Table 2 Characteristics of proteins identified in this study

A: Comparison between patients with active TB and control subjects

		2D-DIGE			LC-MS/MS				
Condition	**Serial Number**	**PDQ SSP#[a]**	**P value**	**+/-[b]**	**Swiss-Plot**	**Mascot search score[e]**	**Protein name**	**Da**	**pI**
Patients versus control subjects	1	HT6102	0.0064	-	RET4_HUMAN	72	Retinol binding protein 4	23010	5.76
	2	HI2406	0.0097	-	FETUA_HUMAN	75	α-2-HS-glycoprotein	39325	5.43
	3	HT5401	0.0331	+	VTDB_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	HI1016	0.0024	-	APOC2_HUMAN	61	Apolipoprotein C-II	11284	4.72

B: Comparison between stimulated and unstimulated conditions in active TB

		2D-DIGE			LC-MS/MS				
Condition	**Serial Number**	**PDQ SSP#**	**P value**	**+/-[c]**	**Swiss-Plot**	**Mascot search score**	**Protein name**	**Da**	**pI**
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
Mtb 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

[a]PDQ 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.
[b]The average density of a spot from 2D-DIGE is higher (+) or lower (-) in patients with active TB than in control subjects.
[c]The average density of the spot from 2D-DIGE is higher (+) or lower (-)under the stimulated condition than that under the unstimulated condition.
[d]The 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 ($P = 0.0014$).
[e]The 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 μg/ml; IQR = 14.4-23.9) were significantly lower than those in control subjects (median = 30.5 μg/ml; IQR = 25.9-40.8; $P < 0.0001$; Figure 5A). Fetuin-A levels in patients with active TB (median = 210.7 μg/ml; IQR = 178.1-235.7) were also significantly lower than those in control subjects (median = 299.4 μg/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

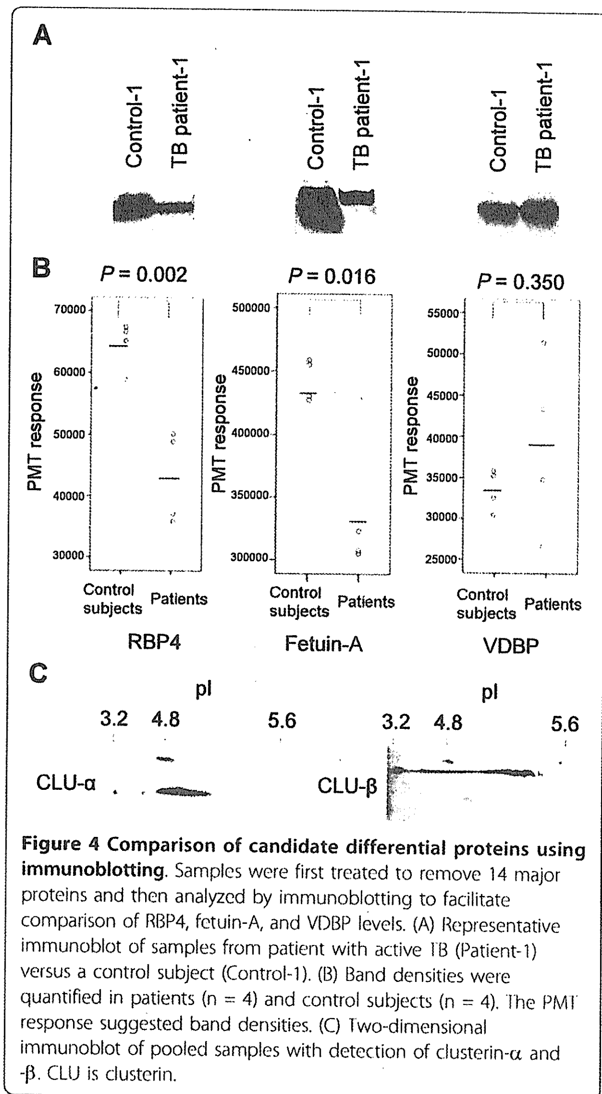

Figure 4 Comparison of candidate differential proteins using immunoblotting. Samples were first treated to remove 14 major proteins and then analyzed by immunoblotting to facilitate comparison of RBP4, fetuin-A, and VDBP levels. (A) Representative immunoblot of samples from patient with active TB (Patient-1) versus a control subject (Control-1). (B) Band densities were quantified in patients (n = 4) and control subjects (n = 4). The PMT response suggested band densities. (C) Two-dimensional immunoblot of pooled samples with detection of clusterin-α and -β. CLU is clusterin.

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.

