9. Yang WS, Lee WJ, Funahashi T, Tanaka S, Matsuzawa Y, et al. (2001) Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. J Clin Endocrinol Metab 86: 3815–3819.
10. Pitombo C, Araújo EP, De Souza CT, Pareja JC, Geloneze B, et al. (2006) Amelioration of diet-induced diabetes mellitus by removal of visceral fat. J Endocrinol 191: 699–706.
11. Klempel MC, Varady KA (2011) Reliability of leptin, but not adiponectin, as a biomarker for diet-induced weight loss in humans. Nutr Rev 69: 145–154.
12. Kuo SM, Halpern MM (2011) Lack of association between body mass index and plasma adiponectin levels in healthy adults. Int J Obes (Lond) 35: 1487–1494.
13. Malli F, Papaioannou AI, Gourgoulianis KI, Daniil Z (2010) The role of leptin in the respiratory system: an overview. Respir Res 11: 152.
14. Klimcakova E, Kovacikova M, Stich V, Langin D (2010) Adipokines and dietary interventions in human obesity. Obes Rev 11: 446–456.
15. Tanaka T, Sakurada S, Kano K, Takahashi E, Yasuda K, et al. (2011) Identification of tuberculosis-associated proteins in whole blood supernatant. BMC Infect Dis 11: 71.
16. Denecke B, Gräber S, Schäfer C, Heiss A, Wöltje M, et al. (2003) Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. Biochem J 376: 135–145.
17. Mori K, Emoto M, Yokoyama H, Araki T, Teramura M, et al. (2006) Association of serum fetuin-A with insulin resistance in type 2 diabetic and nondiabetic subjects. Diabetes Care 29: 468.
18. Stefan N, Hennige AM, Staiger H, Machann J, Schick F, et al. (2006) Alpha2-Heremans-Schmid glycoprotein/fetuin-A is associated with insulin resistance and fat accumulation in the liver in humans. Diabetes Care 29: 853–857.
19. Hennige AM, Staiger H, Wicke C, Machicao F, Fritsche A, et al. (2008) Fetuin-A induces cytokine expression and suppresses adiponectin production. PLoS One 3: e1765.
20. Dasgupta S, Bhattacharya S, Biswas A, Majumdar SS, Mukhopadhyay S, et al. (2010) NF-kappaB mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance. Biochem J 429: 451–462.
21. Gangneux C, Daveau M, Hiron M, Derambure C, Papaconstantinou J, et al. (2003) The inflammation-induced down-regulation of plasma Fetuin-A (alpha2HS-Glycoprotein) in liver results from the loss of interaction between long C/EBP isoforms at two neighbouring binding sites. Nucleic Acids Res 31: 5957–5970.
22. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, et al. (2005) Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 436: 356–362.
23. Vitkova M, Klimcakova E, Kovacikova M, Valle C, Moro C, et al. (2007) Plasma levels and adipose tissue messenger ribonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity. J Clin Endocrinol Metab 92: 2330–2335.
24. Marshall WJ (2008) Nutritional assessment: its role in the provision of nutritional support. J Clin Pathol 61: 1083–1088.
25. van Lettow M, Fawzi WW, Semba RD (2003) Triple trouble: the role of malnutrition in tuberculosis and human immunodeficiency virus co-infection. Nutr Rev 61: 81–90.
26. Schultz DR, Arnold PI (1990) Properties of four acute phase proteins: C-reactive protein, serum amyloid A protein, alpha 1-acid glycoprotein, and fibrinogen. Semin Arthritis Rheum 20: 129–147.
27. Hang NT, Lien LT, Kobayashi N, Shimbo T, Sakurada S, et al. (2011) Analysis of factors lowering sensitivity of interferon-γ release assay for tuberculosis. PLoS One 6: e23806.
28. Sakurada S, Hang NT, Ishizuka N, Toyota E, Hung LD, et al. (2012) Inter-rater agreement in the assessment of abnormal chest X-ray findings for tuberculosis between two Asian countries. BMC Infect Dis 12: 31.
29. Santucci N, D'Attilio L, Kovalevski L, Bozza V, Besedovsky H, et al. (2011) A multifaceted analysis of immune-endocrine-metabolic alterations in patients with pulmonary tuberculosis. PLoS One 6: e26363.
30. Robinson K, Prins J, Venkatesh B (2011) Clinical review: adiponectin biology and its role in inflammation and critical illness. Crit Care 15: 221.
31. Langouche L, Vander Perre S, Frystyk J, Flyvbjerg A, Hansen TK, et al. (2009) Adiponectin, retinol-binding protein 4, and leptin in protracted critical illness of pulmonary origin. Crit Care 13: R112.
32. Walkey AJ, Rice TW, Konter J, Ouchi N, Shibata R, et al. (2010) Plasma adiponectin and mortality in critically ill subjects with acute respiratory failure. Crit Care Med 38: 2329–2334.
33. Moriconi N, Kraenzlin M, Müller B, Keller U, Nusbaumer CP, et al. (2006) Body composition and adiponectin serum concentrations in adult patients with cystic fibrosis. J Clin Endocrinol Metab 91: 1586–1590.
34. Sood A (2010) Obesity, adipokines, and lung disease. J Appl Physiol 108: 744–753.
35. Schwenk A, Hodgson L, Rayner CF, Griffin GE, Macallan DC (2003) Leptin and energy metabolism in pulmonary tuberculosis. Am J Clin Nutr 77: 392–398.
36. van Lettow M, van der Meer JW, West CE, van Crevel R, Semba RD (2005) Interleukin-6 and human immunodeficiency virus load, but not plasma leptin concentration, predict anorexia and wasting in adults with pulmonary tuberculosis in Malawi. J Clin Endocrinol Metab 90: 4771–4776.
37. van Crevel R, Karyadi E, Netea MG, Verhoef H, Nelwan RH, et al. (2002) Decreased plasma leptin concentrations in tuberculosis patients are associated with wasting and inflammation. J Clin Endocrinol Metab 87: 758–763.
38. Buyukoglan H, Gulmez I, Kelestimur F, Kart L, Oymak FS, et al. (2007) Leptin levels in various manifestations of pulmonary tuberculosis. Mediators Inflamm 2007: 64859.
39. Yuksel I, Sencan M, Dokmetas HS, Dokmetas I, Ataseven H, et al. (2003) The relation between serum leptin levels and body fat mass in patients with active lung tuberculosis. Endocr Res 29: 257–264.
40. Cakir B, Yonem A, Guler S, Odabasi E, Demirbas B, et al. (1999) Relation of leptin and tumour necrosis factor alpha to body weight changes in patients with pulmonary tuberculosis. Horm Res 52: 279–283.
41. Bornstein SR, Preas HL, Chrousos GP, Suffredini AF (1998) Circulating leptin levels during acute experimental endotoxemia anti-inflammatory therapy in humans. J Infect Dis 178: 887–890.
42. Kim JH, Lee CT, Yoon HI, Song J, Shin WG, et al. (2010) Relation of ghrelin, leptin and inflammatory markers to nutritional status in active pulmonary tuberculosis. Clin Nutr 29: 512–8.
43. Bruun JM, Pedersen SB, Kristensen K, Richelsen B (2002) Effects of pro-inflammatory cytokines and chemokines on leptin production in human adipose tissue in vitro. Mol Cell Endocrinol 190: 91–99.
44. Arnalich F, Lopez J, Codoceo R, Jim M, Madero R, et al. (1999) Relationship of plasma leptin to plasma cytokines and human survival in sepsis and septic shock. J Infect Dis 180: 908–911.
45. Gualillo O, Eiras S, Lago F, Diéguez C, Casanueva FF (2000) Elevated serum leptin concentrations induced by experimental acute inflammation. Life Sci 67: 2433–2441.
46. Wallace AM, Sattar N, Mcmillan DC (2000) The co-ordinated cytokine/hormone response to acute injury incorporates leptin. Cytokine 12: 1042–1045.
47. Satoh N, Naruse M, Usui T, Tagami T, Suganami T, et al. (2004) Leptin-to-adiponectin ratio as a potential atherogenic index in obese type 2 diabetic patients. Diabetes Care 27: 2488–2490.
48. Wieland CW, Florquin S, Chan ED, Leemans JC, Weijer S, et al. (2005) Pulmonary Mycobacterium tuberculosis infection in leptin-deficient ob/ob mice. Int Immunol 17: 1399–1408.
49. Jahnen-Dechent W, Schäfer C, Ketteler M, McKee MD (2008) Mineral chaperones: a role for fetuin-A and osteopontin in the inhibition and regression of pathologic calcification. J Mol Med (Berl) 86: 379–389.
50. Jersmann HP, Dransfield I, Hart SP (2003) Fetuin/alpha2-HS glycoprotein enhances phagocytosis of apoptotic cells and macropinocytosis by human macrophages. Clin Sci (Lond) 105: 273–278.
51. Koch A, Weiskirchen R, Sanson E, Zimmermann HW, Voigt S, et al. (2010) Circulating retinol binding protein 4 in critically ill patients before specific treatment: prognostic impact and correlation with organ function, metabolism and inflammation. Crit Care 14: R179.
52. Fuhrman MP, Charney P, Mueller CM (2004) Hepatic proteins and nutrition assessment. J Am Diet Assoc 104: 1258–1264.
53. Ingenbleek Y, Van Den Schrieck HG, De Nayer P, De Visscher M (1975) Albumin, transferrin and the thyroxine-binding prealbumin/retinol-binding protein (TBPA-RBP) complex in assessment of malnutrition. Clin Chim Acta 63: 61–67.
54. Gavrila A, Peng CK, Chan JL, Mietus JE, Goldberger AL, et al. (2003) Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. J Clin Endocrinol Metab 88: 2838–2843.

