS_HUMAN	47	Clusterin	52495	5.89
	13	T3107	0.0687 ^d	+	CLUS_HUMAN	50	Clusterin	52495	5.89
	14	T4208	0.0732 ^d	+	EST	-	Clusterin	52495	5.89

^aPDQ SSP# is a PDQuest software standard spot number indicating the unique location of each spot automatically assigned on a 2D gel and is essential for comparing the same spots on different gels.

^bThe average density of a spot from 2D-DIGE is higher (+) or lower (-) in patients with active TB than in control subjects.

^cThe average density of the spot from 2D-DIGE is higher (+) or lower (-) under the stimulated condition than that under the unstimulated condition.

^dThe average density of the 3 spots, T4203, T3107, and T4208, which correspond to a subset of clusterin, was significantly higher in *Mtb*-specific antigen-stimulated than in unstimulated samples ($P = 0.0014$).

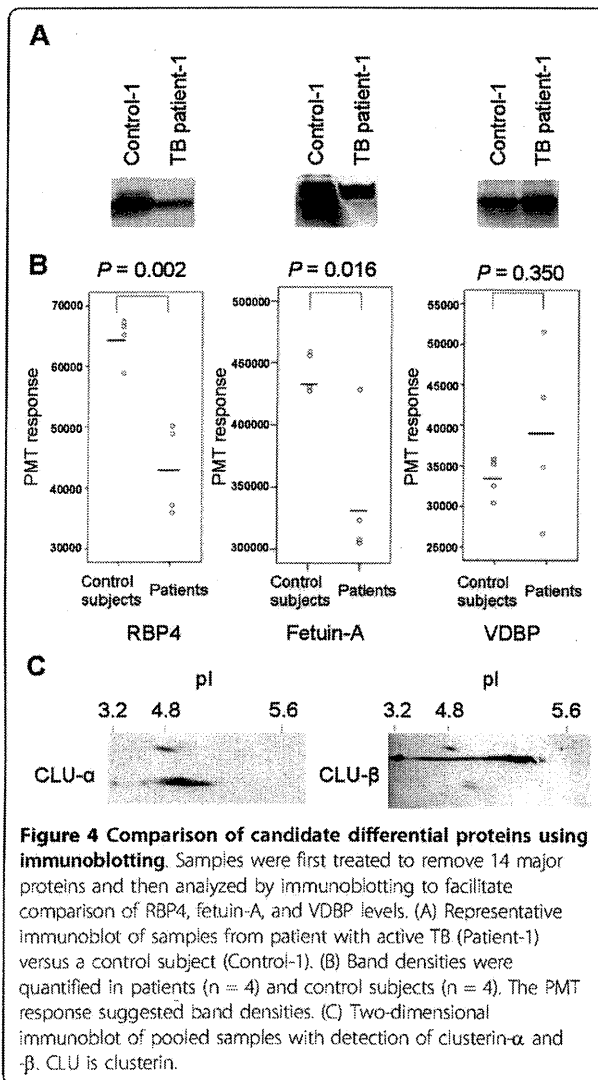
^eThe Mascot search score indicates the degree of compatibility between mass spectra generated by the sample and amino acid sequences within the protein of interest.

We further attempted to verify the differences observed with samples from a different ethnic and regional population, i.e., samples collected from Vietnamese patients. The two proteins identified above were measured in plasma supernatants from Vietnamese patients with active TB and age-, gender-, and ethnicity-matched control subjects. The samples from these Vietnamese patients were obtained from a negative control of IGRA after incubation without stimulants. RBP4 levels in patients with active TB (median = 17.5 μ g/ml; IQR = 14.4-23.9) were significantly lower than those in control subjects (median = 30.5 μ g/ml; IQR = 25.9-40.8; $P < 0.0001$; Figure 5A). Fetuin-A levels in patients with active TB (median = 210.7 μ g/ml; IQR = 178.1-235.7) were also significantly lower than those in control subjects (median = 299.4 μ g/ml; IQR = 265.1-363.2; $P < 0.0001$; Figure 5B). Moreover, both protein levels were not significantly different between IGRA-negative and IGRA-positive subgroups of the control subjects (data not shown).