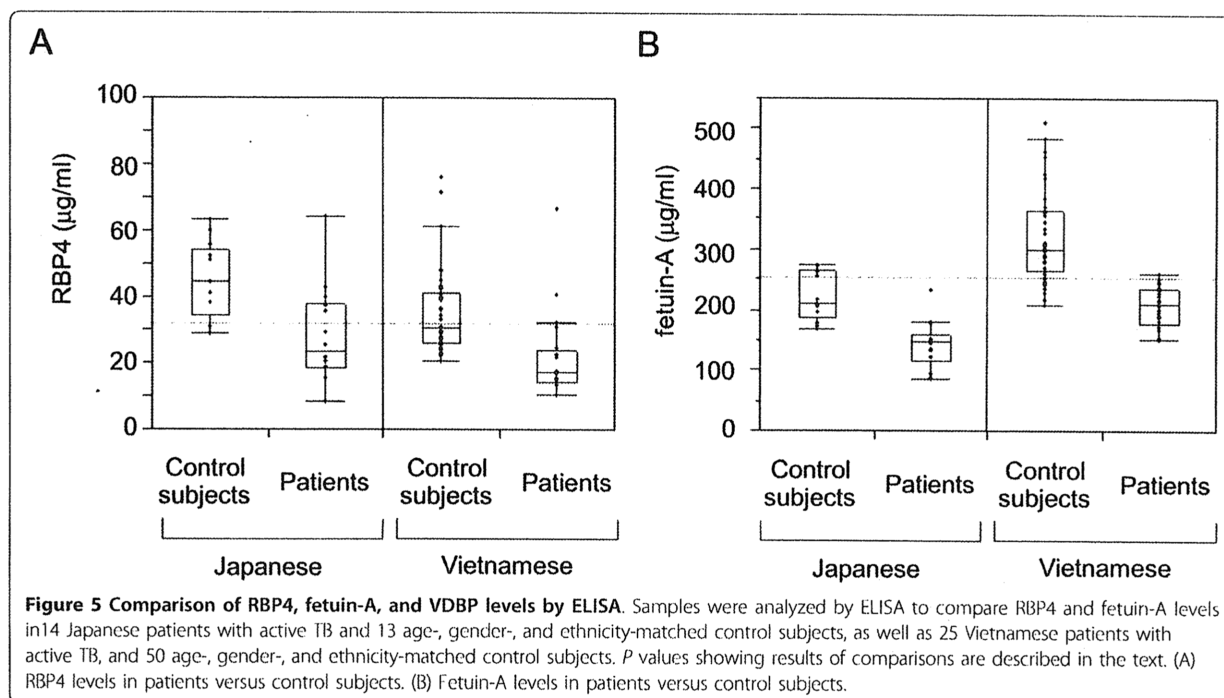

Figure 5 Comparison of RBP4, fetuin-A, and VDBP levels by ELISA. Samples were analyzed by ELISA to compare RBP4 and fetuin-A levels in14 Japanese patients with active TB and 13 age-, gender-, and ethnicity-matched control subjects, as well as 25 Vietnamese patients with active TB, and 50 age-, gender-, and ethnicity-matched control subjects. *P* values showing results of comparisons are described in the text. (A) RBP4 levels in patients versus control subjects. (B) Fetuin-A levels in patients versus control subjects.

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 H12482 (RET4 = RBP4) (E) Mascot search result for H11248 (fetuin-A) (F) Mascot search result for H11240 (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 immunosolvent 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 LTI 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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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-Tube™ (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 QIAamp™ 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/m^2, $P=0.0001$) (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/mm3	442	87.7
	<1,000/mm3	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 TBAg-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).

TBAg-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 (TBAg-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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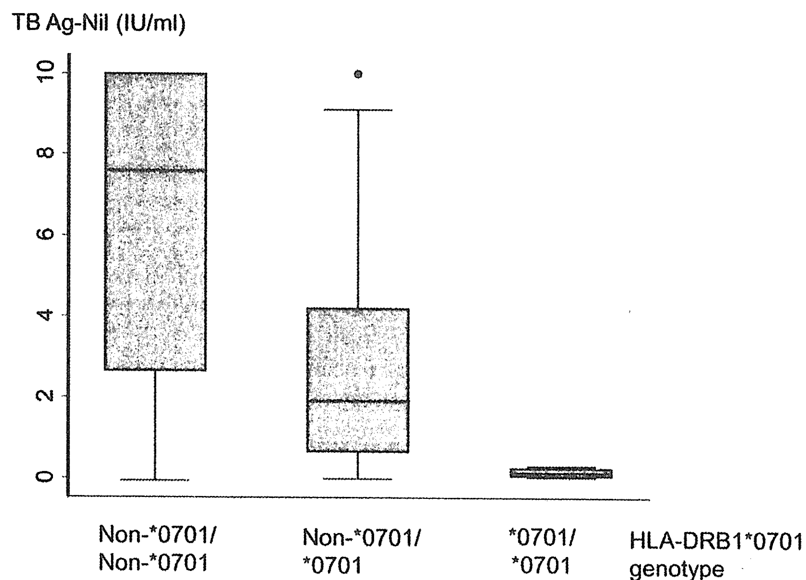

Figure 1. IFN-γ response to TB antigens stratified by HLA-DRB1*0701 genotypes in smear-positive/culture-positive pulmonary TB patients (n = 504). HLA-DRB1*0701 genotype significantly affected TB-Ag specific IFN-γ response (TBAg-Nil) ($P < 0.001$). IFN: Interferon; TB: Tuberculosis; HLA: Human leukocyte 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/m^2, 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 TBAg-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 silico* 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/mm3)	≥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 TBAg-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	1.48–19.79	3/77 (3.9)	1.82	0.29–11.18
	16.0–18.5	11/206 (5.3)	2.65	0.79–8.85	9/206 (4.4)	1.92	0.43–8.48
	≥25.0	0/3 (0.0)	NA	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	1.78–22.92	12/44 (27.3)	99.59	15.85–625.61
HLA-DRB1*0701 (the number of alleles)			5.09†	2.31–11.22		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 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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ORIGINAL INVESTIGATION