TISSUE ANTIGENS
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BRIEF COMMUNICATION

Association of *TLR* polymorphisms with development of tuberculosis in Indonesian females

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Abstract

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and is a major cause of morbidity and mortality worldwide. Many candidate genes have been investigated for a possible association with TB. Toll-like receptors (TLRs) are known to play important roles in human innate immune systems. Polymorphisms in and functions of *TLR*s have been investigated to identify associations with specific infectious diseases, including TB. Here, we examined whether single-nucleotide polymorphisms (SNPs) in *TLRs* and genes in *TLR* signaling were associated with TB susceptibility in Indonesian and Vietnamese populations. A statistically significant association was observed between TB susceptibility in a classified Indonesian female group and rs352139, an SNP located in the intron of *TLR9*, using the genotype (P = 2.76E-04) and recessive (AA *vs* AG+GG, P = 2.48E-04, odds ratio = 1.827, 95% confidence interval = 1.321–2.526) models. Meta-analysis of the Indonesian and Vietnamese populations showed that rs352139 was significantly associated with TB in the recessive model. This finding indicated that a *TLR9* polymorphism might have an important role in the susceptibility to *M. tuberculosis* in Asian populations.

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* and is a major cause of morbidity and mortality worldwide (1). While one third of the world's population is infected with *M. tuberculosis*, only 5%–10% of those infected develop TB during their lifetime (2). Although other pathogens and environmental factors are thought to contribute to the development of TB, host genetic factors are equally important in TB susceptibility. Since a twin study established the importance of a genetic component as a factor in TB susceptibility (3), linkage studies (4–6), a genome-wide association study (7), and many candidate gene studies have been performed, and the candidate gene studies often focused on genes involved in immune function, including human leukocyte antigen (*HLA*) (8), *IL12B1* (9, 10), *TLRs* (11, 12), *SLC11A1* (13, 14), *IFNG* (15, 16), and *CD209* (17, 18). However, relatively few studies have investigated the influence of such genes on TB susceptibility in Southeast Asian countries where the burden of TB is high (1).

TLRs are a family of type 1 transmembrane receptors that are characterized by an extracellular leucine-rich repeat (LRR) domain (19, 20). The LRR domain is thought to be involved in ligand recognition (21), and TLRs are pattern-recognition receptors that recognize pathogen-associated molecular pattern that have an essential function in the innate immune system. The ligand of each human TLR is well characterized, except for the ligand of TLR10. For example, lipopolysaccharides from Gram-negative bacteria are the ligands of TLR4 (22–24), and TLR2 recognizes microbial products, including peptidoglycan from Gram-positive bacteria (25, 26). TLR9 recognizes unmethylated CpG motifs in bacterial DNA (27).

TLRs also have an intracellular Toll/IL-1 receptor (TIR) domain. In the signaling pathway downstream of the TIR domain, myeloid differentiation primary-response protein 88 (MyD88) which has a TIR domain-containing adaptor was characterized to play a crucial role, except for TLR3 (26).

IL-1 receptor-associated kinase is recruited by MyD88, is activated, and then associates with tumor necrosis factor receptor-associated factor 6; this pathway leads to the activation of IkB kinase (IKK) complex. The IKK complex phosphorylates IkB, and phosphorylated IkB induces the activation of the transcription factor NF-kB. Experiments with MyD88-knockout mice showed that the MYD88 knockout resulted in the loss of all immune response against TLRs ligands (28–34).

M. tuberculosis is an intracellular pathogen that infects phagocytic antigen-presenting cells (APCs), including macrophages, monocytes, and dendritic cells. *M. tuberculosis* ligands, including lipoprotein, are recognized by TLRs of APCs, which then secret inflammatory cytokines and chemokines. TLRs are essential as the first line of defense against many pathogens. Genetic studies of humans and animals show associations between *TLR*s and TB. *TLR*-knockout mouse studies indicate that *TLR2*, *TLR4*, and *TLR9* contribute to host resistance to *M. tuberculosis* infection (35–39). Possible associations between genetic variations of *TLR*s, including *TLR2*, *TLR8*, and *TLR9*, and TB have been reported (11, 12). In this study, we examined whether single-nucleotide polymorphisms (SNPs) in *TLR*s and genes downstream of TLRs are associated with TB in two Asian populations, Indonesian and Vietnamese.

Subjects

Samples were collected from individuals (538 pulmonary TB patients and 560 healthy controls) living in the West and East Java provinces of Indonesia. They were collected for identifying polymorphisms and for an association study between TB and human genes (40, 41). The mean age of TB patients was 41.6 years (range, 16-92 years) and the median age was 40 years. Subjects were interviewed about their ethnic background, and only individuals who were confirmed to be of either Javanese or Sundanese–Javanese ethnicity within at least three generations were recruited for the study. Diagnosis of TB was based upon the presence of clinical symptoms, chest X-rays, and microscopic detection of acid-fast bacilli in a Ziehl–Neelsen-stained sputum smear. Percentage of smear-positive TB is 92.3% in Indonesian, 100% in Vietnamese. Genomic DNA was extracted from peripheral lymphocytes using a QIAamp™ DNA Blood Mini Kit (Qiagen, Hilden, Germany). The Research Ethics Committees of Yarsi University, Jakarta, Indonesia and the Faculty of Medicine, University of Tokyo, Japan, approved the study protocols.

For a comparative association study, a Vietnamese population (277 TB patients and 458 controls) whose ethnic backgrounds were Kinh was also analyzed. Samples were collected to investigate HLA characteristics and associations between TB and human genes (42, 43). The mean age was 37.4 years (range, 15–60 years) and the median age was 38 years. Pulmonary TB patients from the Vietnamese population underwent similar physical and laboratory examinations as those from the Indonesian population. Blood samples were taken after obtaining informed consent from each subject. DNA was extracted from whole blood with the QIAamp™ DNA Blood Midi Kit (QIAGEN GmbH, Max Volmer Straße 4, Hilden, Germany). The ethical committees of the Ministry of Health, Vietnam and the International Medical Center of Japan approved the study.

In both subjects, patients and controls from the same family are not involved.

SNP selection

Tag SNPs (64) were selected from CHB (Chinese Beijing) + JPT (Japanese Tokyo) population data from the International HapMap project (http://hapmap.ncbi.nlm.nih.gov/index.html.en). The minor allele frequencies (MAF) of the variants are more than 5%, and each SNP is located in a *TLR* family gene, *MYD88*, *CLEC5A*, or the promoter region of these genes (−5000 to 0 bp). Tag SNPs were selected using TAGGER program in HAPLOVIEW software.

Genotyping methods

DigiTag2 assay

Target SNPs were genotyped using the DigiTag2 assay, which has the potential to simultaneously genotype 96 or 32 SNPs with high accuracy (44). In this study, two sets of 32-plex SNP-typing arrays were used. Multiplex polymerase chain reaction (PCR) was performed first. The reactions were performed in a volume of 10.0 μl containing 5.0 μl of 2× Qiagen Multiplex PCR Master Mix containing HotStarTaq DNA polymerase, multiplex PCR buffer, and deoxynucleoside triphosphate (dNTP) mix (Qiagen Multiplex PCR Kit, Qiagen); 0.5 μl of multiplex primer mix (0.25 pmol, each) (Table S1, supporting information) and 0.5 μl of DNA (1 ng). DNAs were amplified under the following PCR conditions: initial denaturation at 95°C for 15 min and 40 cycles of denaturation at 95°C for 30 s, annealing at 80°C for 1 s, and extension at 68°C for 6 min using a Bio-Rad PTC-200 Peltier thermal cycler (Bio-Rad, Tokyo, Japan).

