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ORIGINAL ARTICLE

Genome-wide SNP-based linkage analysis of tuberculosis in Thais

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Tuberculosis, a potentially fatal infectious disease, affects millions of individuals annually worldwide. Human protective immunity that contains tuberculosis after infection has not been clearly defined. To gain insight into host genetic factors, nonparametric linkage analysis was performed using high-throughput microarray-based single nucleotide polymorphism (SNP) genotyping platform, a GeneChip array comprised 59 860 bi-allelic markers, in 93 Thai families with multiple siblings, 195 individuals affected with tuberculosis. Genotyping revealed a region on chromosome 5q showing suggestive evidence of linkage with tuberculosis (Z(lr) statistics = 3.01, logarithm of odds (LOD) score = 2.29, empirical P-value = 0.0005), and two candidate regions on chromosomes 17p and 20p by an ordered subset analysis using minimum age at onset of tuberculosis as the covariate (maximum LOD score = 2.57 and 3.33, permutation P-value = 0.0187 and 0.0183, respectively). These results imply a new evidence of genetic risk factors for tuberculosis in the Asian population. The significance of these ordered subset results supports a clinicopathological concept that immunological impairment in the disease differs between young and old tuberculosis patients. The linkage information from a specific ethnicity may provide unique candidate regions for the identification of the susceptibility genes and further help elucidate the immunopathogenesis of tuberculosis.
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Introduction

Tuberculosis remains a serious public health problem in the developing world, especially in view of recent outbreaks of virtually untreatable, extensive drug-resistant tuberculosis.[1] A majority of individuals in endemic areas are infected with the pathogen *Mycobacterium tuberculosis* when they reach adulthood. In 2005, around 5 million individuals were diagnosed as having tuberculosis according to a WHO surveillance report.[2]

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Approximately 10% of those who are infected develop tuberculosis in their lifetime. Clinically and epidemiologically, three patterns of disease development after infection are often assumed, depending on the age at onset of tuberculosis and the prevalence of tuberculosis infection: (1) primary tuberculosis in adolescence; (2) reactivation of disease after infection and (3) exogenous re-infection in adulthood.[3] Primary tuberculosis is a disease that develops within the first few years after infection, often because of impaired host immunity, whereas reactivation of disease occurs later in life, after a long period of immune protection against development of the disease in infected individuals, together with the possibility of exogenous re-infection. Identification of a high-risk group corroborated by a specific mechanism for disease development would be one of the most desirable measures for controlling this disease in developing

countries, in which use of chemoprophylaxis for all infected individuals is an unacceptable burden on national tuberculosis control programs.

Contribution of host genetic factors to the development of this infectious disease has been classically observed. Until Koch[4] discovered the tubercle bacilli as the causative agent of tuberculosis, familial aggregation resulted in the initial perception that tuberculosis was a hereditary disease in the 19th century. An early study showed that monozygotic twins have higher risks of tuberculosis than dizygotic twins, given tuberculosis affected one of the twins.[5] Individual genetic variations in innate immunity, adaptive immunity and intracellular bacterial killing ability have roles in different phenotypes of tuberculosis. Disruption of genes in the *IL12/23–IFNG* pathway results in disseminated mycobacterial infection in susceptible infants.[6] Various association studies implicated this as one of the key pathways for containing tuberculosis.[7–9] Mutation in genes with ubiquitination functions have also been implicated as risks in both tuberculosis and leprosy.[10,11] On the basis of databases of genetic association study, nearly 100 genes had been studied for their association with tuberculosis. From these candidate gene association studies, apart from genes in *IL12/23–IFNG* axis, only *SLC11A1* (formerly *NRAMP1*) had been consistently shown evidence of association with tuberculosis.[12]

Prior to candidate gene approach, reverse genetic study in humans is often useful. Susceptibility genes based on novel disease regulatory mechanisms can be identified by hypothesis-free studies, such as genome-wide linkage and genome-wide association studies. In African populations, linkage analysis for tuberculosis earlier demonstrated suggestive evidence for linkage in two regions on chromosome 15 and chromosome X. Subsequent fine mapping of the chromosome 15 regions revealed *UBE3A* as a candidate susceptibility gene for tuberculosis.[10,13] Suggestive evidence of linkage was also found on chromosomes 10q26, 11q12 and 20p12 in the Brazilian population.[14,15] The only significant linkage evidence (logarithm of odds (LOD) = 3.49) to date is a study of 96 Moroccan multiplex families that implicated an autosomal-dominant gene on chromosome 8q12–q13.[16]

Distribution of virulent *M. tuberculosis* strains such as Beijing strain differs among regions of the world,[17] which suggests its adaptation to different human populations.[18] In the context of the host–pathogen relationship, human genetic studies in Asia may also reveal novel susceptibility/resistance genes in this field. In this study, linkage analysis in the Thai population was performed to gain insight into host genetic epidemiology of tuberculosis in Asians.

Results

Description of studied families

The numbers of families with multiple siblings affected with tuberculosis are shown in Table 1. Because of limited genotyping resources, only affected siblings were genotyped, which provided better linkage evidence when compared with genotyping the unaffected individuals in these families.

Table 1 Number of families and sibling pairs in the linkage analysis

	Number of families
Two affected siblings	87
Three affected siblings	5
Four affected siblings	0
Five affected siblings	1
Total families	93
Total number of independent sibling pairs (number of affected siblings-1, per family)	101

A total of 199 individuals from 95 families were originally genotyped using the *Xba*I 50 K microarray system, which is part of the 100 K affymetrix genotyping system. In the relationship analysis of the GRR program, ambiguous relationships were clarified and corrections were made before linkage analysis. Two of the 95 families (four individuals) were excluded because the analysis revealed that the affected individuals were half-siblings on the basis of their genotypes.

A stringent genotype call strategy was used to reduce the number of genotyping errors; criteria for genotype calling were more stringent than the standard criteria proposed by the manufacturer. The average call rate (percentage of successful genotype calls among subjects) was 98.08%. By using more stringent call criteria, the final call rate was 99.48%.

Linkage analysis

Linkage analysis was carried out with MERLIN; analysis without linkage disequilibrium (LD) correction showed inflation of the linkage statistics and noisy linkage spike patterns throughout the genome. LD was taken into account for the analysis by using LD patterns described earlier and MERLIN, which implements a method accounting for LD by inferred haplotypes from single nucleotide polymorphisms (SNPs) that showed LD with each other and used the inferred haplotypes as multi-allelic markers for linkage analysis.

After LD correction, a maximum LOD score of 2.29 was observed on chromosome 5q23.2–31.3 at 138.3 cM, with the LOD-1 support interval between rs1515641 (SNP_A-1673674, 131.9086, 127.2 Mb) and rs252101 (SNP_A-1739565, 141.01 cM, 141.3 Mb) (Table 2 and Supporting information Figures 2a and b). Simulation tests showed this linkage to be genome-wide suggestive evidence of linkage (0.25 peaks per genome scan). Regions that showed LOD score >1 were located on chromosomes 4, 5, 6, 11 and 14 (Table 2). All of these regions could be good candidates for follow-up positional study.

Age at onset analysis

Ordered subset analysis was used to determine whether the evidence for linkage differs according to age at onset. The results at which LOD scores reached the uncorrected significance value (P-value <0.05) are shown (Table 3). Two regions on chromosome 17p13.3–13.1 and chromosome 20p13–12.3 showed significantly higher LOD

Table 2 Chromosome regions with a non parametric LOD score with nominal significance ($P<0.01$)

Chromosome	*Peak SNP*	*Position (cM)*	*Physical position*	*LOD score*[a]	P-*value*
5q23.2–31.3	rs7706155	138.37	135 987 344	2.29	0.0005
	rs9327759	138.39	136 004 959		
	rs2188468	138.42	136 026 947		
11p15	rs1487214	29.85	19 588 715	1.62	0.003
14q32	rs8014257	109.60	98 321 756	1.48	0.005
6p12	rs10484980	78.13	54 559 585	1.33	0.007
4q26	rs2010003	121.27	118 807 065	1.3	0.007

Abbreviation: LOD, logarithm of odds.
[a]Calculated using the Kong and Cox linear model.

Table 3 Ordered subset analysis by minimum age at onset of tuberculosis in the families

Chromosome	*cM*	*Marker*	*No. of families in subset*	*Average age at onset (range)*	*Maximum LOD and (baseline LOD)*	P-*value*
17p13.3–13.1	13.5–13.7	rs2716912 rs10491086	32	19.47 (12–24) years	2.57 (0.14)	0.0187
20p13–12.3	10.6	rs750702	30	19.17 (12–23) years	3.33 (0.64)	0.0183

Abbreviation: LOD, logarithm of odds.

scores (permutation *P*-value at 0.0187 and 0.0183, respectively) (Figures 1a and b).

Discussion

In this study involving 93 Thai families (195 affected individuals), a region on chromosome 5q23.2–31.3 was shown to be a candidate region for tuberculosis susceptibility with suggestive evidence of linkage by genome-wide linkage analysis. From the ordered subset analysis, two regions on chromosome 17p13.3–13.1 and chromosome 20p13–12.3 were shown to have significant linkage with earlier onset of tuberculosis.

In the 5q candidate region, various genes, including a cluster of cytokine genes, *GM-CSF, IL3, IL4, IL5* and *IL13*, have been mapped. *IRF1*, a responsive element in the interferon-γ-mediated pathway, an antimicrobial peptide gene (*LEAP2*), and genes with ubiquitination activities, such as *APBB3* and *SPK1A*, have also been located. This chromosomal region has also been implicated in linkage analyses of the parasitic load of *Schistosoma mansoni*[19] and *Plasmodium falciparum*.[20] In addition, this region on chromosome 5 coincides with the *IBD5* region that showed significant evidence of linkage and association with Crohn's disease, an inflammatory bowel disease closely associated with *Mycobacterium paratuberculosis*.[21] It is interesting to note that the *IBD5* haplotype is distributed differently in Asian and European populations, and that the *IBD5* risk haplotype in Europeans is very rare in Asians.[22] Although candidate genes including *IL4*[23] and *CD14*[24,25] have been studied in this particular region, roles of these genes in tuberculosis remain inconclusive. Small sample size and ethnicity of the study populations may partially explain these equivocal association results. With this linkage evidence, polymorphisms within these genes should be the subjects for further validation in another Asian population.