Association of *IFNGR2* gene polymorphisms with pulmonary tuberculosis among the Vietnamese

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Abstract Interferon-γ (IFN-γ) is a key molecule of T helper 1 (Th1)-immune response against tuberculosis (TB), and rare genetic defects of IFN-γ receptors cause disseminated mycobacterial infection. The aim of the present study was to investigate whether genetic polymorphisms found in the Th1-immune response genes play a role in TB. In our study, DNA samples were collected from two series of cases including 832 patients with new smear-positive TB and 506 unrelated individuals with no history of TB in the general population of Hanoi, Vietnam. Alleles of eight microsatellite markers located around Th1-immune response-related genes and single nucleotide polymorphisms near the promising microsatellites were genotyped. A set of polymorphisms within the interferon gamma receptor 2 gene (*IFNGR2*) showed a significant association with protection against TB ($P = 0.00054$). Resistant alleles tend to be less frequently found in younger age at diagnosis ($P = 0.011$). Luciferase assays revealed high transcriptional activity of the promoter segment in linkage disequilibrium with resistant alleles. We conclude that the polymorphisms of *IFNGR2* may confer resistance to the TB development of newly infected individuals. Contribution of the genetic factors to TB appeared to be different depending on age at diagnosis.

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Introduction

Tuberculosis (TB) remains one of the major health problems worldwide (Lopez et al. 2006): According to an estimate, approximately one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), and more than 9 million people develop active TB disease every year. Of these patients, 80% are from 22 high-burden countries including Vietnam (World Health Organization 2010).

Development of TB has been considered to be a two-stage process, infection with *M. tuberculosis* and progression to disease. In total, 5–10% of immunocompetent individuals initially infected with *M. tuberculosis* develop active TB during their lifetime (Frieden et al. 2003). When young vulnerable individuals fail to inhibit growth of the pathogen, they often develop the disease within 2 years of infection. In the remaining individuals, containment of *M. tuberculosis* is successful, though the agent is not

eliminated completely, which leads to life-long latent infection (Russell 2007). When immune levels are impaired after years of infection, reactivation of dormant bacteria leads to disease manifestation, which contributes to the development of elderly TB, though new TB patients affected by re-infection also have to be taken into account (Tufariello et al. 2003). Protective immunity to control the initial infection, orchestrated by immune cells including T cells and macrophages, is influenced by a variety of factors including genetic predisposition (Möller et al. 2010a).

T helper 1 (Th1)-type immune system is crucial to protection against mycobacterial diseases, in which interferon-γ (IFN-γ) has a key role (Lin and Flynn 2010). Although T cell response to mycobacterial infection in human beings is difficult to be addressed experimentally (Cooper 2009), it is known that genetic defects of Th1 molecules can be found in genes such as interferon gamma receptor 1 (*IFNGR1*), interferon gamma receptor 2 (*IFNGR2*), signal transducer and activator of transcription 1, 91 kDa (*STAT1*), interleukin 12B (*IL12B*) and interleukin 12 receptor, beta 1 (*IL12RB1*) cause severe mycobacterial diseases (Zhang et al. 2008). These observations have highlighted IFN-γ/interleukin-12 (IL-12) axis and their polymorphisms have been investigated in mycobacterial infection: association of promoter polymorphism in *IFNGR1* with TB was reported in African populations in independent studies, whereas association of *IFNGR2* with TB has not been published in the literature (Cooke et al. 2006; Stein et al. 2007). Associations with *IL12B* and *IL12RB1* were not consistently shown (Möller et al. 2010b). In the present study, we analyzed genetic polymorphisms of major Th1 cytokine receptors (*IFNGR1*, *IFNGR2*, *IL12RB1* and *IL12RB2*) and signal transduction molecules (*STAT1* and *STAT4*) in Hanoi–Vietnamese and reported a disease association and functional significance of polymorphisms in *IFNGR2*.

Materials and methods

Study population

The patients and control subjects were recruited in Hanoi, Vietnam (Horie et al. 2007). In total, 832 smear-positive pulmonary TB patients without previous episodes of TB (age 41 ± 14.4, males 77.6%) and 506 healthy volunteers without previous and present history of TB (age 37 ± 10.3, males 50.0%) participated in this study. All of them were unrelated Hanoi, Vietnamese. TB patients were all recruited immediately after the diagnosis was made. The TB panel A (n = 277, age 41 ± 13.5, males 73.3%) was collected in 2003–2004, and the second TB panel B (n = 555, age 41 ± 14.8, males 79.8%) was collected in 2007–2009. Pulmonary physicians diagnosed all the patients as new active pulmonary TB and treated them with anti-TB drugs based on the guidelines of the national TB program. Informed consent was obtained from all participants. The study protocol was approved by the ethics committees of the Ministry of Health, Vietnam and the National Center for Global Health and Medicine, Japan. Since 4 patients in panel A were human immunodeficiency virus (HIV) positive by previously described PCR assay (Panteleeff et al. 1999) with minor modifications and 49 patients in panel B were HIV seropositive (Hang et al. 2011), they were excluded from further analysis.