Discussion

In this study, we identified TB-associated proteins from whole blood supernatants. After the removal of 14 major plasma proteins, RBP4, fetuin-A, and VDBP were initially identified as plasma proteins from unstimulated samples for which the baseline levels differed between the patients and control subjects. Immunoblotting results confirmed the differential expression of RBP4 and fetuin-A between the two groups. Although VDBP has previously been identified as a biomarker for mycobacterial infections in cattle [9], the level of this protein did not differ significantly in our study because of large individual variations. The changes in VDBP levels may not have been accurately immunologically assayed in our study.

Clusterin is a secreted glycoprotein involved in apoptosis, inflammation, and tissue injury. It was differentially expressed in patients with active TB after stimulation and the intensities of the three spots



corresponding to clusterin- α were elevated in whole blood supernatant samples after incubation with *Mtb*-specific antigens. These spots appear to have shifted in both the dimensions on the gel, which suggests small changes in their molecular weights and IEPs. It is conceivable that post-translational modifications, such as degradation and/or deglycosylation, occur via an enzymatic reaction that accompanies immune cell activation. However, we have not demonstrated that this response is observed only when *Mtb*-specific antigens are co-incubated. To determine whether clusterin has a role as a marker of TB or indicates more general response to antigen stimulation, we are currently attempting to find clear and simple methods in detecting these alterations for mass screening.

Subsequent ELISA results for samples from Japanese and Vietnamese subjects confirmed that both plasma

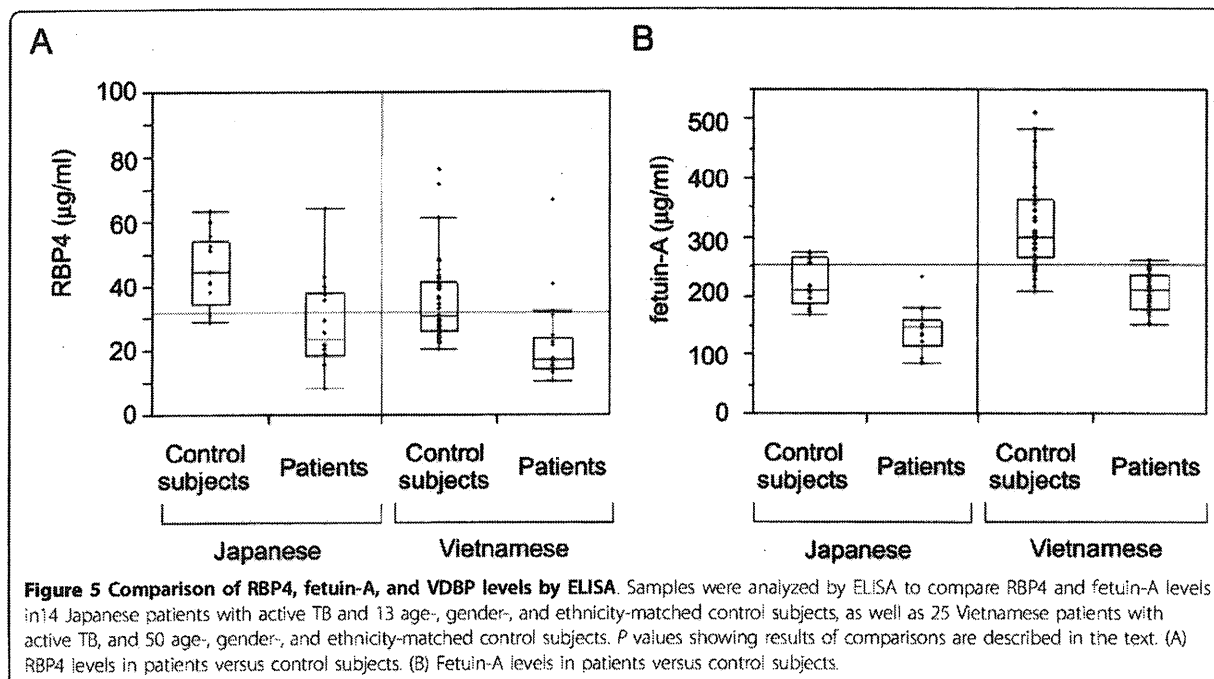
RBP4 and fetuin-A levels were significantly lower in samples collected from patients with active TB than in control subjects, indicating that our findings are reproducible in studies using well-matched control subjects. However, as shown in Figure 5, the average plasma levels of these proteins differed between Japanese and Vietnamese control subjects. This suggests that unknown factors may systemically influence tested populations or the measurement of these markers. Because this variance is crucial in a clinical setting, further basic as well as clinical investigations are necessary to accurately assess these markers.