From the functional viewpoint, this region has been reported to control the differentiation of Th2 lymphocytes, resulting in different levels of interleukin-4 (IL-4) and IL-5 from $CD4^+$ T-cell clones after exposure to parasite antigens, but not to affect the level of interferon-γ.[26] The predilection of this balance for Th2 may also play a role in susceptibility to tuberculosis. The predominant Th2 may partly influence tuberculosis susceptibility by the IL-4 and IL-13 capability of inhibiting autophagy by mycobacterium-infected macrophages.[27] IL-4 has been suggested to be a regulator of the immune response to tuberculosis antigen but not the disease *per se* in Brazilian families with multiple cases of tuberculosis.[23] This study suggests that the Th2 regulatory function of this region may play a more important role in tuberculosis susceptibility in the Thai population than in the Brazilian population. This genetic heterogeneity may reflect the host–pathogen interaction with specific mycobacterium strains: in Thailand, at least 20% of circulating strains are Beijing strains of Asian origin.[28] These virulent strains have been shown to preferentially stimulate Th2 response more than other non-virulent strains.[29]

From the ordered subset analysis of earlier onset of tuberculosis, the maximum linkage statistics (LOD = 3.33) on chromosome 20p were derived from 30 of the 93 families, and the average minimum age at onset of tuberculosis in these families was 19.17 years. The 20p region is of particular interest for tuberculosis susceptibility because it has been shown to have significant evidence of linkage with leprosy.[15,30] The susceptibility gene(s) in this region may play a role in susceptibility to primary tuberculosis in pediatric and adolescent cases. Several promising candidate genes in this region are a cluster of β-defensin genes that play important roles in innate immunity against respiratory infection.[31,32] The suggestive evidence of linkage for the region on chromosome 17p was derived from 32 families with the youngest age at onset, but with a lower maximum LOD score than for the 20p region. Although it is speculative at this stage, a possible candidate in the 17p13.3–13.1 region, *MYBBP1A* was recently identified as

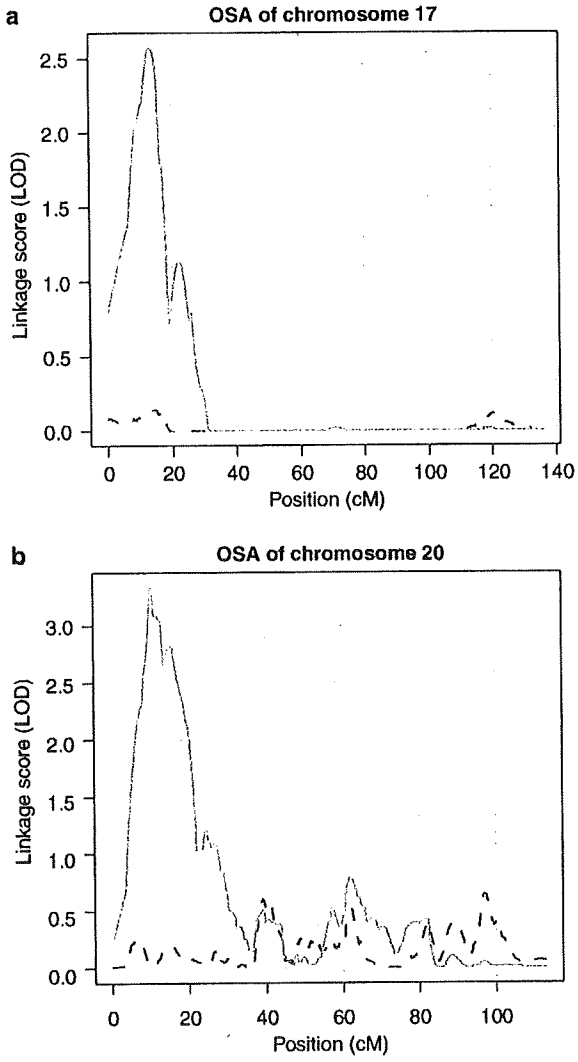

Figure 1 Ordered subset analysis (OSA) results on chromosome 17 and chromosome 20. The solid lines indicate the linkage statistics from the subset of families with younger age at onset. Thirty-two families and 30 families were grouped into the younger age at onset family for chromosome 17 (a) and chromosome 20 (b) respectively. The dotted lines indicate the statistics when all families were included in the analysis. (Image file = Fig1(a)_OSAChr17_black.pdf) (Image file = Fig1(b)_OSAChr20_black.pdf).

a co-repressor of NF-κB.[33] Although these two chromosome regions provide statistical evidence of possible linkage in subsets of these families, small number of families in the ordered subset analysis requires further confirmatory evidence either through additional linkage analysis or association study.

As for the regions on chromosomes 8, 15 and X reported in African populations, we could not find any evidence of linkage. Discordance of the results may be partially explained by the genetic heterogeneity in tuberculosis susceptibility in different human populations, and also by the differences in the distribution of *M. tuberculosis* strains.[18] We could improve the linkage evidence by genotyping more parental samples, but this may not be realistic given the small number of families in whom the parents are alive and available for enrollment. Confirmation of the linkage in another study with a larger sample size with extended genotyping of Asian families with tuberculosis and fine mapping around these candidate regions would be necessary to reinforce our findings in the future. A genome-wide association study provides an alternative tool for selective re-genotyping of the linkage regions and also is capable of revealing common low risk alleles even below the detection limit of linkage analysis. These hypothesis-free, reverse genetic approaches would elucidate the pathogenic mechanism and might enable effective treatment and prevention strategies for tuberculosis in the future.

Materials and methods

Ascertainment and collection of families

Families were ascertained mainly through a tuberculosis surveillance system in Chiang Rai province, the northernmost province of Thailand. Additional families were also recruited through the Central Chest Hospital in Bangkok, Thailand. In total, 95 families with at least two siblings affected with tuberculosis were ascertained. These families included 199 individuals affected with tuberculosis. A parent in a sibling pair family was genotyped because this parent was also affected with tuberculosis. Tuberculosis was diagnosed by clinical characteristics and microbiological confirmation by sputum culture or at least two out of three positive sputum smears. In a minority of cases, the diagnosis was obtained from earlier records in the tuberculosis surveillance system supplemented by an abnormal current chest X-ray. This study was reviewed and approved by The Ethical Review Committee for Research in Human Subjects (Ministry of Public Health, Thailand) and the Institutional Review Board of the International Medical Center of Japan. The patients were tested for HIV using the standard serological test, and HIV-infected patients were not included in this study. Venous blood samples were collected from the patients after individual informed consent was obtained by our field researchers.

Genotyping

Genomic DNA extraction from blood samples was performed using the QIAamp blood midi kit (Qiagen, Hilden, Germany) and quantification was performed using a nanodrop spectrometer to a concentration of 50 ng μl^{-1}. The *Xba*I microarray chip of the mapping 100 K Array (Affymetrix, Santa Clara, CA, USA) was used for genotyping, following the standard genotyping protocol for the GeneChip Mapping 100 K Array. In summary, 5 μl of 50 ng μl^{-1} (250 ng) of genomic DNA were digested with the restriction endonuclease (*Xba*I) and ligated to adaptors with the T4 ligase that recognizes the cohesive four base-pair overhangs. A generic primer that recognizes the adaptor sequence was used to amplify adaptor-ligated DNA fragments. Preferential amplification of fragments in the 250–2000 bp size range was performed by the optimized PCR conditions. The amplified DNA was purified by the DNA amplification clean-up kit (Takara Bio, Shiga, Japan) and then fragmented, labeled and hybridized to the *Xba*I chip of the mapping 100 K set.

Genotype calling algorithm and quality control
Genotype calls for each chip were given by the BRLMM algorithm using a confidence call threshold of 0.3. The BRLMM algorithm is based on modification of the RLMM algorithm, which showed a call accuracy superior to that of the dynamic modeling algorithm.[34] The confidence call threshold was set at a lower threshold than the standard call threshold (0.5) to select only SNPs with higher call confidence and to reduce the effect of genotyping errors in linkage analysis. SNPs with missing rates of more than 5% were excluded from the later analysis. Thus, the average genotype call rate was 99.48% for 52 433 SNPs selected from 58 960 SNPs on the *XbaI* chip.

Quality control and Mendelian error checking
ALOHOROMA was used for quality control and preparation of input files and semi-automated linkage analysis using the Perl script.[35] The gender of each sample was checked with the number of heterozygosities at SNPs on chromosome X, and there was complete concordance between the reported sex and the genotyped sex. PedCheck was used for exclusion of markers that were in Mendelian inconsistence, and 339 genotypes data were deleted.[36]

Markers with significant deviation from Hardy–Weinberg's equilibrium (P-value <0.01) were filtered before the downstream analysis. The relatedness of these samples was analyzed using the GRR program, which analyzed the pair-wise relatedness for each pair of samples in the data set.[37] Mendelian error checking was also performed using MERLIN, with the error automatically wiped out before linkage analysis.[38] The map order and distances between SNP markers were based on the information provided by the manufacturer, with the flanking sequences of each of the SNPs aligned with the May 2004 release of the human genome sequence. LD patterns of these SNPs were determined using Haploview version 3.2. Because the tagging SNP selection algorithm had not been implemented for chromosome X in HAPLOVIEW V 3.2, we used the haplotype inference information based on a spine of strong LD ($D'>0.8$) for correction of LD in X chromosome analysis.[39] LD parameters were calculated by Haploview for every pair of markers situated within 1 Mb from each other. The LD information was used for the selection of tagSNPs to reduce the effect of LD on linkage statistics. TagSNPs were selected using a pairwise tagging strategy with $r^2>0.4$ as the threshold for the selection of independent markers. These tagSNPs in autosomal chromosomes and haplotype inferences in chromosome X were later used in linkage analysis by MERLIN. The final set of markers used for later analysis included 33 365 markers selected as the tagging set ($r^2<0.4$) from all available markers.