Microsatellite markers

We used eight microsatellite markers (*IFNGR1*-MS1, *IFNGR2*-MS1, *IFNGR2*-MS2, *IL12RB1*-MS1, *IL12RB2*-MS1, *IL12RB2*-MS2, *STAT1*-MS1 and *STAT4*-MS1) located in the major Th1-immune response genes (Tanaka et al. 2005) for screening of genetic polymorphisms associated with active TB. A part of the samples, 98 TB patients from the TB panel A and 200 controls were analyzed as described under (Tanaka et al. 2005).

Single nucleotide polymorphisms (SNP) screening in *IFNGR2* of Vietnamese samples

Forty-eight control samples were subjected to PCR amplifications of promoter and seven exon regions of *IFNGR2* and their sequences were analyzed for polymorphisms. GC content of genomic sequence upstream of the translation initiation codon was high (78.2% of nucleotides −1 to −500), and PCR condition was optimized for GC-rich template. The genomic DNA was extracted from anticoagulated blood with QIAamp DNA midi kit (QIAGEN, Hamburg, Germany). PCR was performed using TaKaRa LA Taq with GC buffer I (TaKaRa, Shiga, Japan) with primers 5′-CTCCCAACAGGCGTCAAACGACATGGTG-3′ and 5′-TGGTCCCTGCTCCACCGCTGCTACTACAAA-3′. PCR cycling condition was 40 cycles of 95°C for 30 s, 67°C for 30 s and 72°C for 2 min. Amplified products (1,607 bp) were purified and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using 3100 Genetic Analyzer (Applied Biosystems), with primers 5′-AGCTTAATATGTACTTTGGGG-3′ and 5′-CACCCACTCTGAGCACCCGG-3′. This method was also used for the typing of three promoter SNPs, rs8134145, rs8126756 and rs17882748. Sequencing primers that have the allelic variant at their 3′-end 5′-GGAGGGGTGGGGGCTCCAGGGAAA-3′, 5′-GCAGGGCCCGCCTCTTCCCGAGCA-3′ and 5′-GGGCTCCAGGGAAAGCCCGGGGGT-3′ were also designed, and allele-specific sequencing was

performed to directly determine the haplotypes of the three promoter SNPs.

Selection of representative SNPs around *IFNGR2* and genotyping

Representative SNPs around *IFNGR2* were selected from HapMap database (The International HapMap Consortium 2005). SNP genotype data of Han Chinese in Beijing (CHB) encompassing 350 kb from *IL10RB* to *CRYZL1* were analyzed by Haploview 4.2 (Barrett et al. 2005), and 27 representative SNPs were chosen based on the method of block-by-block tags in linkage disequilibrium (LD) blocks determined by confidence interval method (Gabriel et al. 2002). The *IFNGR2* SNPs identified as mentioned above and selected SNPs were genotyped in 273 TB patients of panel A and 506 controls. Genotyping was performed by the Digitag2 assay that has previously been described in another study (Nishida et al. 2007).

Rapid amplification of cDNA end (5′-RACE) of *IFNGR2*

The exact 5′ end of exon 1 was confirmed with FirstChoice RLM-RACE Kit (Ambion, Austin, TX, USA) using total RNA of THP-1 cells (ATCC TIB-202) stimulated with 10 ng/ml of phorbol myristate acetate (Schwende et al. 1996), U937 cells (ATCC CRL-1593.2) and Jurkat cells (ATCC TIB-152).

Luciferase assay

Promoter region of *IFNGR2* (Rhee et al. 1996) consisting of 1,167 bp (position −1,172 to −6 of initiation codon) was amplified by PCR and inserted into *Xho* I and *Bgl* II sites of pGL4.10 vector (Promega, Madison, WI, USA). Three plasmids of the observed haplotypes (CCC, ATC, ATT of rs8134145, rs8126756 and rs17882748) were constructed, and their sequences were confirmed to be devoid of any additional nucleotide difference. Reporter plasmids were mixed with pRL-TK (Promega) and transfected to Jurkat human T-cell leukemia cells with Lipofectamin LTX (Invitrogen, Carlsbad, CA, USA) in triplicate. Cells were harvested after 24 h and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). The transfection experiments were repeated twice with three independent subclones of each plasmid.