No significant differences were observed in RBP4 and fetuin-A levels in samples from IGRA-positive and -negative control subjects. This suggests that these proteins levels are not affected by latent TB infection, but that they presumably change during disease progression via an unknown mechanism.

Intriguingly, the literature supports the idea that RBP4 and fetuin-A are functionally significant since they may be involved in macrophage activation [10-12]. Retinoic acid has been shown to stimulate and induce monocyte differentiation, leading to inhibition of *Mtb* multiplication in human macrophages [13]. RBP4 is the specific carrier protein for retinol (vitamin A) and has recently been described as an adipokine that contributes to insulin resistance [13]. This protein is believed to modulate pathophysiological processes during bacterial infection. Fetuin-A was originally identified as a fetal protein and has been shown to affect the development of many mammalian tissues. Moreover, the results of *in situ* mRNA hybridization and immunocytochemical studies in adult sheep have revealed that the main sites of fetuin-A expression are hepatocytes and monocytes or macrophages in the spleen and bone marrow [14]. Fetuin-A is known to modulate various immune and metabolic responses. Previous reports have shown that fetuin-A deactivates macrophages, acts as an opsonin for cationic-deactivating molecules including spermine [15], reduces TNF- α production and inflammatory responses [16], and enhances phagocytosis of apoptotic cells and macropinocytosis by human macrophages [17]. On the other hand, this protein is known to be a potent inhibitor of systemic calcification [18] and is associated with the incidence of diabetes mellitus [19].

Our study is the first to highlight the relationship between these two markers and TB, even though these marker levels may be affected by endogenous or exogenous factors and are presumably nonspecific to TB given their relative abundance in plasma and the broad spectrum of functional significance proposed in the above references.

Nevertheless, performing a prospective cohort study may help clarify the role of these proteins in TB.



If within-individual variation in baseline levels is relatively small, it can be used to monitor the course of disease before, during, and after treatment. Further clinical studies on various conditions may better characterize these proteins. Single use of these markers or their combined use with other promising biomarkers may be a useful tool to aid the development of new effective therapies and vaccines.

Conclusions

We identified three TB-associated proteins, RBP4, fetuin-A, and clusterin, in whole blood supernatants using a proteomic approach. We subsequently showed that both plasma RBP4 and fetuin-A levels are significantly and reproducibly lower in patients with active TB than in control subjects. These findings may help us understand and monitor the disease process in TB.

Additional material

Additional file 1: Figure S1 - Mascot search results-Information about the identified proteins obtained using the Mascot server. (A) Mascot search result for T2116 (clusterin) (B) Mascot Search Result for T2103 (clusterin) EST (C) Mascot search result for T1486 (clusterin) (D) Mascot search result for HT2482 (RET4 = RBP4) (E) Mascot search result for HT1248 (fetuin-A) (F) Mascot search result for HT1240 (VDBP).

Abbreviations

TB: Tuberculosis; *Mtb*: *Mycobacterium tuberculosis*; IFN- γ : interferon-gamma; IRGA: interferon-gamma-release assay; 2D-DIGE: two-dimensional difference

gel electrophoresis; LC-MS: liquid chromatography-mass spectrometry; ELISA: enzyme-linked immunosorbent assay; IEF: isoelectric focusing; RBP-4: retinol binding protein-4; VDBP: vitamin D binding protein; IQR: interquartile range; ESTs: expressed sequence tags;

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Authors' contributions

TT carried out the plasma proteome studies, participated in 2D-DIGE studies, a part of immunoassays and drafted the manuscript. SS conceived of the study, and participated in the study planning and coordination and helped to draft the manuscript. KK carried out the LC-MS/MS analysis. ET, KY and

HH helped to design the study. YK participated in the study design and overall supervision. NK, NTLH and LTL participated in management and analysis of data. IM and MH participated in the acquisition of data. TU helped to draft the manuscript. NK participated in the design of the study, performed statistical analysis and have given final approval of the version to be published. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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