Linkage analysis
Nonparametric multipoint linkage analysis was carried out by MERLIN using all markers that passed the quality control criteria. LD was accounted for in the analysis by reanalyzing the data using haplotype block information derived from HAPLOVIEW (spine of strong LD, $D'>0.8$) on chromosome X and reanalyzing by using only tagSNPs ($r^2>0.4$ in the 1 MB region) in autosomal regions to reduce the effect of LD on linkage analysis. Population allele frequencies of these SNPs were inferred from unrelated individuals in the samples by MERLIN.

MERLIN recently provided a method to account for LD by determining the haplotype of SNPs in strong LD and using inferred haplotypes as multiallelic markers.[40] Effects of LD on the linkage analysis were investigated by comparison of nonparametric linkage statistics with and without haplotype block information and tagSNPs for linkage analysis (Supporting information Figure 2a and b). The significant levels of linkage statistics were assessed by 10 000 gene dropping simulation. The simulation was carried out with data from chromosome 22 to reduce the computation time. The numbers for each of the linkage statistics from the simulations were multiplied by 44 to compensate for the expected size of the whole genome compared with chromosome 22. The suggestive and significant evidences of linkage by this simulation were observed at LOD score levels of 1.79 and 3.12, respectively.

Ordered subset analysis with age at onset
Age at onset was taken into account for linkage analysis using the age at diagnosis of tuberculosis. When the age at diagnosis was not available, the patient's current age was imputed to replace the age at diagnosis. Only nine data points were imputed using the current age at ascertainment. Our ascertainment scheme only identified patients who developed tuberculosis within the past 10 years, and the current age at ascertainment should be fairly accurate for imputation. The age at onset was analyzed as a covariate using ordered subset analysis by the FLOSS program.[41] The minimum age at onset of tuberculosis was used as the covariate because it is more likely that tuberculosis occurred at a younger age caused by immunological impairment to tuberculosis rather than by repetitive external mycobacterium exposures or endogenous immune senescence when compared with tuberculosis in the elderly population. FLOSS accepts the MERLIN LOD score outputs per family for reanalyzing the linkage statistics by ordering the families by the supplied covariate. The linkage analysis was re-performed by adding one family at a time, and the highest linkage score from the ordered subset analysis was chosen as the plausible linkage when age at onset presented in the disease model. The significance of the ordered subset analysis was calculated by simulation through re-sampling of equal numbers of families that provided the maximum linkage statistics from all available families, and then the number of times the re-sampling statistics exceeded the highest observed statistics was counted. The significant levels of the ordered subset analysis can be considered chromosome-wide with a permutation P-value <0.025 and genome-wide significant with a permutation P-value <0.001. This analysis technique is robust against heterogeneity and should be useful in analysis of linkage in tuberculosis that showed two peaks of higher incidence in two different age groups, that is young adult and elderly populations.

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No evidence for association between the interferon regulatory factor 1 (*IRF1*) gene and clinical tuberculosis

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SUMMARY

Interferon regulatory factor 1 is a transcription factor involved in initiating a vigorous Th1 response during *Mycobacterium tuberculosis* infection. Therefore, we considered it as a possible candidate gene for certain polymorphisms to confer susceptibility to develop clinical tuberculosis. However, all polymorphisms with minor allele frequencies higher than 5% and haplotype frequencies in two Southeast Asian populations (Indonesian and Vietnamese) turned out not to be associated with pulmonary tuberculosis.

1. Introduction

Tuberculosis (TB) is still a major health problem worldwide. Although, it is mainly active in developing countries, it obtained a new impact in developed countries when linked to immune suppressions as for example after HIV infections.[48,7] About one third of the world's population is predicted to be infected by *Mycobacterium tuberculosis (M. tuberculosis)*[11] and it causes nearly 2 million deaths per year. However, of all infected subjects, only 5–10% develop clinical disease.[49]

The immune mechanisms in TB are well studied and it is known that the IL-12/IFN-γ axis plays a major role for the control and elimination of *M. tuberculosis*.[45,46] Although T cells, especially γδ T cells, are involved during the immune response,[16] macrophages play one of the most important roles and their activation status is crucial for controlling the infection.

During the last few years, resistance to tuberculosis was found to be connected to specific polymorphisms of genes of some of the major immune components. Specific single nucleotide polymorphisms (SNPs) in the *IL-12* or IL-12 receptor (*IL-12R*) gene, for instance, were related to higher susceptibility of tuberculosis.[3] Specific alleles of the *IFNG* gene led to higher amounts of IFN-γ,[30] and the presence of a low IFN-g producing genotype was overpresented in tuberculosis-infected patients.[44] Defects of the IFN-γ receptor led to higher susceptibility of mycobacterial infections,[17] and some alleles of the *IFNG* gene were associated to disease.[10,25,34] SNPs directly affecting the genes of macrophage activation, such as *SP110* in human[42] homologue to *Ipr1* in mice[29] and *MIF* (macrophage inhibitory factor)[13] first discovered as a proinflammatory T-cell cytokine[26] were also shown to have an impact in the control and elimination of mycobacterial infection.

Although some important factors for mycobacterial resistance are already known, it is essential to learn more about the fine tuning of the immune response in order to have better tools for manipulating the immune system in areas with a high incidence of mycobacterial infections such as Indonesia that ranks 3rd among 22-high burden TB countries in the world. In this regard, transcription factors play a very important role. Interferon regulatory factors (IRFs) are known to be important for the initiation and fine tuning of immune responses.[36,47] IRFs are a tightly regulatory network of gene regulation for genes that are important for anti-bacterial immunity.[14,40,41] Among those, IRF1 is known to play an important role in promoting an anti-bacterial immune response by

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targeting *NOS2*, *GBP1* and *gp91PHOX* genes,[18,21] promoting a vigorous cellular response by targeting IL-12[39] and IL-18,[9] influencing the hematopoietic cell development by targeting IL-15.[27] MyD88 associated IRF1 is a potent activator for IFN-β, NOS and IL-12p35.[24] IRF1 is essential to develop a T helper type 1 (Th1) response[22] and, thus, is important during *M. tuberculosis* infection.[31,33] It was also up-regulated upon infection with *M. tuberculosis* in the mice.[20] Therefore, we chose this transcription factor as our target gene to examine their possible association to the susceptibility to TB.

Human *IRF1* gene is a minus strand spanning 7.72 kb with a 495 bp promoter region and it has been assigned to 5q31.1,[14] a region contains the cytokine gene cluster and is frequently deleted in the malignant cells of patients with myelodysplasia and myeloid leukemia.[1] It encodes a 36.5 kDa protein. *IRF1* genomic sequence consists of 10 exons and there are 94 SNPs reported in publicly accessible genome database/GenBank.

2. Material and methods

2.1. Patients and control subjects

A case-control study was designed in 2006 by using diagnosed pulmonary TB patients ($n = 192$) from the West Java province of Indonesia. The patients were between the ages of 15 and 68 years old and their ethnic background was Javanese and Sundanese–Javanese, West Java with comparable sex ratio. Diagnosis of TB was based upon the presence of clinical symptoms, chest X-rays and microscopic detection of acid-fast bacilli in Ziehl–Nehlsen stained sputum smear.

In the same period, community healthy controls were collected from people of the same age, gender and area ($n = 192$). Controls had the same interview with standard questions and underwent the same physical examinations. Investigations were approved by the Ethical Committees of Yarsi University, Jakarta, Indonesia and Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

In the study of the Vietnamese population, 162 pulmonary TB patients underwent similar physical and laboratory examinations for TB as Indonesian were recruited to participate. As control, 132 healthy unrelated subjects were randomly selected from 20 communes of Hanoi, Vietnam, and blood samples were taken after obtaining the informed consent from each subject. Ethical approval for this research was obtained from the ethical committees of both the Ministry of Health of Vietnam and the International Medical Center of Japan.

2.2. Variation screening and Genotyping of SNPs on IRF1

Genomic DNA was extracted from peripheral blood using a commercial kit (QiaAmp Blood Mini Kit, Qiagen). Polymerase chain reactions were performed in a final volume of 15 μl, containing 10 ng genomic DNA, 1.5 pmol of each primer, 2 mM of dNTP, 10× PCR buffer, 5× GC-rich solution and 0.6 U FastStart Taq Polymerase (Roche Applied Science, Mannheim, Germany). Each PCR was performed with a hot-start procedure at 95 °C for 10 min. The amplification process was carried out using 40 cycles of denaturation at 94 °C for 1 min, with annealing temperature between 59.2 °C and 62.9 °C for 30 s, and elongation at 72 °C for 1 min followed by a final elongation at the last cycle at 72 °C for 10 min.

Table 1
Genome-specific primer pairs with the location on the gene and the direction.

Gene Region	Abbr.	Sense (5′ → 3′)	Antisense (5′ → 3′)
Promoter region	P	ccccttctcctcctctgttc	ttgcctcgactaaggagtgg
Exon1/intron1	E1I1	ctcgccactccttagtcgag	aaaggcgtactcacctctgc
Intron1/exon2/intron2	I1I2	gtcaggaaggcgtagaatgg	ccagagtgactggtgcaaga
Intron2	I2	ggcttagcagaggacaaacg	cacagactttggggctgagt
Exon3/intron3	E3I3	gtctcagactcagccccaaa	cagagagccacagtggtcaa
Intron4	I4	ctggcaaaagcatctgtgaa	cagagagccacagtggtcaa
Exon4/exon5/exon6/intron6	E4I6	agtgtcaccgggagtacctg	ccacaggtcaaggttgtgtg
Exon7/exon8/intron7	E7I7	gctgtcagcagcactctcc	ctgtactgcagcccactctg
Intron8	I8	tgggtagctgctgttgtcac	tggccattttcacaatctca
Intron8/exon9/exon10	I9E10	aaatggccaagggtgtgata	gctcagaggaaaaagc
Intron9	I9	gaaccacgtagggatggaga	aggtggcatccatgttcttc

Part of the PCR products was subjected to electrophoresis in a 2% agarose gel to verify specific amplification. After purification of the PCR product using 10 U Exo1 and 1 U Sap, direct sequencing was performed using a commercial kit (BigDye Terminator ver. 3.1, Applied Biosystems) with an automated sequencer (ABI 3730, Applied Biosystems).