Statistical analysis

Disease associations with markers were assessed by Chi-square test or Fisher's exact test, and *P* values less than 0.05 were considered significant. Statistical analysis was performed using Stata version 10 (StataCorp, College Station, TX, USA). When necessary, *P* values were subjected to Bonferroni's correction for multiple comparisons. To determine whether genotype frequencies in the populations are compatible with Hardy–Weinberg equilibrium, Hardy–Weinberg exact tests were carried out using the program Arlequin version 3.11 (Excoffier et al. 2007). To assess pairwise LD between polymorphisms, we calculated Lewontin's D' and r square (r^2) for polymorphisms by Haploview version 4.2 (Barrett et al. 2005). TB disease associated with genetic variations was assessed by odds ratios unadjusted or adjusted for sex, age at recruitment and its interaction using logistic models. Tendency of having resistant alleles in the order of age at diagnosis was also tested using a similar logistic model within the TB group. Difference in luciferase activity between the haplotype under consideration and the other haplotypes was assessed by Wilcoxon rank sum/Mann–Whitney *U* test.

Results

Microsatellite markers

Microsatellite marker *IFNGR2*-MS1 located in 5′-upstream region of *IFNGR2* showed significant association with TB even after Bonferroni's correction (Table 1) and the frequency of *IFNGR2*-MS1-325 allele was significantly lower in TB patients than in controls (Supplementary table 1). *IFNGR2*-MS2, the other microsatellite was located in intron 2 of *IFNGR2* and the frequency of *IFNGR2*-MS2-252 allele was also lower in TB patients than in controls (uncorrected $P = 0.0024$), but not significant after Bonferroni's correction. *IFNGR2*-MS1-325 allele and *IFNGR2*-MS2-252 allele were in LD ($D' = 0.91$, $r^2 = 0.64$).

Screening of genetic polymorphisms in *IFNGR2*

Forty-eight control samples were subjected to PCR amplifications of promoter and seven exons of *IFNGR2* and their sequences were analyzed for possible polymorphisms. In the exonic sequences of *IFNGR2*, a non-synonymous SNP, rs9808753 was found in exon 2, and another SNP, rs1059293 was shown in 3′-untranslated region (UTR) of exon 7, while there were no SNPs in exon–intron boundaries. In the 5′ region up to −850 bp of the translation initiation codon, three SNPs, rs8134145, rs8126756 and rs17882748 were also identified.

Genotyping of selected SNPs around *IFNGR2*

Association of microsatellite markers of *IFNGR2* with TB prompted us to identify relevant SNPs that may show

Table 1 Association results for microsatellite markers

Marker	Locus	No. of alleles (total)	No. of alleles (<5% grouped[a])	*P* value[b] (2 × m)	Minimum *P* value[b] (2 × 2)	*P* value[c] Corrected	*P* value HWE
IFNGR1-MS1	6q23.3	14	7	0.419	0.0549	NS	0.4858
IFNGR2-MS1	21q22.11	8	5	0.016	0.0009	0.036	0.2762
IFNGR2-MS2		6	4	0.013	0.0024	NS	0.0326
IL12RB1-MS1	19p13.1	4	4	0.366	0.1600	NS	0.1606
IL12RB2-MS1	1p31.3-p31.2	12	6	0.155	0.0267	NS	0.7499
IL12RB2-MS2		6	4	0.540	0.2228	NS	0.7289
STAT1-MS1	2q32.2	13	5	0.563	0.3010	NS	0.0254
STAT4-MS1	2q32.2-q32.3	11	5	0.232	0.1046	NS	0.5243

NS not significant, *HWE* Hardy–Weinberg equilibrium

[a] Alleles with frequencies less than 5% were grouped

[b] Fisher's exact test

[c] Bonferroni's correction. 2 × 2 minimum *P* value was multiplied by the numbers of total alleles (40)