After multiplex PCR, multiplex oligonucleotide ligation reaction was performed with the multiplex PCR products. Prior to the encoding reaction, 32 non-labeled common probes were simultaneously phosphorylated at the 5′ end in a volume of 40.0 μl containing 10× protruding end kinase buffer, adenosine triphosphate (30 mM), polynucleotide kinase (40 U), and 3′ query probes for 32 SNP sites (4 pmol) (Kination Kit, Toyobo, Osaka, Japan) (Table S2). The reaction mixtures were incubated for 37°C for 30 min and then for 95°C for 3 min using a Bio-Rad PTC-200 Peltier thermal cycler (Bio-Rad). The encoding reaction was prepared by mixing 3.0 μl of the multiplex PCR products with 15.0 μl of *Taq* DNA ligase buffer containing 20 mM Tris–HCl (pH 7.6), 25 mM

potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol (DTT), 1 mM nicotinomide adenosine dinucleotide, and 0.1% Triton X-100 (New England Biolabs, Beverly, MA) with 15 fmol of probes (64 5′-query probes and 32 3′-phosphorylated common probes) and 5 U *Taq* DNA ligase. The encoding reaction was run under the following conditions: (i) 95°C for 5 min, (ii) 50°C for 1 min, and (iii) 58°C 60 min using a Bio-Rad PTC-200 Peltier thermal cycler (Bio-Rad).

For the labeling reaction, 1.0 μl of ligation products was directly mixed in 12.0 μl of *Ex Taq* buffer containing 20 mM Tris–HCl (pH 8.0), 100 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% glycerol, and 2.5 mM of each dNTP (TaKaRa, Otsu, Japan) with CodeMix (60 fmol each), primer mix (6 pmol each), and 0.6 U *Ex Taq* polymerase. The labeling reaction was run under the following conditions: 95°C for 1 min followed by 25 cycles of 95°C for 30 s, 55°C for 6 min, and 72°C for 30 s using a Bio-Rad PTC-200 Peltier thermal cycler (Bio-Rad).

Finally, hybridization reactions were performed using a DNA microarray that had 32 separated areas on the same glass slide, and each of the separated areas contained oligonucleotide probes for 32 SNPs. A hybridization mixture was prepared by mixing 7.5 μl of the labeled products in 18.0 μl of hybridization buffer containing 0.5× standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 15% formamide, and 1 mM EDTA with 1.5 μl of hybridization control. The hybridization control was prepared with 3.75 fmol of Alexa555-labeled D1_100 and Alexa647-labeled D1_100. Then 8.0 μl of the hybridization mixture was applied to each area on the DNA microarray. Hybridization was carried out for 60 min at 37°C in a hybridization oven (ThermoStat plus, Eppendorf). After hybridization, the microarray was washed in washing buffer (0.1× SSC and 0.1% SDS) for 3 min, subsequently washed in distilled water for 1 min, and dried by centrifugation at 900 × g for 1 min. The hybridization image was scanned using a DNA chip scanner, and fluorescence image analysis was performed using the GenePix 4000B unit and GenePix Pro 4.1 software packages (Axon Instruments, Union City, CA). The genotype calls were determined using the SNPSTAR software (version 0.0.1.0, Olympus, Tokyo, Japan).

Variation screening

Variation screening was carried out using the samples from 16 TB patients from the Indonesian subjects. To cover SNPs located in *TLR9* region, all exons, an intron, and the promoter region (−1300 to 0 bp) of *TLR9* were directly sequenced. The primers used in this assay were designed and analyzed by the free software Primer3 and are shown in Table S3 and Figure 1. PCR of samples was performed in a volume of 15.0 μl containing 1.5 μl 10× buffer with 15 mM $MgCl_2$ (Roche, Basel, Schweiz), 0.6 μl of $MgCl_2$ (Roche), 3 μl of 5×

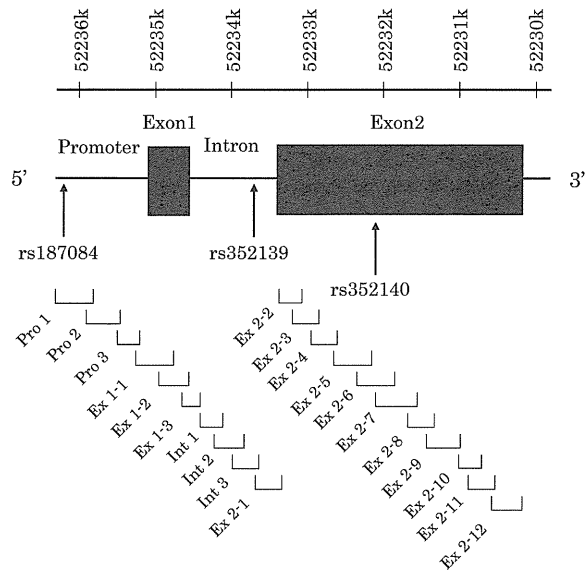

Figure 1 Primer design for *TLR9* variation screening in the Indonesian population. Primers were designed to cover the *TLR9* region, including the promoter region (−1300 to 0 bp). Primer sequences for each region are shown in Table S3. The arrows indicate the single-nucleotide polymorphism discovered in this variation screening.

G–C rich solution (Roche), 1.2 μl of dNTPs (0.20 mM each) (TaKaRa), 0.3 μl of forward primer (3 pmol) (Sigma-aldrich), 0.3 μl of reverse primer (3 pmol) (Sigma-aldrich), 0.75 U FastStart *Taq* Polymerase (Roche), and 2.0 μl of DNA (4 ng). DNAs were amplified under the following PCR conditions: (i) 95°C for 3 min; (ii) 95°C for 30 s; (iii) 60°C for 30 s; (iv) 72°C for 1 min; steps (ii) to (iv) were repeated for 39 cycles; (v) 72°C for 5 min; and (vi) 20°C preservation.

Sequencing reactions were performed in a volume of 15.0 μl containing 2.0 μl of 5× Sequencing Buffer (Applied Biosystems), 1.5 μl of BigDye Terminator version 3.1 (Applied Biosystems), 0.48 μl of primer (4.8 pmol) (Sigma-Aldrich, St. Louis, MO), and 1.5 μl of PCR product. This reaction was carried out under the following condition: (i) 96°C for 3 min; (ii) 96°C for 10 s; (iii) 50°C for 5 s; (iv) 60°C for 3 min; steps (ii) to (iv) were repeated for 24 cycles; and (v) 4°C preservation. Sequences were read with an automated sequencer (ABI 3100 and ABI 3730, Applied Biosystems, Foster City, CA).

Comparative association study

For an ethnic comparison, rs352139 was also examined in a Vietnamese population. This SNP was genotyped by direct sequencing. The primers used in the assay, 5′-GCCAGG GATTGGTTAAGTGA-3′ and 5′-GTTTGACGATGCGGTT GTAG-3′, were designed by the free software Primer3. PCR was performed under the same conditions used for the variation screening.

Statistical analysis

Hardy–Weinberg equilibrium (HWE) tests were performed in controls by Fisher exact test. A P-value of less than 0.01 was considered to indicate deviation from HWE. For SNPs located on X chromosome, subjects were classified by sex and HWE P-values were calculated using only female controls. Association analysis between SNPs and TB was also performed by Fisher exact test. A P-value of less than 0.05 was considered to be statistically significant. Bonferroni corrections for multiple comparisons were applied; the level of significance was $P < 8.62E\text{-}04$, based on the number of successfully genotyped SNPs (58 of 64). Odds ratio (OR) with 95% confidence interval (CI) were calculated for each SNP for evaluating the relative risk. Subjects were classified by sex and age (40 years old) and analyzed with respective to these classifications. For the comparative association study, the Mantel–Haenszel test was conducted.

SNP analysis with DigiTag2 assay

Of the 64 SNP selected, 58 SNPs were successfully genotyped with DigiTag2 assay using Indonesian population. Table S4 shows the genotype count of all SNPs examined in this study. SNP rs12668558 was excluded from further analysis because it alone showed deviation from HWE ($P = 8.56E\text{-}36$). SNP rs352139 in the recessive model had the lowest P-value ($P = 2.20E\text{-}03$) of all SNPs using all models (Tables S1 and S4), but no SNP had a significant P-value with any of the models when data from males and females were combined. Next, data were classified by sex and age of the subjects (Tables S5–S8). SNPs on autosomal chromosomes and X chromosome of male subjects are presented in table (Table S6). SNP rs352139 showed a statistically significant association with TB susceptibility in females in the genotype ($P = 2.76E\text{-}04$) and recessive models (AA *vs* AG+GG, $P = 2.48E\text{-}04$, OR = 1.827, 95% CI = 1.321–2.526) (Table 1). No other SNPs showed any significant association with TB susceptibility for any groups in any model.