Variation screening was done using 192 Indonesian controls. All exons and introns of *IRF1* as well as the promoter region (−500 bp to 0 bp) were read by direct sequencing. Then, only SNPs that had minor allele frequencies over 5% were genotyped in all case and control subjects. The specific primers used for variation screening and genotyping were listed in Table 1. Primers I4 and I9 were used only for re-genotyping of low called SNPs.

The promoter region of *IRF1* seemed to be one of the most promising sites for TB associated SNPs,[38] because it potentially influenced the outcome of a Th1 response during viral infection.[35] There were numeral GC-rich boxes with SP1 and NF-κB binding.[38] Therefore, we used samples of the Vietnamese population to double-check the five polymorphisms in the promoter, which had a minor allele frequency higher than 5%.

2.3. Statistical analysis

Allele and genotype frequencies were checked for deviation from the Hardy–Weinberg equilibrium and the differences of allele and genotype frequencies between cases and controls were analyzed. Haplotype frequencies were estimated by the maximum-likelihood method using the Haploview ver. 4.1 software. The haplotype structure with linkage disequilibrium (LD) values was also constructed using the same software. The permutation p value was calculated with 5000× permutation test.

Association analysis was done using the chi-square test. Meta-analysis on the promoter alleles in Indonesian and Vietnamese was also done using a previously described method.[23] The statistical power was assessed according to disease prevalence, minor allele frequency, significant level and odds ratio according to our previous study.[28]

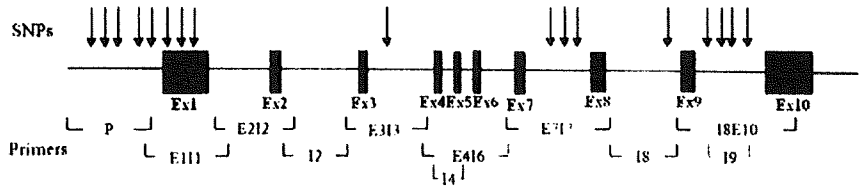

Figure 1. Structure of the *IRF1* gene. Gene structure including exons and introns with the sites of the 17 SNPs and the annealing position of the primers.

Table 2
Location and frequency of *IRF1* SNP alleles and genotypes in Indonesian case and control samples.

Name	Location	Allele or genotype	Frequency in cases (%)	Frequency in controls (%)	*p*-Value
rs2549005	Promoter	G	243 (63.9)	244 (67)	0.376
		A	137 (36)	120 (32.9)	
		GG	75 (39.4)	77 (40.1)	0.626
		GA	93 (48.9)	87 (45.3)	
		AA	22 (11.5)	28 (14.6)	
rs2549006	Promoter	C	243 (63.9)	244 (67)	0.376
		T	137 (36)	120 (32.9)	
		CC	75 (39.4)	76 (39.5)	0.652
		CT	93 (48.9)	88 (45.8)	
		TT	22 (11.5)	28 (14.6)	
rs2706384	Promoter	A	241 (63.7)	240 (62.5)	0.719
		C	137 (36.2)	144 (37.5)	
		AA	74 (39.1)	76 (39.5)	0.649
		AC	93 (49.2)	88 (45.8)	
		CC	22 (11.6)	28 (14.5)	
rs2549007	Promote	G	241 (63.7)	241 (62.7)	0.775
		A	137 (36.2)	143 (37.2)	
		GG	74 (39.1)	77 (40.1)	0.618
		GA	93 (49.2)	87 (45.3)	
		AA	22 (11.6)	28 (14.5)	
rs2549008	Promoter	C	324 (90.5)	327 (90.8)	0.879
		T	34 (9.4)	33 (9.1)	
		CC	145 (81)	147 (81.6)	0.872
		CT	34 (18.9)	33 (18.3)	
		TT	0 (0)	0 (0)	
rs1124211	Exon 1	C	357 (93.0)	360 (93.8)	0.055
		G	25 (6.5)	18 (4.7)	
		CC	167 (87.0)	174 (90.6)	0.137
		CG	23 (12.0)	12 (6.3)	
		GG	1 (0.5)	3 (1.6)	
rs10900809	Exon 1	C	253 (65.9)	238 (62.0)	0.400
		T	131 (34.1)	140 (36.5)	
		CC	82 (42.7)	77 (40.1)	0.258
		CT	89 (46.4)	84 (43.8)	
		TT	21 (10.9)	28 (14.6)	
rs960757	Exon 1	C	357 (93.0)	360 (93.8)	0.288
		T	25 (6.5)	18 (4.7)	
		CC	167 (87.0)	174 (90.6)	0.137
		CT	23 (12.0)	12 (6.3)	
		TT	1 (0.5)	3 (1.6)	
rs2070723	Intron 3	T	244 (63.5)	231 (60.1)	0.211
		C	134 (34.9)	153 (39.8)	
		TT	78 (40.6)	74 (38.5)	0.100
		TC	88 (45.8)	83 (43.2)	
		CC	23 (12.0)	35 (18.2)	
rs10214312	Intron 7	A	251 (65.4)	238 (62.0)	0.378
		C	133 (34.6)	144 (37.5)	
		AA	82 (42.7)	78 (40.6)	0.232
		AC	87 (45.3)	82 (42.7)	
		CC	23 (12.0)	31 (16.1)	
rs2070725	Intron 7	G	249 (64.8)	236 (61.5)	0.379
		A	135 (35.2)	146 (38.0)	
		GG	82 (42.7)	71 (37.0)	0.269
		GA	85 (44.3)	94 (49.0)	
		AA	25 (13.0)	26 (13.5)	
rs17848415	Intron 7	A	241 (64.4)	236 (62.4)	0.568
		C	133 (35.5)	142 (37.5)	
		AA	76 (40.6)	74 (39.1)	0.468
		AC	89 (47.5)	88 (46.5)	
		CC	22 (11.7)	27 (14.2)	
rs2070727	Intron 8	G	241 (64.4)	233 (61.6)	0.427
		T	133 (35.5)	145 (38.3)	
		GG	76 (40.6)	72 (38)	0.384
		GT	89 (47.5)	89 (47)	
		TT	22 (11.7)	28 (14.8)	
rs2070728	Intron 9	G	239 (64.2)	235 (62.1)	0.555
		A	133 (35.7)	143 (37.8)	
		GG	75 (40.3)	73 (38.6)	0.480
		GA	89 (47.8)	89 (47)	
		AA	22 (11.8)	27 (14.2)	
rs7701588	Intron 9	T	235 (61.2)	245 (63.8)	0.301
		C	149 (38.8)	133 (34.6)	
		TT	72 (37.5)	77 (40.1)	0.248
		TC	91 (47.4)	91 (47.4)	
		CC	29 (15.1)	21 (10.9)	
rs2070729	Intron 9	T	247 (64.3)	233 (60.7)	0.443
		G	137 (35.7)	145 (37.8)	
		TT	78 (40.6)	74 (38.5)	0.272
		TG	91 (47.4)	85 (44.3)	
		GG	23 (12.0)	30 (15.6)	
rs2070730	Intron 9	C	247 (64.3)	233 (60.7)	0.443
		T	137 (35.7)	145(37.8)	
		CC	78 (40.6)	74 (38.5)	0.272
		CT	91 (47.4)	85 (44.3)	
		TT	23 (12.0)	30 (15.6)	

(continued)

3. Results

3.1. Variation screening and association analyses in the Indonesian population

Of the 94 SNPs registered in the *IRF* gene in GenBank database, only 17 of those SNPs had a minor allele frequency over 5% and these SNPs were subjected to analysis for a possible association with clinical TB. No polymorphisms were newly discovered. Of these 17 SNPs, five were found in the promoter region, three were in exon 1, one was in intron 3, three was in intron 7, one was in intron 8 and the last four were situated in intron 9 (Figure 1). All polymorphisms were in accordance with the Hardy–Weinberg equilibrium (data not shown). Their description, location and allele frequency were shown in Table 2. Any single SNP did not show significant association with the disease.

3.2. Haplotype analyses in the Indonesian population

Haploblocks were constructed according to solid spine method (Figure 2). The first block consisted of the six SNPs composed of three SNPs of intron 10, one of intron 9 and two of intron 8. The second block was constructed from the remaining SNPs. The haplotype blocks were mapped in Table 3. Of the haplotype blocks, 6 tag SNPs (rs2070730, rs7701588, rs2070727 in the block 1 and rs2070725, rs960757, rs2549005 in the block 2) were selected for further association analyses (Figure 2).

Strong LD was observed in most region of the gene involving all the 17 SNPs. Four pairs of SNPs (rs2070730 and rs2070729, rs960757 and rs11242115, rs2549008 and rs2549005 and rs2706384 and rs2549006) showed complete LD with $r^2 = 1$. In the promoter region, all 5 SNPs showed maximal LD with $D' = 1$ (Figure 2). LD and haplotype analyses were carried out based on the genotypes of 17 SNPs in control and case samples. There was no significant association between any haplotype and TB (Table 3).