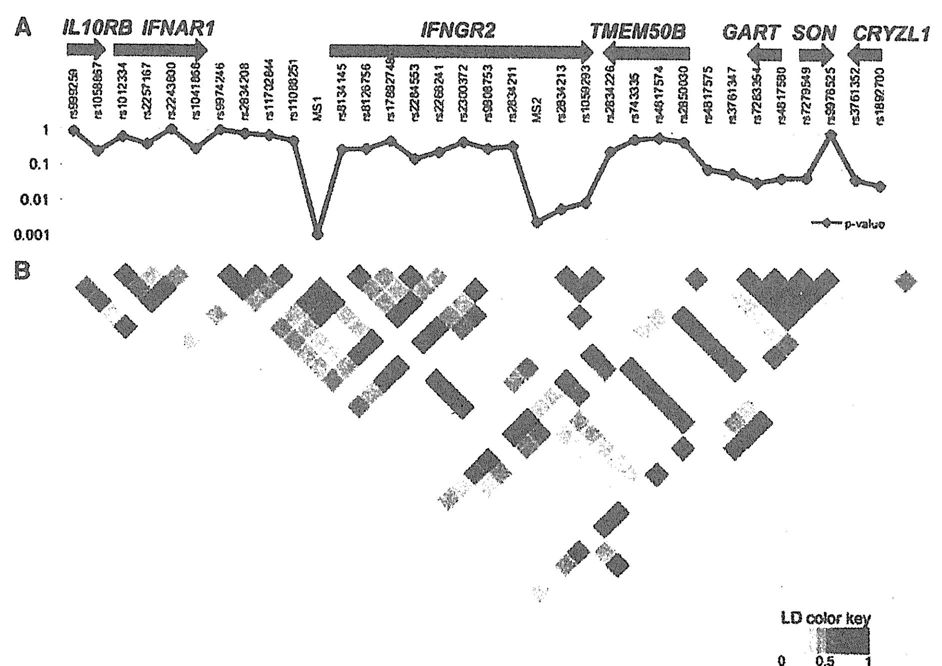

Fig. 1 Association *P* values and pairwise LD of genotyped polymorphisms around *IFNGR2* region. **a** Association results for 32 SNPs in 273 patients (panel A) and 506 controls are shown. *P* values of microsatellite markers in 98 patients and 200 controls are also included. Positions of genes are shown on the top. **b** Pairwise LD (r^2) between 32 SNPs and 2 microsatellite markers determined by the Haploview program is shown. In the calculation of pairwise LD, microsatellite alleles except for one showing the smallest *P* value were grouped, and the microsatellite locus was regarded as having only two alleles

further association. We genotyped 27 SNPs selected around *IFNGR2*, 3 SNPs in the 5′ upstream region, a non-synonymous SNP in exon 2, and a 3′UTR SNP in exon 7 of *IFNGR2* in 273 TB patients (panel A) and 506 controls (Fig. 1, Supplementary table 2). The rs2834213 SNP in intron 2 and the rs1059293 SNP in 3′UTR were associated with TB ($P = 0.0073$, OR 0.69 95% confidence interval [CI] 0.52–0.91; $P = 0.0088$, OR 0.70 95% CI 0.54–0.92). These SNPs were in Hardy–Weinberg equilibrium in the control group. We confirmed that SNPs in other nearby genes were not associated with the disease (Supplementary table 2). As expected, the resistant G allele of rs2834213 and C allele of rs1059293 were both in LD with *IFNGR2*-MS1-325 allele and *IFNGR2*-MS2-252 allele (Supplementary Fig. 1). Particularly, the resistant G allele of rs2834213 in intron 2 was in high LD with *IFNGR2*-MS1-325 allele ($D' = 0.94$, $r^2 = 0.83$), that is located 1.9 kb upstream of the translation initiation codon.

Directly determined haplotypes consisting of three SNPs in the 5′ GC-rich region of *IFNGR2*

In addition to single SNPs associated with the disease, we also characterized a set of SNPs in the 5′ GC-rich region of

the gene, rs8134145, rs8126756 and rs17882748, since these three SNPs are closely located within 300 bp upstream of the transcription start site as discussed below, which may influence *IFNGR2* expression. When we directly determined haplotypes of three 5′ SNPs by allele-specific sequencing in 273 patients and in 506 controls, three common haplotypes (CCC, ATC and ATT) accounted for 99.7% of chromosomes. The haplotype ATC was in high LD with the intron 2 SNP rs2834213 ($D' = 0.97$, $r^2 = 0.82$), and frequencies of the ATC haplotype were significantly lower in patients than in controls ($P = 0.036$, OR 0.76 95% CI 0.58–0.99). Haplotypes carrying SNPs in the entire *IFNGR2* region and their frequencies were estimated in 273 patients and in 506 controls. Consequently, the G allele of the intron 2 SNP rs2834213, the C allele of the 3′UTR SNP rs1059293 and the directly determined haplotype ATC, are uniquely contained in the same haplotype as shown in Supplementary table 3.

Transcription start site (TSS) of *IFNGR2*

In the public database, the aforementioned 5′ SNPs, rs8134145, rs8126756, and rs17882748 are regarded as variants in 5′ UTR, since TSS of the reference cDNA sequence (NM_005534.3) is located at position −648 of the translation initiation codon. However, multiple TSS were actually reported in *IFNGR2,* the positions of which were distributed from the initiation codon to almost 990 bp upstream, presumably due to cell type differences (Rhee et al. 1996). For this reason, we determined the 5′ ends by 5′ RACE in our study. As a result, TSS obtained from all immune cell lines tested were 121 bp upstream of the initiation codon. Thus, the positions of the three SNPs were calculated as −295, −285 and −8 from the TSS, indicating that they are promoter variants in these cell types.

Association results of TB panel B

We selected the intron 2 SNP, rs2834213 as a representative SNP for the disease-resistant polymorphisms and genotyped 503 patients in TB panel B, which were compared with the original control subjects ($N = 506$) in Table 2. The G allele of rs2834213 was significantly associated with TB in panel B ($P = 0.0025$, OR 0.71 95% CI 0.57–0.89). In a logistic model to assess possible confounders, adjusted odds ratios was compared with non-adjusted odds ratios for the G allele, which were hardly affected by sex, age at recruitment and its interaction term, indicating that the *IFNGR2* SNP remained significantly associated with TB in dominant and recessive models respectively ($P = 0.016$ and $P = 0.004$; table not shown).