Variation screening for *TLR9*

SNP rs352139, which showed significant association with TB susceptibility in Indonesian females, is located in the intron of *TLR9* on chromosome 3. According to HapMap CHB + JPT data, SNPs located within *TLR9* and in the proxy of the gene are in strong linkage disequilibrium ($r^2 \geq 0.94$) with rs352139, but otherwise they had low MAF less than 4% (Figure 2). To find polymorphisms with MAF of at least 5%, variation screening was conducted using 16 TB patient samples from the Indonesian participants.

Although five SNPs with MAF of more than 1% are registered in CHB + JPT population in HapMap database, only three SNPs were observed in the Indonesian population in this study; rs187084 in the promoter region, rs352139 in the *TLR9* intron, and rs352140 in *TLR9* exon2. No new polymorphism was identified.

Comparative association study

SNP rs352139 showed no deviation from HWE ($P = 0.649$). Possible associations for genotype, allele, dominant, and recessive models were analyzed using direct sequencing data from the Vietnamese participants. The lowest P-value was observed in the recessive model in (AA *vs* AG+GG, $P = 0.0939$, OR = 1.294, 95% CI = 0.958–1.747) (Table 1). The data were further classified by sex, but no model resulted in a significant association for males or females. Compared with the results in Indonesian, the minor allele (G) showed susceptibility to TB in both populations, and the allele and recessive models showed the same tendencies in all groups (Table 1). Meta-analysis by Mantel–Haenszel test showed significant P-values for recessive model in all samples ($P = 6.45E\text{-}04$) and the female group ($P = 7.38E\text{-}04$) (Table 1).

SNP rs352139, which is located in the intron of *TLR9*, showed a significant association with TB in the genotype and recessive models in the Indonesian females in this study. It was reported that this SNP was associated with TB in the allele model in an African–American population (11). This SNP showed a tendency to associate with TB all participants (both sexes and both Indonesian and Vietnamese) in addition to the Indonesian female group. Moreover, it is interesting that a *TLR9* polymorphism was reportedly associated with pulmonary TB in females in a South Indian population (45); the present finding on Indonesian females agrees with these reports, supporting that *TLR9* is a susceptibility genes to TB.

TLR9 is expressed in endosome and recognizes unmethylated CpG motifs in bacterial DNA (27). The interaction of TLR9 with unmethylated CpG induces the secretion of proinflammatory cytokines (39). Sex hormone differences may have been responsible for the difference between the male and female groups in their association between rs352139 and TB. Reportedly, the female sex steroid hormone progesterone inhibits CpG-induced, *TLR9*-mediated inflammatory responses in plasmacytoid dendritic cells (46). Additionally, the levels of interferon-α in response to TLR7 stimulation was significantly higher in female peripheral blood lymphocytes (PBLs) than male PBLs (47). However, the studies on interactions between *TLR*s and sex are still in development, and further research is necessary to confirm our present results.

In the variation screening for *TLR9*, only three SNPs, including rs352139, were observed and all the SNPs showed strong linkage disequilibrium (LD) with each other according to CHB + JPT population data (Figure 2). Thus, it may be that one of these three SNPs was the causative SNP responsible for association between *TLR9* and development of TB.

In the comparative association study of rs352139, no significant association was observed between rs352139 and TB in

Table 1 rs352139 genotype frequencies among TB patients and healthy controls in Indonesian and Vietnamese populations

	Indonesian				Vietnamese				Mantel–Haenszel	
	TB patients	Healthy controls	OR (95% CI)	*P*-Value	TB patients	Healthy controls	OR (95% CI)	*P*-Value	OR (95% CI)	*P*-Value
All	*N* = 538 (frequency)	*N* = 560 (frequency)			*N* = 277 (frequency)	*N* = 458 (frequency)				
Genotype										
AA	199 (0.371)	259 (0.463)	—	—	123 (0.446)	232 (0.510)	—	—	—	—
AG	279 (0.520)	233 (0.416)	—	—	125 (0.453)	183 (0.402)	—	—	—	—
GG	59 (0.110)	68 (0.121)	—	0.00222	28 (0.101)	40 (0.088)	—	0.239	—	—
Allele										
A	677 (0.630)	751 (0.671)	1.193	—	371 (0.672)	647 (0.711)	1.200	—	1.196	—
G	397 (0.370)	369 (0.329)	(1.001–1.423)	0.0489	181 (0.328)	263 (0.289)	(0.955–1.508)	0.127	(1.041–1.375)	0.0130
Dominant										
AA+AG	478 (0.890)	492 (0.879)	0.893	—	248 (0.899)	415 (0.912)	1.171	—	0.981	—
GG	59 (0.110)	68 (0.121)	(0.616–1.294)	0.5721	28 (0.101)	40 (0.088)	(0.705–1.947)	0.600	(0.727–1.323)	0.958
Recessive										
AA	199 (0.371)	259 (0.463)	1.461	—	123 (0.446)	232 (0.510)	1.294	—	1.393	—
AG+GG	338 (0.629)	301 (0.538)	(1.148–1.860)	0.00220	153 (0.554)	223 (0.490)	(0.958–1.747)	0.0939	(1.154–1.682)	0.000645
Female	*N* = 228 (frequency)	*N* = 560(frequency)			*N* = 68 (frequency)	*N* = 458 (frequency)				
Genotype										
AA	73 (0.320)	259 (0.463)	—	—	32 (0.471)	232 (0.510)	—	—	—	—
AG	130 (0.570)	233 (0.416)	—	—	30 (0.441)	183 (0.402)	—	—	—	—
GG	25 (0.110)	68 (0.121)	—	0.000276	6 (0.088)	40 (0.088)	—	0.804	—	—
Allele										
A	276 (0.605)	751 (0.671)	1.327	—	94 (0.691)	647 (0.711)	1.099	—	1.267	—
G	180 (0.395)	369 (0.329)	(1.060–1.663)	0.0144	42 (0.309)	263 (0.289)	(0.743–1.625)	0.686	(1.042–1.539)	0.0202
Dominant										
AA+AG	203 (0.890)	492 (0.879)	0.891	—	62 (0.912)	415 (0.912)	1.004	—	0.915	—
GG	25 (0.110)	68 (0.121)	(0.548–1.450)	0.715	6 (0.088)	40 (0.088)	(0.409–2.466)	1	(0.596–1.405)	0.765
Recessive										
AA	73 (0.320)	259 (0.463)	1.827	—	32 (0.471)	232 (0.510)	1.170	—	1.611	—
AG+GG	155 (0.680)	301 (0.538)	(1.321–2.526)	0.000248	36 (0.529)	223 (0.490)	(0.703–1.950)	0.604	(1.227–2.116)	0.000738
Male	*N* = 289 (frequency)	*N* = 560 (frequency)			*N* = 203 (frequency)	*N* = 458 (frequency)				
Genotype										
AA	120 (0.417)	259 (0.463)	—	—	88 (0.436)	232 (0.510)	—	—	—	—
AG	136 (0.472)	233 (0.416)	—	—	92 (0.455)	183 (0.402)	—	—	—	—
GG	32 (0.111)	68 (0.121)	—	0.307	22 (0.109)	40 (0.088)	—	0.196	—	—

Table 1 Continued

	Indonesian				Vietnamese				Mantel–Haenszel	
	TB patients	Healthy controls	OR (95% CI)	*P*-Value	TB patients	Healthy controls	OR (95% CI)	*P*-Value	OR (95% CI)	*P*-Value
Allele										
A	376 (0.653)	751 (0.671)	1.083	—	268 (0.663)	647 (0.711)	1.248	—	1.148	—
G	200 (0.347)	369 (0.329)	(0.876–1.338)	0.480	136 (0.337)	263 (0.289)	(0.971–1.605)	0.0910	(0.977–1.350)	0.103
Dominant										
AA+AG	256 (0.889)	492 (0.879)	0.904	—	180 (0.891)	415 (0.912)	1.268	—	1.031	—
GG	32 (0.111)	68 (0.121)	(0.579–1.413)	0.736	22 (0.109)	40 (0.088)	(0.732–2.195)	0.389	(0.726–1.457)	0.933
Recessive										
AA	120 (0.417)	259 (0.463)	1.205	—	88 (0.436)	232 (0.510)	1.348	—	1.264	—
AG+GG	168 (0.583)	301 (0.538)	(0.904–1.605)	0.215	114 (0.564)	223 (0.490)	(0.966–1.881)	0.0907	(1.017–1.571)	0.0400

CI, confidence interval; OR, odds ratio; TB, tuberculosis.