3.3. Association analyses in the Vietnamese population

Five polymorphisms in the promoter region were analyzed also in Vietnamese samples. Similar as in the Indonesian samples, there was no association between the polymorphisms and the disease (Table 4). LD structure and haplotype structure were well preserved

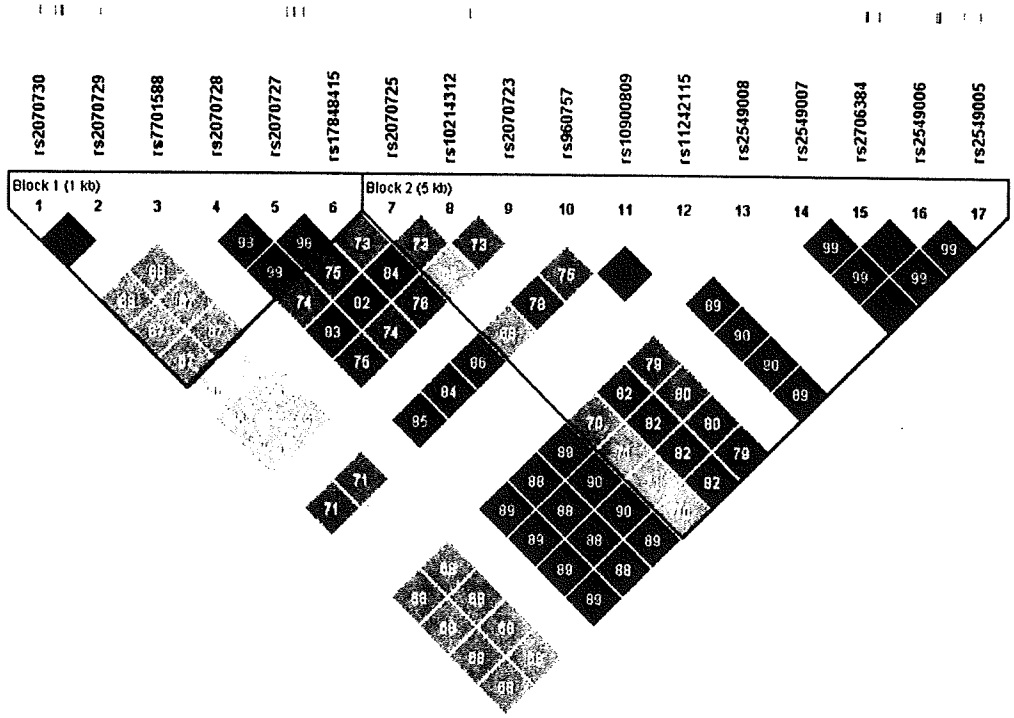

Figure 2. Haplotype block structure in the *IRF1* gene in Indonesian. The extent of the LD (r^2) between SNPs is shown by the color of the box, where black boxes indicate a high r^2 (with the percentage written inside) and white boxes showing a lower r^2. SNPs number 1, 3, 5, 7, 10 and 13 were selected as tag SNPs.

as Indonesian samples (Figure 3), and no haplotype was found to be associated with TB (Table 4).

3.4. Meta-analysis of promoter polymorphisms

Using 5 SNPs found in the promoter region, we also performed meta-analysis on both data of Indonesian and Vietnamese (Table 5). All promoter SNPs showed no significant association with tuberculosis as well.

4. Discussion

Pathogenesis of TB has been extensively studied, however, lack of understanding of the details of how the immune response mechanisms control and eliminate the pathogen is still remained,[45,46] resulting the disease as a major cause of death worldwide and as a single agent surpassed only by malaria in the number of deaths per year.[49]

During an immune response there are numeral cells involved during the recognition and activation phase, as well as in the

Table 3
Haplotype frequency in Indonesian case and control samples.

Haplotype	Frequency in cases (%)	Frequency in controls (%)	Permutation p-value
Block 1 (rs2070730–rs7701588–rs2070727)			
C–C–G	38.3	30.0	0.1782
T–T–T	34.0	31.3	0.9980
C–T–G	24.5	24.8	1.0000
T–T–G	1.6	7.0	0.0036
Block 2 (rs2070725–rs960757–rs2549008)			
G–C–T	45.7	41.4	1.0000
A–C–C	31.7	28.3	0.9914
G–C–C	8.3	7.0	0.9740
G–T–C	6.5	3.8	0.6368

Table 4
Location and frequency of *IRF1* promoter SNP alleles, genotypes and haplotypes in Vietnamese case and control samples.

Name	Location	Allele or genotype	Frequency in cases (%)	Frequency in control (%)	p-Value
rs2549005	Promoter	G	182 (56.2)	160 (60.6)	0.278
		A	142 (43.8)	104 (39.4)	
		GG	57 (35.2)	50 (37.9)	0.421
		GA	68 (42.0)	60 (45.5)	
		AA	37 (22.8)	22 (16.7)	
rs2549006	Promoter	C	182 (56.2)	158 (59.8)	0.369
		T	142 (43.8)	106 (40.2)	
		CC	57 (35.2)	49 (37.1)	0.516
		CT	68 (42.0)	60 (45.5)	
		TT	37 (22.8)	23 (17.4)	
rs2706384	Promoter	A	182 (56.2)	160 (60.6)	0.278
		C	142 (43.8)	104 (39.4)	
		AA	57 (35.2)	50 (37.9)	0.421
		AC	68 (42.0)	60 (45.5)	
		CC	37 (22.8)	22 (16.7)	
rs2549007	Promoter	G	181 (55.9)	160 (60.6)	0.247
		A	143 (44.1)	104 (39.4)	
		GG	56 (34.6)	50 (37.9)	0.419
		GA	69 (42.6)	60 (45.5)	
		AA	37 (22.8)	22 (16.7)	
rs2549008	Promoter	C	295 (91.0)	239 (90.5)	0.942
		T	29 (9.0)	23 (8.7)	
		CC	135 (83.3)	109 (82.6)	0.917
		CT	25 (15.4)	21 (15.9)	
		TT	2 (1.2)	1 (0.8)	

rs2549008-rs2549007	Frequency in cases (%)	Frequency in controls (%)	Permutation p-value
C–G	89.8	96.4	0.7990
C–A	76.7	64.5	0.7824
T–G	9.2	9.8	1.0000

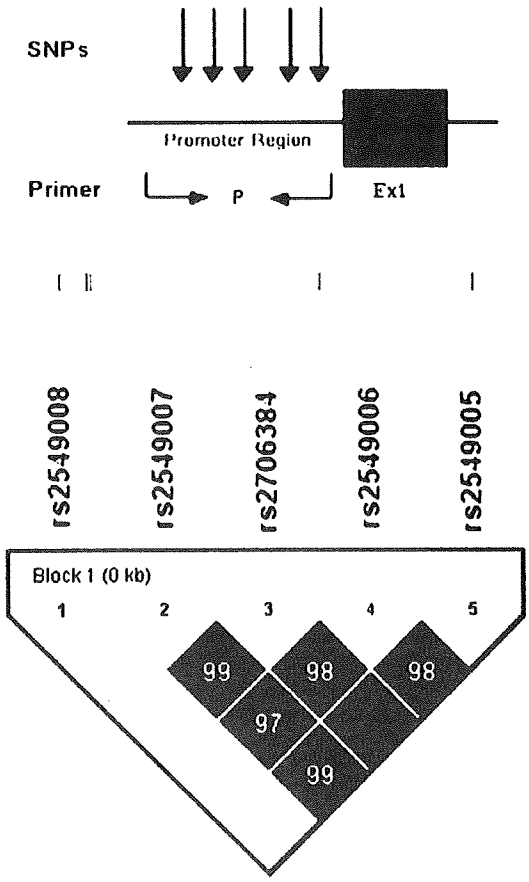

Figure 3. Haplotype structure of polymorphisms found in the promoter of the *IRF1* gene in Vietnamese. The extent of the LD (r^2) between SNPs is shown by the color of the box, where black boxes indicate a high r^2 (with the percentage written inside) and white boxes showing a lower r^2. SNPs number 1 and 2 were selected as tag SNPs.

Table 5
Meta-analysis of *IRF1* promoter polymorphisms in Indonesian and Vietnamese.

Name	Location	Allele or genotype	Frequency in cases (%)	Frequency in control (%)	*p*-Value
rs2549005	Promoter	G	425 (60.4)	404 (64.3)	0.137
		A	279 (39.6)	224 (35.7)	
		GG	132 (37.5)	127 (39.2)	0.854
		GA	161 (45.7)	147 (45.4)	
		AA	59 (16.8)	50 (15.4)	
rs2549006	Promoter	C	425 (60.4)	402 (64.0)	0.171
		T	279 (39.6)	226 (36.0)	
		CC	132 (37.5)	125 (38.6)	0.923
		CT	161 (45.7)	148 (45.7)	
		TT	59 (16.8)	51 (15.7)	
rs2706384	Promoter	A	423 (60.3)	400 (61.7)	0.580
		C	279 (839.7)	248 (38.3)	
		AA	131 (37.3)	126 (38.9)	0.857
		AC	161 (45.9)	148 (45.7)	
		CC	59 (16.8)	50 (15.4)	
rs2549007	Promoter	G	422 (60.1)	401 (61.9)	0,506
		A	280 (39.9)	247 (38.1)	
		GG	130 (37.0)	127 (39.2)	0.808
		GA	162 (46.2)	147 (45.4)	
		AA	59 (16.8)	50 (15.4)	
rs2549008	Promoter	C	619 (90.8)	566 (91.0)	0.883
		T	63 (9.2)	56 (9.0)	
		CC	280 (82.1)	256 (82.3)	0.883
		CT	59 (17.3)	54 (17.4)	
		TT	2 (0.6)	1 (0.3)	

effector phase. Dendritic cells (DC) and macrophages recognize the pathogen by their innate pattern recognition receptors, especially toll-like receptor (TLR) 2 and 4.[32,1,8,6] This leads to an early production of IL-12 and TNF-α, which in turn activates natural killer (NK) cells to produce IFN-γ.[15,43,2] Antigen-presenting cells also migrate to the lymph nodes to activate naïve T cells. This, together with high production of IL-12, leads to a Th1 immune response. These T cells are responsible for high amounts of IFN-γ production during the adaptive phase.[15] IFN-γ together with TNF-α is very important in the activation of macrophages, especially when they are infected by *M. tuberculosis*.[46]

This knowledge helped to investigate specific components of the immune system for polymorphisms. Indeed, polymorphisms in *IL-12*,[34] *IL-12R*,[3] *IFNG*,[30,44] *IFNGR*[17,10,25,34] and in effector functions of macrophages[29,42] led to an increased susceptibility to TB.