Furthermore, we set up another logistic model to examine the relationship between having the TB-resistant

Table 2 Association results of rs2834213 A/G SNP

Sample	Allele (frequency)		Genotype (%)			*P* value			OR (95% CI)		
	A	G	A/A	A/G	G/G	Allele	Genotype		Allele	Genotype	
							Dominant	Recessive		Dominant	Recessive
TB panel A	452 (0.837)	88 (0.163)	186 (68.9)	80 (29.6)	4 (1.5)	0.0073	0.047	0.0050	0.69 (0.52–0.91)	0.73 (0.57–0.92)	0.25 (0.08–0.72)
TB panel B	838 (0.833)	168 (0.167)	347 (67.0)	144 (28.6)	12 (2.4)	0.0025	0.015	0.0068	0.71 (0.57–0.89)	0.72 (0.56–0.95)	0.40 (0.20–0.80)
TB combined	1290 (0.834)	256 (0.166)	533 (69.0)	224 (29.0)	16 (2.1)	0.00054	0.0075	0.00048	0.70 (0.57–0.86)	0.73 (0.57–0.92)	0.35 (0.18–0.65)
Controls	786 (0.780)	222 (0.220)	311 (61.7)	164 (32.5)	29 (5.8)						

TB tuberculosis, *OR* odds ratio, *CI* confidence interval

Table 3 Tendency of having G allele (rs2834213) in the order of age strata at the time of diagnosis ($N = 757$)

Age at diagnosis (year)	GA or GG genotype (n/N)	(%)	Odds ratio per 10-year change* (95% CI)
16–25	35/124	28.2	0.88 (0.79–0.98)
26–35	43/171	25.1	
36–45	53/165	32.1	
46–55	54/171	31.6	
56–65	35/87	40.2	
65–	15/39	38.5	

* In a logistic model, the trend of having the G allele was calculated as odds ratio when the patients are 10-years younger at the time of diagnosis ($P = 0.019$)

G alleles (as binary outcome) and age at diagnosis (as a continuous variable). In patients from panel A and B ($n = 757$), the TB-resistant G allele was less frequently found, as the age at diagnosis was younger ($P = 0.011$). Similarly, in the age-stratified analysis, when the patients are 10 years younger at the time of diagnosis, the odds ratio (OR) for having the G allele was 0.88 (95% CI, 0.79–0.98) and this trend remained significant ($P = 0.019$) (Table 3).

Luciferase assay

We constructed plasmids containing 5′ fragments in which only nucleotide sequences of the three promoter SNPs rs8134145, rs8126756, and rs17882748 are different and measured transcriptional activity of the three promoter segments (CCC, ATC, and ATT) in Jurkat human T-cell leukemia cells. Consequently, the resistant ATC haplotype had significantly higher transcriptional activity than CCC haplotype and ATT haplotype ($P = 0.037$ respectively) by Mann–Whitney U test (Fig. 2).

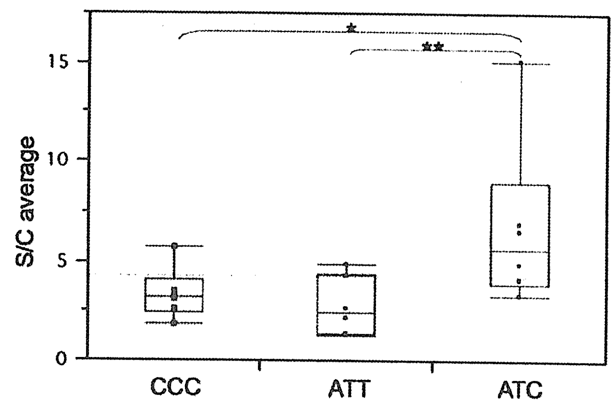

Fig. 2 Dual luciferase reporter assays. The ratios of Firefly luciferase activity (signal S) to Renilla luciferase activity (control C) are displayed using *box* and *whisker plots*. Three subcloned plasmids were prepared, and each subcloned plasmid was tested in triplicate and S/C values were averaged. The experiments were carried out twice independently. As a result, six independent S/C values were obtained for each haplotype. ATC haplotype showed significantly higher transcriptional activity than CCC haplotype and ATT haplotype (*, **$P = 0.037$, respectively) by Mann–Whitney U test. No significant difference was observed between CCC and ATT haplotypes (data not shown)

Discussion

IFN-γ plays a crucial role in host defense against intracellular pathogens mainly through activation of macrophages and regulation of Th1 cell response (Boehm et al. 1997). IL-12 released from dendritic cells and macrophages drives production of IFN-γ via IL-12 receptors, IL12RB1 and IL12RB2, on Th1 cells and subsequent activation of STAT4. In turn, IFN-γ binds to IFN-γ receptors composed of IFNGR1 and IFNGR2 subunits and transduces STAT1 signals to target cells (Bach et al. 1997).

In this study, we first screened eight microsatellite markers within the genes encoding these Th1 cytokine receptors and signal transducers, and demonstrated that the *IFNGR2* marker alleles showed significant association with active TB. SNPs around the gene were analyzed and a strong disease association with the intron 2 SNP rs2834213, the 3′UTR SNP rs1059293 and the 5′ promoter segment characterized by three SNPs was thus demonstrated. Possible influence of population substructure was kept to a minimum, since their ethnicity was Hanoi Vietnamese in which more than 99% were the Kinh people (Hoa et al. 2008).