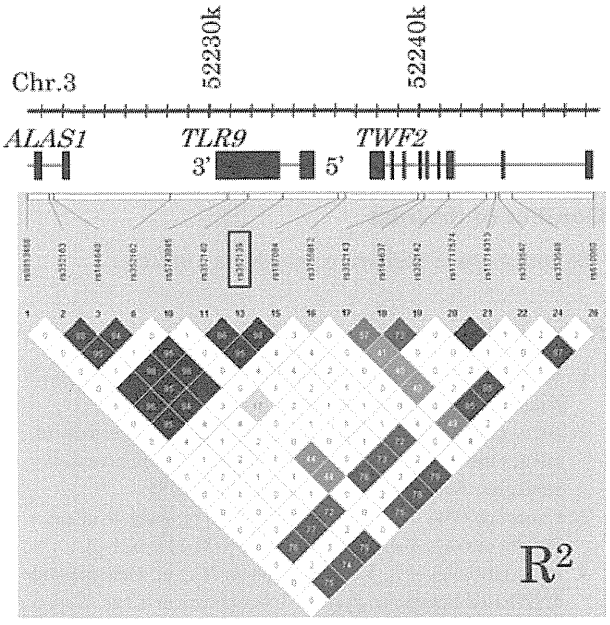

Figure 2 *LD block structure in TLR9 and in the proximity of the gene.* LD block structure in *TLR9* and in the proximity of the gene from the CHB (Chinese Beijing) + JPT (Japanese Tokyo) population of HapMap data (by HAPLOVIEW software version 4.2). The extent of the R-squared is shown in the blocks. The single-nucleotide polymorphisms surrounded with red squares were analyzed in the DigiTag2 assay.

the Vietnamese population, but both populations showed the same tendencies in allele and recessive models. With Mantel–Haenszel test, statistically significant association between rs352139 and TB was observed in the recessive model not only in the female group but also across all Vietnamese and Indonesian participants. Thus, this SNP may have an important role in the development of TB across different populations. Meanwhile, the number of Vietnamese female TB patients ($N = 68$) was much smaller than that of Indonesian female TB patients ($N = 228$). To confirm that possibility, larger number of Vietnamese female TB patients should be analyzed.

In conclusion, a *TLR9* polymorphism was associated with TB in the Indonesian population and also likely in a Vietnamese. Since a previous study in African–American population also described the same association (11), the *TLR9* polymorphism may play an important role in human innate immunity to TB.

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Conflict of interest

The authors have declared no conflicting interests.

References

1. WHO. *Global tuberculosis control: epidemiology, strategy, financing. Geneva: WHO, 2009.*
2. Barry CE III, Boshoff HI, Dartois V et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* 2009: **7**: 845–55.
3. Comstock GW. Tuberculosis in twins: a re-analysis of the Prophit survey. *Am Rev Respir Dis* 1978: **117**: 621–4.
4. Mahasirimongkol S, Yanai H, Nishida N et al. Genome-wide SNP-based linkage analysis of tuberculosis in Thais. *Genes Immun* 2009: **10**: 77–83.
5. Miller EN, Jamieson SE, Joberty C et al. Genome-wide scans for leprosy and tuberculosis susceptibility genes in Brazilians. *Genes Immun* 2004: **5**: 63–7.
6. Jamieson SE, Miller EN, Black GF et al. Evidence for a cluster of genes on chromosome 17q11-q21 controlling susceptibility to tuberculosis and leprosy in Brazilians. *Genes Immun* 2004: **5**: 46–57.
7. Thye T, Vannberg FO, Wong SH et al. Genome-wide association analyses identifies a susceptibility locus for tuberculosis on chromosome 18q11.2. *Nat Genet* 2010: **42**: 739–41.
8. Lombard Z, Brune AE, Hoal EG et al. HLA class II disease associations in southern Africa. *Tissue Antigens* 2006: **67**: 97–110.
9. Remus N, El Baghdadi J, Fieschi C et al. Association of IL12RB1 polymorphisms with pulmonary tuberculosis in adults in Morocco. *J Infect Dis* 2004: **190**: 580–7.
10. Lee HW, Lee HS, Kim DK et al. Lack of an association between interleukin-12 receptor beta1 polymorphisms and tuberculosis in Koreans. *Respiration* 2005: **72**: 365–8.
11. Velez DR, Wejse C, Stryjewski ME et al. Variants in toll-like receptors 2 and 9 influence susceptibility to pulmonary tuberculosis in Caucasians, African-Americans, and West Africans. *Hum Genet* 2009: **127**: 65–73.
12. Davila S, Hibberd ML, Hari Dass R et al. Genetic association and expression studies indicate a role of toll-like receptor 8 in pulmonary tuberculosis. *PLoS Genet* 2008: **4**: e1000218.
13. Li HT, Zhang TT, Zhou YQ, Huang QH, Huang J. SLC11A1 (formerly NRAMP1) gene polymorphisms and tuberculosis susceptibility: a meta-analysis. *Int J Tuberc Lung Dis* 2006: **10**: 3–12.
14. van Crevel R, Parwati I, Sahiratmadja E et al. Infection with Mycobacterium tuberculosis Beijing genotype strains is associated with polymorphisms in SLC11A1/NRAMP1 in Indonesian patients with tuberculosis. *J Infect Dis* 2009: **200**: 1671–4.
15. Pacheco AG, Cardoso CC, Moraes MO. IFNG +874T/A, IL10 -1082G/A and TNF -308G/A polymorphisms in association with tuberculosis susceptibility: a meta-analysis study. *Hum Genet* 2008: **123**: 477–84.
16. Sahiratmadja E, Baak-Pablo R, de Visser AW et al. Association of polymorphisms in IL-12/IFN-gamma pathway genes with susceptibility to pulmonary tuberculosis in Indonesia. *Tuberculosis (Edinb)* 2007: **87**: 303–11.
17. Barreiro LB, Neyrolles O, Babb CL et al. Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. *PLoS Med* 2006: **3**: e20.
18. Vannberg FO, Chapman SJ, Khor CC et al. CD209 genetic polymorphism and tuberculosis disease. *PLoS One* 2008: **3**: e1388.
19. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A* 1998: **95**: 588–93.
20. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997: **388**: 394–7.
21. Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* 2001: **11**: 725–32.
22. Poltorak A, He X, Smirnova I et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998: **282**: 2085–8.
23. Qureshi ST, Lariviere L, Leveque G et al. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J Exp Med* 1999: **189**: 615–25.
24. Hoshino K, Takeuchi O, Kawai T et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 1999: **162**: 3749–52.
25. Takeuchi O, Hoshino K, Kawai T et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999: **11**: 443–51.
26. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 1999: **274**: 17406–9.
27. Hemmi H, Takeuchi O, Kawai T et al. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000: **408**: 740–5.
28. Takeuchi O, Kaufmann A, Grote K et al. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J Immunol* 2000: **164**: 554–7.
29. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999: **11**: 115–22.
30. Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, Akira S. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int Immunol* 2000: **12**: 113–7.
31. Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis

factor receptor-associated factor (TRAF)6. *J Exp Med* 2000: **192**: 595–600.
32. Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr Biol* 2000: **10**: 1139–42.
33. Hemmi H, Kaisho T, Takeuchi O et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* 2002: **3**: 196–200.
34. Hayashi F, Smith KD, Ozinsky A et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001: **410**: 1099–103.
35. Reiling N, Holscher C, Fehrenbach A et al. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J Immunol* 2002: **169**: 3480–4.
36. Drennan MB, Nicolle D, Quesniaux VJ et al. Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. *Am J Pathol* 2004: **164**: 49–57.
37. Sugawara I, Yamada H, Li C, Mizuno S, Takeuchi O, Akira S. Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol Immunol* 2003: **47**: 327–36.
38. Abel B, Thieblemont N, Quesniaux VJ et al. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J Immunol* 2002: **169**: 3155–62.
39. Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A, Sher A. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med* 2005: **202**: 1715–24.
40. Yuliwulandari R, Sachrowardi Q, Nakajima H et al. Association of HLA-A, -B, and -DRB1 with pulmonary tuberculosis in western Javanese Indonesia *Hum Immunol* 2010: **71**: 697–701.
41. Yuliwulandari R, Sachrowardi Q, Nishida N et al. Polymorphisms of promoter and coding regions of the arylamine N-acetyltransferase 2 (NAT2) gene in the Indonesian population: proposal for a new nomenclature. *J Hum Genet* 2008: **53**: 201–9.
42. Hoa BK, Hang NT, Kashiwase K et al. HLA-A, -B, -C, -DRB1 and -DQB1 alleles and haplotypes in the Kinh population in Vietnam. *Tissue Antigens* 2008: **71**: 127–34.
43. Vollstedt S, Yuliwulandari R, Okamoto K et al. No evidence for association between the interferon regulatory factor 1 (IRF1) gene and clinical tuberculosis. *Tuberculosis (Edinb)* 2009: **89**: 71–6.
44. Nishida N, Tanabe T, Takasu M, Suyama A, Tokunaga K. Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. *Anal Biochem* 2007: **364**: 78–85.
45. Selvaraj P, Harishankar M, Singh B, Jawahar MS, Banurekha VV. Toll-like receptor and TIRAP gene polymorphisms in pulmonary tuberculosis patients of South India. *Tuberculosis (Edinb)* 2010: **90**: 306–10.
46. Hughes GC, Thomas S, Li C, Kaja MK, Clark EA. Cutting edge: progesterone regulates IFN-alpha production by plasmacytoid dendritic cells. *J Immunol* 2008: **180**: 2029–33.
47. Berghofer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 ligands induce higher IFN-alpha production in females. *J Immunol* 2006: **177**: 2088–96.