Although knowledge has expanded considerably, it is still not enough to control infection and disease. For this goal we need more insights into how these components and cytokines are regulated and how to use this knowledge as tools to manipulate the immune systems for vaccinations and treatments. In this study we approached this question by analyzing *IRF1* gene. This transcription factor does not only play an important role in the induction of IL-12,[39] an early component for a vigorous Th1 response, it is also essential for NK cell development[27] and during T cell differentiation itself.[22] However, our study showed that polymorphisms in this gene did not play a role in the outcome of clinical TB. Using 192 samples each for case and control of Western Javanese Indonesia with the incidence of TB 100/100,000 population per year, we found that 90% of statistical power with a significance level of 0.05 will be obtained if the susceptible allele showing OR > 1.63 with average MAF of 31%.[28] Or in other words, at least 143 samples each are needed for case and control to get statistical power >80% in the studied population. In this study, both populations showed neither single polymorphisms nor specific haplotypes led to an increased susceptibility to TB. Since the control samples in Vietnamese were only 132, this insignificant result could be due to low statistical power resulted from the small sample size. Then we also did meta-analysis of SNPs in promoter region in both Indonesian and Vietnamese population. Again no significant results were shown. Therefore, it is likely that the result of this study could produce conclusive result.[5] This is not surprising because transcription factors such as IRFs are redundant to some extent. IRF5 for example is another factor, which plays an important role in developing a Th1 response. Polymorphisms in *IRF5* gene have been reported to be associated with susceptibility to systemic lupus erythematosus.[12,19,37] However, thus far no reports have been published on its possible association with TB.

Although there was no association between polymorphisms in *IRF1* gene and TB, this transcription factor plays an important role in the transcription of *IL-12* which leads to the activation of Th1 cells essential for host defense mechanisms against TB. Investigations on the other transcription factors related to the immune pathway would be worthy to further elucidate the pathogenesis of TB. In addition, it is very important that research in this field should be continued in order to learn more about the subtle regulations of the immune response and, to gain knowledge for efficiently prohibiting the fatal outcome of this pathogen's activity.

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Prevalence and Risk Factors for Tuberculosis Infection among Hospital Workers in Hanoi, Viet Nam

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Abstract

Background: Transmission of tuberculosis (TB) to health care workers (HCWs) is a global issue. Although effective infection control measures are expected to reduce nosocomial TB, HCWs' infection has not been assessed enough in TB high burden countries. We conducted a cross-sectional study to determine the prevalence of TB infection and its risk factors among HCWs in Hanoi, Viet Nam.

Methodology/Principal Findings: A total of 300 HCWs including all staff members in a municipal TB referral hospital received an interferon-gamma release assay (IGRA), QuantiFERON-TB Gold In-Tube™, followed by one- and two-step tuberculin skin test (TST) and a questionnaire-based interview. Agreement between the tests was evaluated by kappa statistics. Risk factors for TB infection were analyzed using a logistic regression model. Among the participants aged from 20 to 58 years (median = 40), prevalence of TB infection estimated by IGRA, one- and two-step TST was 47.3%, 61.1% and 66.3% respectively. Although the levels of overall agreement between IGRA and TST were moderate, the degree of agreement was low in the group with BCG history (kappa = 0.29). Working in TB hospital was associated with twofold increase in odds of TB infection estimated by IGRA. Increased age, low educational level and the high body mass index also demonstrated high odds ratios of IGRA positivity.

Conclusions/Significance: Prevalence of TB infection estimated by either IGRA or TST is high among HCWs in the hospital environment for TB care in Viet Nam and an infection control program should be reinforced. In communities with heterogeneous history of BCG vaccination, IGRA seems to estimate TB infection more accurately than any other criteria using TST.

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Introduction

Transmission of *Mycobacterium tuberculosis* (MTB) in health care facilities is a problem worldwide [1–3]. Occupational tuberculosis (TB) can lead to the loss of skilled workers and impact health care service adversely, which has serious consequences in association with recent spread of multi-drug resistant (MDR) MTB strains [1]. Effective infection control measures are expected to reduce nosocomial TB [3–6]. In this sense, estimation of prevalence and risk for TB infection among health care workers (HCWs) involved in TB care is one of the essential steps to review and reinforce TB control measures.

In TB high burden countries, however, occupational risk for TB has often been neglected and concealed by the high prevalence in the general population. Furthermore, in those countries, widespread use of BCG vaccination has interfered with interpretation of tuberculin skin testing (TST) [1,7], which was the only measure to detect TB infection until recently.

A newly developed diagnostic test designated as the interferon-gamma release assay (IGRA) uses a principle that MTB-specific antigens provoke immune reaction in the whole blood after TB infection [8]. With the advent of IGRA, many investigators have reported that latent TB infection could be detected more specifically than using TST [9–11]. QuantiFERON-TB Gold test, an ELISA-based IGRA, is also recommended by the US Centers for Disease Control and Prevention (CDC) for initial and sequential-testing of latent TB infection among HCWs [12].

Viet Nam is one of the 22 TB high burden countries defined by WHO, with prevalence of TB being 227/100,000 population and drug resistance TB is ever-increasing [13]. In Hanoi, the capital of Viet Nam, prevalence of smear positive pulmonary TB is 146/100,000 population [14] and the annual risk of TB infection reported from the suburban area is 0.8% [15]. Despite the high burden, little is known about TB infection among HCWs. We conducted this study to estimate the prevalence and risk factors for TB infection among HCWs in a crowded TB referral hospital together with an adjacent general hospital in Hanoi, Viet Nam, by comparing IGRA with conventional TST one- and two-step methods.

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 International Medical Center of Japan respectively.

Study design and setting

We conducted a cross-sectional study in November 2007 in two hospitals adjacently located in the same block of Hai Ba Trung District in Hanoi city (Figure 1). A 110-bed "TB hospital", which receives 2,000 TB in-patients and 46,000 turns of examination per year, is mainly assigned for taking care of TB patients in the entire city. The other is a 460-bed "non-TB hospital", which is a general hospital but transfers all TB-suspected patients to the aforementioned TB hospital.

Participants and data collection

Sample size was determined by the number of all staff members in the TB hospital, since our goal was to clarify the situation of all available HCWs working in the environment. The same categories of departments, such as outpatient clinic, intensive care unit, departments of internal medicine, laboratory and administration were selected from the non-TB hospital and the equivalent number was randomly extracted from each category. Demographic information, history of BCG vaccination and factors potentially associated with TB exposure were collected by an interview using a structured questionnaire. Those factors included job category, duration of working, practice of wearing mask, and professional or household contact with TB patients. For all participants, the blood was collected for IGRA, then TST was administered but not for those with pregnancy, breast-feeding or allergy to tuberculin. To rule out active TB, chest X-ray was taken for all participants with positive IGRA results. Sputum test was performed for participants with productive cough. They also had the chance of receiving INH for treatment of latent TB infection if they wished, after consultation with TB doctors there.

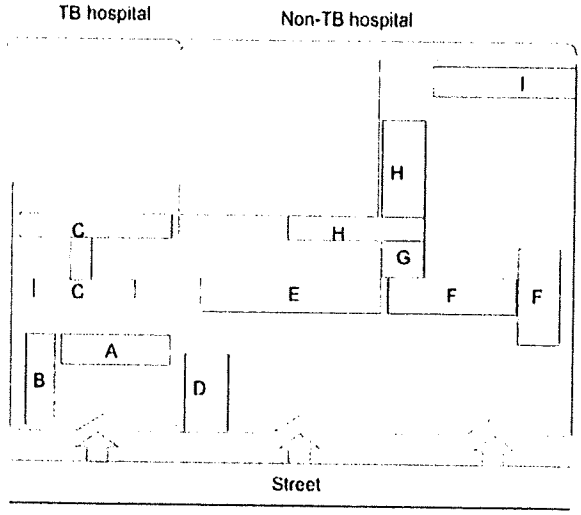

Figure 1. Allocation of two hospitals. TB: Tuberculosis. TB hospital buildings: A = Administration; B = Outpatient clinic and Laboratory; C = Wards and Imaging diagnosis. Non-TB hospital buildings: D = Emergency; E = Wards; F = Laboratory; G = Administration and Imaging diagnosis; H = Out-patient clinic and Wards; I = Imaging diagnosis.
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TST and IGRA

As the first TST, a 5-tuberculin unit dose of Purified Protein Derivatives (Pasteur institute, Nha Trang, Viet Nam), authorized by the Ministry of Health of Viet Nam, was administered by well-trained technicians. Diameter of the induration size was measured after 48 to 72 hours, using a standardized ruler. If the size was less than 10 mm, the second administration with the same dosage was given after 14 days and the results were interpreted similarly (the second TST). From experience in Viet Nam [15], a cut-off value of 10 mm was used in this study, unless otherwise specified. Possible effects of changing cut-off values from 11 mm to 15 mm were also evaluated.

IGRA for TB is a method to measure interferon-gamma induced by MTB-specific antigens (TB antigen) to detect infection. In this study, the newest version of ELISA-based IGRA, QuantiFERON-TB Gold In-Tube™ (Cellestis, Victoria, Australia), was used. One milliliter of the whole blood was collected separately in each heparin-containing tube pre-coated with nil for negative control, mitogen for positive control, and TB antigen. After 18-hour incubation in 37°C, each tube was centrifuged and plasma was harvested. Concentration of interferon-gamma in the plasma was measured using the ELISA method and calculated using analytical software recommended by the manufacturer. The cut-off value of interferon-gamma concentration was 0.35 IU/ml calculated from TB antigen minus negative control. Based on the algorithm of the software, the result was considered to be indeterminate in one of the following two conditions: the nil value itself was higher than 8.0 IU/ml, or mitogen minus nil value was less than 0.5 IU/ml in addition to TB antigen minus nil value was less than 0.35 IU/ml. The testing procedure was carefully monitored [16] and quality control of the test was done in each run, following the manufacturer's instruction.

Statistical analysis

To compare proportions in two groups, chi-squared test was used. Mantel-Haenszel method for stratified data was also attempted. Agreement between TST and IGRA was quantified using kappa statistic. Symmetry test equivalent to McNemar test was used to evaluate the symmetry of discordant results, TST+/IGRA- and TST-/IGRA+. To determine whether history of BCG vaccination or other factors interprets discordant results, unadjusted and adjusted odds ratios were calculated using a logistic regression model. The associations between potential risk factors and TB infection estimated by IGRA positivity were also evaluated by multivariate analysis using a logistic regression model, with IGRA result as outcome and factors possibly related to tuberculosis infection as independent variables. Biologically significant variables such as sex and other variables showing p

values <0.20 in the univariate analysis were included in the multivariate model. All statistical analyses were performed using Stata version 10 (StataCorp, College Station, TX) and $p<0.05$ was considered to be statistically significant.