To our knowledge, this is the first report of *IFNGR2* polymorphisms associated with TB. Intron 2 SNP, rs2834213 was most robustly associated with TB, but its biological importance is currently unclear. Indeed, it was not located near the splice sites (5,582 nucleotides downstream of splice donor site and 877 nucleotides upstream of splice acceptor site). A SNP in 3′UTR of exon 7 rs1059293 was in strong LD with the rs2834213 and also associated with TB, but it was 99 nucleotides upstream of polyadenylation signal. *IFNGR2* did not carry any non-synonymous SNP in high LD with rs2834213.

In an attempt to search functional polymorphism(s) in strong LD with the intron SNP (rs2834213) further, we identified the 300 bp promoter segment containing three SNPs. HapMap database does not have data of the three promoter SNPs, presumably due to high GC content that hinders high throughput genotyping method. The direct haplotyping revealed that it was also associated with the disease as well as the intron 2 SNP. Although we demonstrated that the promoter ATC haplotype showing an inverse disease association has high transcriptional activity in vitro and may confer resistance to TB, we could not

conclude which polymorphism around *IFNGR2* is primarily responsible for the disease until the functional roles of other SNPs showing more robust association are fully studied.

Among previous TB association studies with Th1-related genes, CC genotype at the -56 C/T SNP (rs2234711) of *IFNGR1* was repeatedly associated with TB in African populations (Cooke et al. 2006; Stein et al. 2007). In our study, *IFNGR1*-MS1-158 allele was in strong LD with −56 SNP (Tanaka et al. 2005), but this *IFNGR1* marker allele was not associated with TB. The lack of association is presumably because of insufficient power to detect weak genetic effects. Otherwise, it could be due to population-specific LD, when the true causative variant was not −56 SNP itself.

Experimental data have shown that IFNGR2 is a key regulator for IFN-γ-STAT1 signaling in T cells (Schroder et al. 2004; Regis et al. 2006). During the development of Th1 cells, *IFNGR2* transcription is reduced in the IFN-γ rich condition and this reduction alleviates a potentially harmful anti-proliferative action of IFN-γ-STAT1 signaling. However, IFNGR2 expression is not completely suppressed, because temporary activation of STAT1 is still necessary for Th1 system. *IFNGR2* transcription is thus fine-tuned during the Th1 differentiation process. In the promoter region, the transcriptional activity of the resistant haplotype ATC was higher than the other two common haplotypes in the Jurkat T cell line at baseline levels. Although physiological modulation of *IFNGR2* expression is not easily simulated in a single cell-type model, this segment may have a potential to influence Th1 function through *IFNGR2* regulation.

In this study, another interesting finding is that the resistant allele tend to be less frequently observed in younger patients at the time of diagnosis, a surrogate for age at onset in new patients. This effect was moderate but significant. The allele frequency in older age at diagnosis nearly reached the level of the control population. It is likely that the elderly kept latent infection of *M. tuberculosis* for long years, and the age-associated decline in immune response caused development of active TB, while the younger patients developed active TB soon after initial infection (Tufariello et al. 2003). In intermediate or low burden countries, there are more elderly patients and the effect of the resistant allele of *IFNGR2* may be smaller.

Moreover, in African countries with high rates of TB and HIV co-infection, HIV is the strongest risk factor for TB development (Reid et al. 2006). By contrast, the proportion of HIV-positive TB patients is only 8.8% in the Vietnamese TB panel B and 1.4% in TB panel A, therefore possible effect of the resistant allele on HIV infection could not be determined in this study. In the previous reports, other polymorphisms of *IFNGR2* were associated with liver fibrosis of chronic hepatitis C virus infection and with viremia of hepatitis B virus infection (Nalpas et al. 2010; Huang et al. 2011). Because IFN-γ is a key cytokine for the control of infectious diseases, association of *IFNGR2* polymorphisms with HIV infection needs be clarified.

One limitation in our study is a single control panel of the Vietnamese population. Results of the first case–control set were only partially confirmed because of incomplete independence of the two study sets, though sample size itself was not small. Another limitation is that our control panel may include asymptomatic individuals with latent TB infection, because performing tuberculin testing is not common in Vietnam. Considering two-stage process of infection with the pathogen and progression to disease, we cannot directly specify which stage of TB was more affected by *IFNGR2* in our study population. Future use of interferon gamma release assays to detect latent infection of *M. tuberculosis* in this field might be helpful to arrive at a solution (Pai et al. 2008). Because of the complexity of LD structure and the age-dependent effect as regards these variations, carefully conducted studies should be undertaken to reproduce our results in other populations. Validation studies by re-sequencing are also warranted. In non-Asian populations, however, the LD of rs2834213 does not appear to reach the promoter region of *IFNGR2* (data not shown), indicating that the functional promoter haplotype may not be easily found in disease marker association studies by the conventional tag SNP-based approach in other populations.

We conclude that the polymorphisms of *IFNGR2* may confer resistance to TB in Vietnam. It appeared to be different depending on age at diagnosis. Further functional studies are needed to elucidate the genetic susceptibility to TB, fully considering complicated immune process regarding early or late onset of the disease.

Ethical standards We declare that these experiments comply with the current laws of Japan and Vietnam.

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Conflict of interest The authors declare that they have no conflict of interest.

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