Supporting Information

The following supporting information is available for this article:

Table S1. Primers for DigiTag2 assay.

Table S2. Genotyping probes for DigiTag2 assay.

Table S3. Primers for TLR9 variation screening.

Table S4. Association study of TLR SNP (toll-like receptor single-nucleotide polymorphism) sets among tuberculosis patients and healthy controls in Indonesian.

Table S5. Association study of TLR SNP (toll-like receptor single-nucleotide polymorphism) sets among tuberculosis patients and healthy controls in Indonesian classified by female.

Table S6. Association study of TLR SNP (toll-like receptor single-nucleotide polymorphism) sets in autosomal chromosomes and X chromosome among tuberculosis patients and healthy controls in Indonesian classified by male.

Table S7. Association study of TLR SNP (toll-like receptor single-nucleotide polymorphism) sets among tuberculosis patients and healthy controls in Indonesian classified by young (<40 years old).

Table S8. Association study of TLR SNP (toll-like receptor single-nucleotide polymorphism) sets among tuberculosis patients and healthy controls in Indonesian classified by old (>40 years old).

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RESEARCH ARTICLE Open Access

Inter-rater agreement in the assessment of abnormal chest X-ray findings for tuberculosis between two Asian countries

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Abstract

Background: Inter-rater agreement in the interpretation of chest X-ray (CXR) films is crucial for clinical and epidemiological studies of tuberculosis. We compared the readings of CXR films used for a survey of tuberculosis between raters from two Asian countries.

Methods: Of the 11,624 people enrolled in a prevalence survey in Hanoi, Viet Nam, in 2003, we studied 258 individuals whose CXR films did not exclude the possibility of active tuberculosis. Follow-up films obtained from accessible individuals in 2006 were also analyzed. Two Japanese and two Vietnamese raters read the CXR films based on a coding system proposed by Den Boon et al. and another system newly developed in this study. Inter-rater agreement was evaluated by kappa statistics. Marginal homogeneity was evaluated by the generalized estimating equation (GEE).

Results: CXR findings suspected of tuberculosis differed between the four raters. The frequencies of infiltrates and fibrosis/scarring detected on the films significantly differed between the raters from the two countries ($P < 0.0001$ and $P = 0.0082$, respectively, by GEE). The definition of findings such as primary cavity, used in the coding systems also affected the degree of agreement.

Conclusions: CXR findings were inconsistent between the raters with different backgrounds. High inter-rater agreement is a component necessary for an optimal CXR coding system, particularly in international studies. An analysis of reading results and a thorough discussion to achieve a consensus would be necessary to achieve further consistency and high quality of reading.

Background

Despite its several disadvantages, chest radiography remains an important supporting tool in tuberculosis (TB) surveys and clinical management of active disease [1-3]. Chest X-ray (CXR) findings should be carefully assessed because of its potential problems such as low specificity and insufficient reproducibility [4].

In this context, reading methods that are less influenced by raters are required and several CXR coding systems have been proposed [5-7]. In general, complex interpretation codes hamper intra- and inter-rater agreement and simple codes are preferred [6,7], because reproducible and validated coding system may be useful in monitoring disease in clinical and epidemiological studies [8,9].

Previous studies suggest that variability in CXR interpretation among raters is attributed to subjective reading accompanied by insufficient experience or different professional background of the raters [7,10-12]. However, the relationship between agreement levels and relevant factors that may cause disagreement, particularly influence of medical background including different national origins has not been characterized.

In the present study, Vietnamese and Japanese raters studied the readings of suspected TB lesions on CXR films taken during a survey of TB prevalence in Hanoi,

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Viet Nam [3]. The follow-up films were also compared with the initial films. As analytical tools, two different types of coding systems were used: One was previously reported by another group [5] and the other was newly developed in this study. The aim of the study was to highlight inter-rater agreement between raters with different medical backgrounds. We also attempted to characterize the optimal codes or coding systems used in international studies for a simple and objective evaluation of CXR findings suspected of TB.

Methods

Ethics approval

This study was approved by the ethics committees of the Ministry of Health, Viet Nam and the National Center for Global Health and Medicine (formerly, International Medical Center of Japan). Written informed consent was obtained from each participant prior to the investigations, including the prevalence survey and the follow-up study.

Study population

A population-based TB prevalence survey of 11,624 people aged 15 and over was conducted in Hanoi in 2003 as reported previously [3]. Briefly, subjects suspected of having active TB based on CXR or on symptoms underwent sputum smear microscopy and/or mycobacterial culture. Details of HIV status were not obtained from the study subjects. According to the report of World Health Organization during this period, estimated prevalence of HIV co-infection in new TB patients aged 15-49 was relatively low (2.8%) in Viet Nam [13].

Barring 317 individuals, active TB was radiographically excluded for the rest. Of these 317 individuals, 22 (6.9%) were diagnosed by bacteriological methods, including sputum culture [3]. In 2004, individuals who presented with radiographic findings during the initial survey were advised to undergo sputum smear and culture tests following the World Health Organization recommendation [14,15]. In the 2006 follow-up, in which the same group of individuals was recalled for plain chest radiographic examination (AGFA X-ray film, Beijing, China; Shimadzu UD 150L-30V, Kyoto, Japan) and sputum test, including direct smear and culture. Using a questionnaire, we collected information regarding individual history, additional examinations performed, and treatment for TB undergone after the initial survey. Demographic information (including addresses) collected during the prevalence survey was used to trace the target group in the follow-up period.

The CXR films analyzed in this study were those in which active TB had not been radiographically excluded during the prevalence survey and were those taken during the follow-up in 2006. In total, 258 of the 317 films in the prevalence survey and 93 follow-up films were available at the time of analysis in this study. The rest of TB-suspected films in the prevalence survey were missing.

CXR coding systems and reading of films

Two coding systems were used to classify the CXR findings. The chest radiograph reading and recording system (CRRS) was developed in 2005 to detect TB and other forms of lung disease [5]. Profusion score and details of abnormalities unrelated to TB were omitted. All the other coding items of this system were retained. A Japan-Vietnam CXR coding system (JVCS) (Figure 1) consisting of rather simple codes was also used: We newly developed this system, considering a registration form used in a public payment system for TB treatment expenses in Japan and reading practice in Viet Nam. CRRS classifies parenchymal abnormalities as primary or secondary lesions depending on the significance of the lesion. In contrast, JVCS does not consider the significance of the lesion, though it records pleural effusion and thickening separately. Additionally, CRRS classifies nodules based on their size and calcification, whereas JVCS separately records nodules and calcification.

Two Japanese pulmonary physicians (E.T. and N.K.) and two Vietnamese radiologists (L.D.H. and P.T.C.) read the CXR films. These readers were different from those who read the CXR films during the initial survey. All CXR films were first read using CRRS. After the completion of readings by CRRS, CXR films were read using JVCS without the results of CRRS being made known to the readers. Each reader was also blinded to the others' readings and clinical information. Instruction and training regarding the two coding systems were given prior to the actual reading. The four raters were asked to reach a consensus while assessing 10 standard films from Japan and another 10 films from Viet Nam.