Results

Characteristics of study population

As shown in Table S1, a total of 300 HCWs of the two hospitals participated in our study and the majority of these were female. The median age was 40 years old, ranging from 20 to 58. Educational levels depended on job categories, but two thirds were at pre-university level or lower. More than one third of the participants had a history of BCG vaccination, of which more than 95% had actual BCG scar (data not shown).

Participants to the study included all of the 150 HCWs working in the TB hospital and 150 of 803 HCWs from the non-TB hospital (Table S1). Two thirds of HCWs in the TB hospital were less than 40 years old and this proportion was larger than in the non-TB hospital ($p<0.0001$, table not shown).

Study flow

As shown in Figure 2, all 300 participants provided blood for IGRA, while 288 of the 300 received TST. Out of them, 112 (38.9%) HCWs whose induration size of the first TST was less than 10 mm took the second TST. IGRA results were indeterminate in 35 (11.7%) individuals, in which 33 received TST and 2 did not. Since IGRA-TST data sets were analyzable when positive or negative results were obtained from both tests, these 33 IGRA indeterminate results were subtracted from 288 TST results, making 255 valid data sets. For check-up of active pulmonary TB, 131 of 142 IGRA-positive HCWs took chest radiography. Spontaneously cured tuberculosis was not completely excluded in 11 individuals (data not shown). Although active TB could not be ruled out from the chest radiography in one individual, all of the IGRA-positive individuals did not report any signs or symptoms in the follow-up period and were regarded as having latent TB infection. TST results were not emphasized in making this decision, because we expected that false-positive TST results due to previous BCG vaccination were not clinically negligible. None of them agreed to take INH treatment.

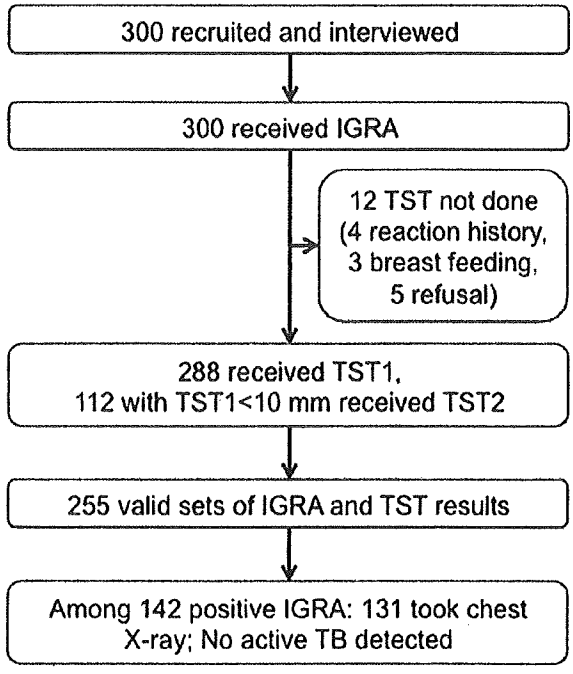

Figure 2. Study flow diagram. IGRA: Interferon-gamma release assay; TST1: The first Tuberculin skin test; TST2: The second Tuberculin skin test; TB: Tuberculosis.
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TST and IGRA positivity

TST measurements were obtained in 288 individuals. With a cut-off value of 10 mm, 176 of 288 (61.1%) were positive after the initial injection as a result of conventional "one-step" TST (Table not shown). Of 112 participants with negative TST initially, 15 turned into positive after the second injection, increasing the overall positivity up to 66.3% as a result of "two-step" TST. When 15 mm of induration size was used as the cut-off value, positive results were decreased to half (29.5%). Positive or negative IGRA results were obtained in 265 out of 300 individuals (88.3%). With the cut-off point of IGRA described in the method section, 142 were positive out of the total 300 tested (47.3%).

Since age distribution was rather different between the two hospitals, data stratified by age of each hospital was shown in Table 1. In the TB hospital, positive TST results with one-step TST and 10-mm cut-off value accounted for 54.9%, 72.7%, 86.5% and 85.7% in groups of 20–29, 30–39, 40–49 and ≥50 years old respectively. IGRA results in the same hospital revealed positive in 38.2%, 47.9%, 51.3% and 87.5% for the corresponding age groups. The proportion of TST ≥10 mm was higher in the TB hospital than in the non-TB hospital by the Mantel-Haenszel test. IGRA positivity in the TB hospital had a similar tendency as compared with that in the non-TB hospital when stratified by age, although the difference did not reach statistical significance (Table 1).

Agreement between TST and IGRA

TST using different cut-off values were compared with IGRA in 255 sets of data (Table 2). Overall kappa values showed moderate agreement (kappa = 0.4 to 0.6) between TST and IGRA, whereas high cut-off values such as 13 mm and 15 mm of TST did not further increase the degree of agreement. As compared with one-step TST with the cut-off value of 10 mm (agreement rate = 72.5%, kappa = 0.44), two-step TST did not have any favorable effect on the degree of agreement (agreement rate = 71.0%, kappa = 0.41). In the group with BCG history, the degree of agreement was rather low, in contrast to the group without BCG history (kappa = 0.29 vs. 0.55) (Table 2).

These findings prompted us to investigate the source of disagreement. With the cut-off value of 10 mm, the number of TST+/IGRA- individuals was disproportionately larger than that of TST-/IGRA+ individuals, which was statistically significant by the symmetry test ($p = 0.0008$). This disproportion was predominant in the subgroup with BCG history, but not in that without BCG history ($p = 0.0013$ vs. 0.20 respectively), when the same cut-off value was applied. Conversely, TST-/IGRA+, the other type of discordance, was strikingly increased when cut-off values of 13 or 15-mm were used ($p = 0.0008$ and $p<0.0001$ respectively) (Table 2).

Consequently, we investigated more deeply into factors associated with discordant results. In univariate analysis, BCG vaccination showed a significant association with TST+/IGRA- discordant results, with OR = 2.34 (95%CI, 1.14–4.81), when the other combinations were set as controls [10,11]. In multivariate analysis, when age, working hospital and working

Table 1. Proportion of IGRA and TST positivity, stratified by age.

		Non-TB hospital		TB hospital		*p* value
		No. positive/No. tested	(%)	No. positive/No. tested	(%)	
IGRA						
	20–29*	6/26	(23.1)	21/55	(38.2)	
	30–39	4/13	(30.8)	23/48	(47.9)	
	40–49	43/87	(49.4)	20/39	(51.3)	
	≥50	18/24	(75.0)	7/8	(87.5)	
	Combined**					0.13
One-step TST, ≥10 mm						
	20–29*	7/26	(26.9)	28/51	(54.9)	
	30–39	5/13	(38.5)	32/44	(72.7)	
	40–49	47/86	(54.7)	32/37	(86.5)	
	≥50	19/24	(79.2)	6/7	(85.7)	
	Combined**					<0.0001
One-step TST, ≥15 mm						
	20–29*	2/26	(7.7)	5/51	(9.8)	
	30–39	5/13	(38.5)	15/44	(34.1)	
	40–49	27/86	(31.4)	17/37	(45.9)	
	≥50	12/24	(50.0)	2/7	(28.6)	
	Combined**					0.45
Two-step TST, ≥10 mm						
	20–29*	8/26	(30.8)	28/51	(54.9)	
	30–39	5/13	(38.5)	33/44	(75.0)	
	40–49	60/86	(69.8)	32/37	(86.5)	
	≥50	19/24	(79.2)	6/7	(85.7)	
	Combined**					0.0004

TB: Tuberculosis; IGRA: Interferon-gamma release assay; TST: Tuberculin skin test.
*Years old.
**Mantel-Haenszel test for stratified data.
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Table 2. Agreement between IGRA and TST using different cut-off values of TST.

		One-step TST						Two-step TST
		≥10 (mm)*			≥11 (mm)*	≥13 (mm)*	≥15 (mm)*	≥10 (mm)*
			BCG (−)**	BCG (+)**				
TST+/IGRA+ (n)		114	44	39	102	86	69	119
TST+/IGRA- (n)		49	14	27	30	21	13	58
TST-/IGRA+ (n)		21	8	8	33	49	66	16
TST-/IGRA- (n)		71	33	23	90	99	107	62
Agreement, %		72.5	77.8	63.9	75.3	72.5	69.0	71.0
Kappa (SE)		0.44 (0.06)	0.55 (0.10)	0.29 (0.09)	0.50 (0.06)	0.46 (0.06)	0.39 (0.06)	0.41 (0.06)
Symmetry test***	Chi-squared value	11.2	1.64	10.3	0.14	11.2	35.6	23.8
	p value	0.0008	0.20	0.0013	0.71	0.0008	<0.0001	<0.0001

IGRA: Interferon-gamma release assay; TST: Tuberculin skin test; SE: Standard error; BCG (−): Without history of BCG vaccination; BCG (+): With history of BCG vaccination;
*N = 255; all subjects with valid data sets.
**N = 99 and 97 for BCG (−) and BCG (+) groups, respectively.
***Equivalent to McNemar test for evaluation of the symmetry of TST+/IGRA- and TST-/IGRA+.
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duration were included in the model, BCG was the only parameter showing significant association with this discordance (OR = 2.26 [95%CI, 1.09–4.71]) (Table not shown). No factors analyzed in this study showed association with TST-/IGRA+ discordance.

Factors associated with IGRA positivity

We tried to identify factors associated with IGRA positivity. In univariate analysis, non-occupational factors such as age and the high body mass index (BMI) were significantly associated with having a positive IGRA result (OR = 1.05 [95%CI, 1.02–1.07] per one year and OR = 5.10 [95%CI, 1.45–17.99], respectively) (Table 3), whereas occupational factors including job category, working duration, and mask use did not show significant associations with IGRA positivity (Table 3).