Statistical analysis

We adopted a double entry system of data entry. JMP version 7.0.1 (SAS Institute Inc., Cary, NC, USA) and SAS version 9.1 (SAS Institute Inc.) were used for analysis. Kappa statistics were used to investigate inter-rater agreement on the presence or absence of lesions of interest. We adopted the following guidelines for interpretation of kappa coefficients: < 0, poor agreement; 0-0.20, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, good; and 0.81-1.00, very good [16-18]. Weighted kappa was used to assess inter-rater agreement on variables with more than two categories. McNemar's test or its extension, Bowker's test of symmetry, was used to investigate the symmetry of disagreement between two raters, which tests whether the frequency of an abnormality detected by one rater is significantly different from that by another rater. The generalized estimation equation (GEE) was also used to test the

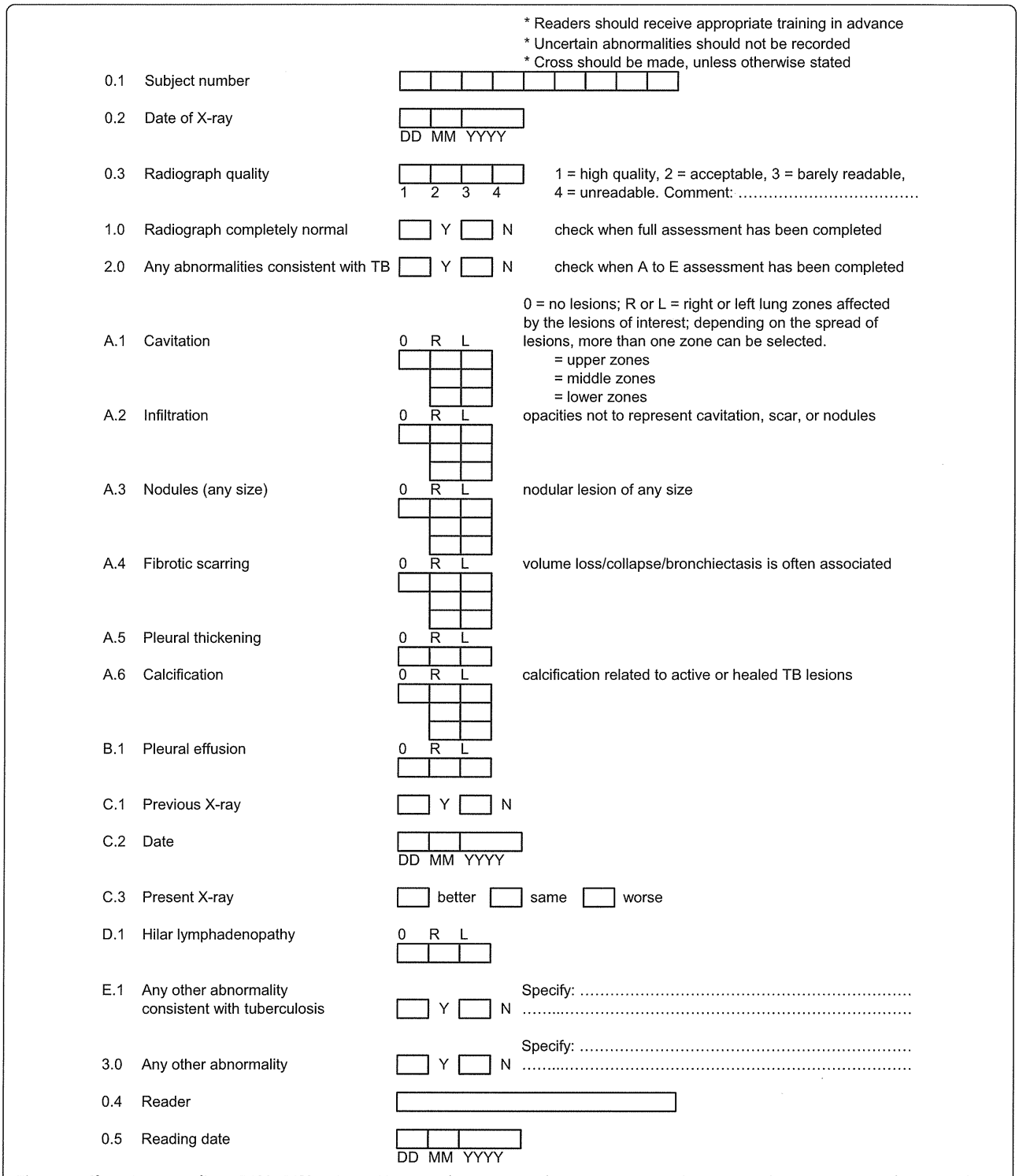

Figure 1 Chest X-ray coding: JVCS. JVCS = Japan-Vietnam chest X-ray coding system; DD = date in two digits; MM = month in two digits; YYYY = year in four digits; Y = yes; N = no; R = right; L = left.

similarities in frequencies of positive findings between groups of raters (marginal homogeneity). No symmetry or non-marginal homogeneity was considered to be significant when $P < 0.05$.

Results

Follow-up after TB prevalence survey

In 2004, one year after the prevalence survey, 204 (64.4%) of the 317 individuals who presented with

radiographic findings of suspected TB underwent a sputum smear test, one of whom tested positive. The initial CXR film of this case showed infiltrates, fibrosis/scarring, and calcification. The follow-up radiograph in 2006 showed improvement after treatment.

In the follow-up in 2006, 93 individuals were checked, one of whom was diagnosed by smear and culture as TB positive (Figure 2). Besides calcification, which was seen in the initial CXR film, infiltrates were present in the follow-up film. All raters evaluated this case as "worse" based on the radiographic findings.

In total, five individuals were reported to have active TB during the 3-year follow-up period. Two were diagnosed bacteriologically and three were diagnosed based on self-reported TB episodes. All the films were randomly mixed in the study set.

Inter-rater agreement on CXR findings

Using the two coding systems, four raters assessed the 258 films taken during the 2003 prevalence survey; two raters assessed the 93 films taken in the 2006 follow-up. A total of 2,436 readings were conducted (Figure 2).

Agreement levels regarding overall parenchymal abnormalities assessed by CRRS varied. Their kappa values were interpreted as fair to good, ranging from 0.24 to 0.63, from the following six comparisons: a comparison between the two Japanese raters (JP-JP); four comparisons between Japanese and Vietnamese raters (JP-VN (1) to (4)); and a comparison between the two Vietnamese raters (VN-VN) (Table 1). Agreement levels regarding calcification also varied. They were considered as fair to good with JVCS and slight to fair with CRRS. Kappa values for pleural effusion with JVCS were interpreted as moderate to good, ranging from 0.54 to 0.77, indicating high level of agreement irrespective of country or rater.

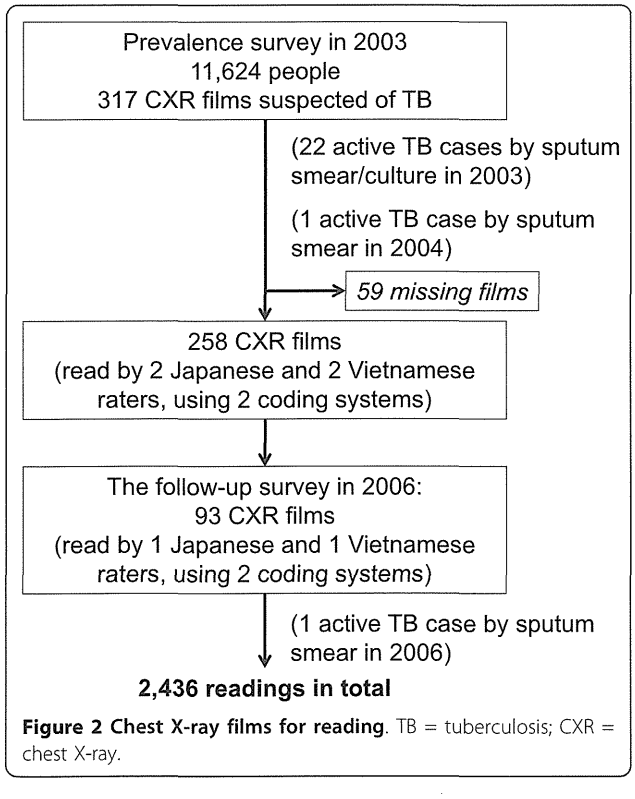

Figure 2 Chest X-ray films for reading. TB = tuberculosis; CXR = chest X-ray.

Major parenchymal findings, cavity, fibrosis/scarring, infiltrates, and nodules were assessed in a similar way, as shown in Table 2. Agreement levels regarding primary and secondary cavities in CRRS were rather low (kappa values ranged from -0.02 to 0.36) except for relatively high agreement levels regarding a primary cavity between the Japanese raters (kappa = 0.60), and a secondary cavity between the Vietnamese raters (kappa = 0.43). Cavitation was, thus, mainly classified as a primary lesion by the Japanese raters and as a secondary lesion by the Vietnamese raters.

Although agreement levels relating to fibrosis/scarring were also low, kappa values for secondary fibrosis/scarring with CRRS revealed fair levels of agreement between raters from the same country (kappa = 0.28 [JP-JP] and 0.22 [VN-VN]), but revealed only slight agreement between raters from different countries (kappa = 0.11 to 0.20 [JP-VN]). Among all Japanese-Vietnamese pairs, the Vietnamese raters specified secondary fibrosis/scars more frequently than the Japanese raters ($P = 0.0001$ or $P < 0.0001$ by McNemar test). The frequency of positive findings of secondary fibrosis with CRRS by both Vietnamese raters was 26/255 (10.2%), whereas that by both Japanese raters was only 7/245 (2.9%) (Table not shown). The frequency of positive findings of fibrosis/scarring with JVCS by both Vietnamese raters (56/255 = 22.0%) also tended to be higher than that by both Japanese raters (42/245 = 17.1%). GEE further confirmed the significant difference in frequencies of fibrosis/scarring between raters from different countries ($P = 0.0082$).