In multivariate analysis, significantly increased odds of IGRA positivity were observed with non-occupational factors such as increase in age (OR = 1.06 [95%CI, 1.00–1.11]), high BMI (OR = 4.18 [95%CI, 1.14–15.36]), education lower or equal to high school level (OR = 4.28 [95%CI, 1.28–14.27]) and pre-university level (OR = 3.54 [95%CI, 1.18–10.59]). Among occupational factors tested, working in TB hospital was the only parameter showing the significant association (OR = 1.94 [95%CI, 1.04–3.64]) (Table 3).

Discussion

Our study demonstrated the high prevalence of latent TB infection estimated by either TST or IGRA positivity among hospital workers and higher risk of infection adjusted for age and other factors in the TB hospital than in a general hospital in Hanoi, Viet Nam. Disagreement between TST and IGRA positivity was largely affected by BCG vaccination history and it was not improved by changing cut-off values of TST. As far as we know, this is the first report on TB infection among HCWs evaluated by IGRA in Southeast Asia.

The overall prevalence of IGRA positivity among HCWs in our study (47.3%) is high and comparable to previous estimates from India, Russia and Georgia (40.0%, 40.8%, and 60.0% respectively) [17–19]. Direct comparison is difficult among the studies, because in the previous studies particularly the Russian one, detailed information about age strata has not been shown, which strongly affects the prevalence of TB infection.

The prevalence of TST positivity in our study population was higher than that of IGRA. High false-positive TST reaction due to BCG vaccination given after infancy has been reported [7,20], especially in individuals less than 40 years old [21]. In fact, the degree of TST/IGRA agreement was low in the group with BCG vaccination in our study, with a significant disproportional increase in TST+/IGRA- over TST-/IGRA+. Furthermore,

Table 3. Logistic regression analysis results for the associations between potential risk factors and IGRA positivity (n = 265).

		Proportion of positive results		Uni-variate		Multi-variate	
		n	(%)	Odds Ratio	(95%CI)	Odds Ratio	(95%CI)
Non-occupational factors:							
Age	/year	NA*	NA*	1.05	(1.02–1.07)	1.06	(1.00–1.11)
Sex	Female	102/197	(51.8)	1.00	(reference)	1.00	(reference)
	Male	40/68	(58.8)	1.33	(0.76–2.32)	1.10	(0.56–2.16)
BMI	18.5≤<25.0	114/223	(51.1)	1.00	(reference)	1.00	(reference)
	<18.5	12/23	(52.2)	1.04	(0.44–2.46)	1.50	(0.57–3.94)
	25.0≤	16/19	(84.2)	5.10	(1.45–17.99)	4.18	(1.14–15.36)
Education	University and higher	47/93	(50.5)	1.00	(reference)	1.00	(reference)
	High school and lower	25/36	(69.4)	2.22	(0.98–5.04)	4.28	(1.28–14.27)
	Pre-university	70/136	(51.5)	1.04	(0.61–1.76)	3.54	(1.18–10.59)
Occupational factors:							
Hospital	Non-TB	71/136	(52.2)	1.00	(reference)	1.00	(reference)
	TB	71/129	(55.0)	1.12	(0.69–1.82)	1.94	(1.04–3.64)
Job	Others	45/74	(60.8)	1.00	(reference)	1.00	(reference)
	Doctor	38/66	(57.6)	0.88	(0.45–1.72)	2.60	(0.82–8.29)
	Nurse	45/98	(45.9)	0.55	(0.30–1.01)	0.78	(0.28–2.19)
	Technician	14/27	(51.9)	0.69	(0.29–1.69)	1.02	(0.31–3.35)
Working years	<2	12/29	(41.4)	1.00	(reference)	1.00	(reference)
	2≤<5	20/47	(42.6)	1.05	(0.41–2.68)	0.94	(0.34–2.58)
	5≤<10	22/44	(50.0)	1.42	(0.55–3.65)	0.85	(0.28–2.56)
	10≤	88/145	(60.7)	2.19	(0.97–4.92)	0.91	(0.27–3.13)
Mask use	Frequently	65/124	(52.4)	1.00	(reference)	1.00	(reference)
	Occasionally	40/82	(48.8)	0.86	(0.50–1.51)	1.02	(0.55–1.88)
	Rarely/never	37/59	(62.7)	1.53	(0.81–2.88)	1.78	(0.81–3.94)

*NA = Not applicable.

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among parameters tested in our study, BCG history was the only factor to be associated with TST+/IGRA-discordance in univariate and multivariate analysis. Our finding is consistent with the previous reports [10,11], but different from that of another recent study, where BCG did not account for this discordance [22]. This may be simply due to difference in age of BCG vaccination. Involvement of other unknown factors for the discordance cannot be excluded. Exposure to nontuberculous mycobacterium might be another factor for TST+/IGRA- discordance [11], although nontuberculous mycobacterium is rarely found among smear-positive patients in Viet Nam (unpublished data).

These findings indicate that IGRA is more advantageous than TST with different cut-off values [23]. In Viet Nam, BCG vaccination has been included in the Extended Program of Immunization since 1986 and given within one month after birth. Before this point of time, there were no national guidelines and BCG vaccination was sporadically implemented in several areas and mostly given during childhood. In the heterogeneous background of BCG vaccination, it seems difficult to interpret TST result of the present Vietnamese HCWs even with a higher cut-off value as recommended elsewhere [21,24]. High agreement level between TST and IGRA in a study from India [17] is probably attributed to the fact that most of their participants were vaccinated at birth.

On the other hand, our study did not find significant associations between BCG history and TST-/IGRA+ discordant results and this finding is consistent with the previous reports [10,11,22]. Age was associated with TST-/IGRA+ in one study [10] but this was not confirmed in our study.

The CDC [3] and others [25] recommend performing a two-step TST on all newly employed HCWs to identify HCWs who have had MTB infection. Two-step TST is known to evoke remote infection, weak response by nontuberculous mycobacteria, past BCG or other factors, while IGRA appears to reflect recent rather than remote MTB infection [23]. In our study, the influence of two-step TST on TST/IGRA discordance was not much different from that of one-step TST.

In the TB hospital, the proportion of young HCWs who should lower the overall IGRA positivity was larger than in non-TB hospital. Despite this fact, the IGRA positivity in TB hospital was not low. Occupational factors as well as non-occupational factors have been expected to be associated with latent TB infection. In multivariate analysis using a logistic regression model, working in the TB hospital was significantly associated with twofold increase in odds of TB infection estimated by IGRA. Although previous studies have shown that occupational factors, such as working duration and job category, confer a risk on IGRA positivity [17–19], our results did not support their data. Working duration is closely related to age and it was difficult to assess its independent effect on TB infection in our study. On the other hand, our data imply that many staff members pursuing a variety of job in the TB hospital might have a considerable chance of exposure to infectious droplet nuclei. While non-TB hospital is a large hospital including an eleven-story building and located in a site with a large yard, the TB hospital is smaller and more enclosed, where all TB patients and HCWs share the same ambulatory route from the entrance (Figure 1). Personal protective equipment used is mostly surgical mask, which cannot prevent the transmission effectively. This finding suggests that the overall working environment and currently used administrative measures should be reconsidered. The cores of infection control programs should be understood deeply to avoid health-care associated infection of TB or MDR-TB at the worst, when a number of MDR-TB patients are hospitalized for treatment.

Non-occupational risk factors for IGRA positivity have been shown in several studies [17,19]. Age reflects cumulative exposure to MTB and it was significantly associated with IGRA positivity in our study. Education levels may indicate potential risk of TB infection in non-working environment as well as high risk of nosocomial infection. Although these two risks were not separately assessed in our study design, training may be necessary to increase awareness of prevention of nosocomial infection towards workers with low educational levels. Possible risk of high BMI for TB infection was unexpected, but the effect was highest among all covariates. In fact, high BMI was associated with both TST and IGRA positivity. TB development associated with diabetes accompanied by overweight is known [26], but the relationship between overweight and TB infection itself has not been reported. The results may have been produced by chance. Another independent investigation is necessary to determine whether it can be reproducible.

Among the HCWs with positive results of IGRA, no one took INH for treatment of latent TB infection. INH treatment is a safe and low-cost intervention and recommended by WHO [27] and others [28]. However, in typical health care facilities of TB high burden countries where the risk of TB exposure is high and continuous, HCWs still doubt significance of one-time INH treatment.

Our study has several limitations. Firstly, we were not able to evaluate the risk of infection from non-working environment as mentioned above, although the prevalence of TB infection could be estimated roughly from the annual risk of TB infection based on the TST surveys using a formula recommended elsewhere [29]. In Viet Nam, the infection rate was too different between areas to estimate it (data not shown). Secondly, we did not measure HIV infection in our study population. According to the data from a household survey in Ho Chi Minh city, estimated prevalence of HIV infection there is 0.7% in 2005 [30]. We assume that the prevalence is lower in Hanoi. Thirdly, it was not possible to identify the cause of indeterminate cases of IGRA. We re-performed ELISA for all preserved plasma samples with indeterminate results and obtained completely the same results. In addition, we have paid careful attention to maintain the high quality of this test [16]. All of the indeterminate cases showed low response to both TB antigen and mitogen and the pattern of TST measurements in IGRA indeterminate cases was similar to that of IGRA negative cases. For this reason, while calculating the IGRA positivity we did not include indeterminate cases in the numerator but did include them in the denominator, although our results might have underestimated the true proportion.

In conclusion, there is a potential high risk of TB infection among HCWs, particularly those working in TB health facilities in a TB high burden country. Prompt attention is necessary to prevent TB infection among HCWs, preparing for recent spread of MDR-TB in resource-limited settings. For this purpose, IGRA seems appropriate to estimate latent TB infection accurately, contributing to improve infection control strategy especially for young vulnerable HCWs who have heterogeneous history of BCG vaccination after birth.

Supporting Information

Table S1 Characteristics of population studied. TB: Tuberculosis. *Others mainly consist of administrative staff and pharmacists.

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Author Contributions

Conceived and designed the experiments: LTL NTLH NK HY ET SS PHT VCC NH KH LAT NK. Performed the experiments: NTLH VCC IM. Analyzed the data: NTLH AN TM NK. Contributed reagents/materials/analysis tools: LTL NK HY ET SS PHT IM LAT NK. Wrote the paper: LTL NTLH NK.

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