Agreement levels regarding infiltrates between the two raters from the same country were considered as moderate (kappa = 0.49 [JP-JP] and 0.57 [VN-VN]) and as fair between two raters from different countries (kappa = 0.21 to 0.30 [JP-VN]) according to JVCS (Table 2). The Japanese raters detected infiltrates more frequently than the Vietnamese raters ($P < 0.0001$ by McNemar test) in all comparisons. The frequency of positive findings of primary infiltrates with CRRS by both Japanese raters was 68/245 (27.8%), whereas that by both Vietnamese raters was only 22/255 (8.6%) (Table not shown). The frequency of positive findings of infiltrates with JVCS by both Japanese raters (119/245 = 48.6%) also tended to be higher than that by both Vietnamese raters (46/255 = 18.0%). The different frequencies of positive

Table 1 Inter-rater agreement with respect to general and parenchymal findings for each coding system (n = 258)

Item	Coding system	Inter-rater agreement Kappa with 95% confidence interval and the absolute number of films (−/-+/+-/++)					
		JP-JP	JP-VN				VN-VN
			[1]	[2]	[3]	[4]	
Total number	JVCS	(245)	(246)	(245)	(245)	(244)	(255)
of tested films	CRRS	(245)	(245)	(246)	(246)	(245)	(255)
Parenchymal	JVCS	NA	NA	NA	NA	NA	NA
abnormality	CRRS	0.63 [0.51-0.75]	0.24 [0.16-0.32]	0.50 [0.38-0.62]	0.25 [0.16-0.34]	0.58 [0.46-0.70]	0.27 [0.17-0.37]
		(32/21/7/185)	(9/44/0/192)	(24/29/6/187)	(7/32/2/205)	(22/17/8/198)	(6/3/24/222)
Calcification*	JVCS	0.62 [0.49-0.75]	0.47 [0.35-0.59]	0.21 [0.12-0.30]	0.55 [0.42-0.68]	0.30 [0.21-0.39]	0.26 [0.17-0.35]
		(188/13/14/30)	(187/14/22/23)	(198/2/38/7)	(190/12/18/25)	(201/0/34/9)	(215/2/31/7)
	CRRS	0.28 [0.15-0.41]	0.35 [0.23-0.47]	0.15 [0.04-0.26]	0.36 [0.25-0.47]	0.36 [0.25-0.47]	0.17 [0.09-0.25]
		(219/10/11/5)	(206/23/6/10)	(226/4/14/2)	(208/23/5/10)	(228/2/11/4)	(219/2/30/4)
Pleural	JVCS	0.58 [0.45-0.71]	0.77 [0.64-0.90]	0.66 [0.54-0.78]	0.64 [0.51-0.77]	0.54 [0.41-0.67]	0.73 [0.61-0.85]
effusion		(222/8/5/10)	(226/5/2/13)	(222/8/3/12)	(221/6/6/12)	(217/9/7/11)	(230/6/4/15)
Pleural	JVCS	0.35 [0.23-0.47]	0.22 [0.14-0.30]	0.45 [0.33-0.57]	0.28 [0.18-0.38]	0.45 [0.32-0.58]	0.31 [0.22-0.40]
thickening		(158/39/19/29)	(88/110/2/46)	(161/36/14/34)	(84/93/6/62)	(148/28/26/42)	(87/4/95/69)
Pleural	CRRS	0.30 [0.20-0.40]	0.16 [0.09-0.23]	0.46 [0.34-0.58]	0.45 [0.33-0.57]	0.54 [0.42-0.66]	0.32 [0.22-0.42]
abnormalities		(130/73/6/36)	(83/120/3/39)	(159/44/7/36)	(76/60/10/100)	(124/12/42/67)	(81/6/91/77)
Pleural effusion/	CRRS	0.48 [0.36-0.60]	0.34 [0.22-0.46]	0.55 [0.43-0.67]	0.49 [0.36-0.62]	0.67 [0.55-0.79]	0.48 [0.36-0.60]
thickening**		(176/35/6/28)	(168/43/11/23)	(189/22/9/26)	(156/26/23/41)	(176/6/22/41)	(173/14/33/35)

* In CRRS, calcification here indicates calcified granuloma only

**In CRRS, pleural effusion and thickening are combined

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *TB* tuberculosis, *NA* not applicable, *JP-JP* a comparison between the Japanese raters; *JP-VN* [1] to [4] comparisons between Japanese-Vietnamese raters, *VN-VN* a comparison between the Vietnamese raters, (−/-+/+-/++) (negative findings by both raters/positive findings only by the second rater/positive findings only by the first rater/positive findings by both raters)

infiltrate readings between the raters from the two countries were also confirmed by using GEE ($P < 0.0001$).

The levels of inter-rater agreement were considered slight to fair for nodules, irrespective of the raters' home country or the coding system used.

Table 2 Inter-rater agreement with respect to parenchymal findings for each coding system (n = 258)

Item	Coding system	Inter-rater agreement Kappa with 95% confidence interval					
		JP-JP	JP-VN				VN-VN
			[1]	[2]	[3]	[4]	
Total number	JVCS	(245)	(246)	(245)	(245)	(244)	(255)
of tested films	CRRS	(245)	(245)	(246)	(246)	(245)	(255)
Cavity	JVCS	0.44 [0.32-0.56]	0.36 [0.25-0.47]	0.47 [0.34-0.64]	0.30 [0.20-0.40]	0.50 [0.38-0.62]	0.52 [0.40-0.64]
	CRRS primary *	0.60 [0.48-0.72]	0.10 [0.03-0.17]	0.28 [0.18-0.38]	0.06 [-0.02-0.14]	0.36 [0.25-0.47]	0.15 [0.04-0.26]
	CRRS secondary	-0.02 [-0.14-0.10]	0.04 [0.00-0.08]	0.06 [0.01-0.11]	0.00 [-0.05-0.05]	0.04 [-0.03-0.11]	0.43 [0.32-0.54]
Fibrosis/scar	JVCS	0.30 [0.17-0.43]	0.19 [0.07-0.31]	0.34 [0.22-0.46]	0.18 [0.05-0.31]	0.34 [0.23-0.45]	0.31 [0.20-0.42]
	CRRS primary	0.31 [0.18-0.44]	0.02 [-0.02-0.06]	0.27 [0.14-0.40]	-0.02 [-0.07-0.03]	0.15 [0.02-0.28]	0.03 [-0.01-0.07]
	CRRS secondary	0.28 [0.16-0.40]	0.20 [0.10-0.30]	0.11 [0.02-0.20]	0.14 [0.03-0.25]	0.16 [0.06-0.26]	0.22 [0.10-0.34]
Infiltrate	JVCS	0.49 [0.37-0.61]	0.30 [0.20-0.40]	0.27 [0.18-0.36]	0.22 [0.13-0.31]	0.21 [0.13-0.29]	0.57 [0.45-0.69]
	CRRS primary	0.33 [0.21-0.45]	0.24 [0.15-0.33]	0.31 [0.19-0.43]	0.15 [0.08-0.22]	0.22 [0.12-0.32]	0.41 [0.30-0.52]
	CRRS secondary	-0.05 [-0.18-0.08]	0.13 [0.03-0.23]	-0.02 [-0.12-0.08]	-0.04 [-0.14-0.06]	-0.02 [-0.12-0.08]	0.02 [-0.03-0.07]
Nodule	JVCS	0.27 [0.14-0.40]	0.11 [0.05-0.17]	0.26 [0.14-0.38]	0.09 [0.03-0.15]	0.31 [0.20-0.42]	0.19 [0.11-0.27]
	CRRS primary	0.37 [0.25-0.49]	0.13 [0.06-0.20]	0.40 [0.28-0.52]	0.09 [0.03-0.15]	0.24 [0.12-0.36]	0.21 [0.13-0.29]
	CRRS secondary	0.22 [0.10-0.34]	0.22 [0.11-0.33]	0.14 [0.02-0.26]	0.13 [0.03-0.23]	0.29 [0.16-0.42]	0.22 [0.12-0.32]

* Primary and secondary lesions are described in CRRS

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *TB* tuberculosis, *NA* not applicable, *JP-JP* a comparison between the Japanese raters, *JP-VN* [1] to [4] comparisons between Japanese-Vietnamese raters, *VN-VN* a comparison between the Vietnamese raters

An overall assessment of CXR changes after 3 years was conducted by one of the two raters from each country. Agreement was moderate for both coding systems (weighted kappa = 0.47 and 0.40). The Japanese rater indicated deterioration more frequently than the Vietnamese rater (Table 3); this difference was considered highly significant for both JVCS and CRRS by the symmetry test (P = 0.0002 and 0.0008, respectively, by Bowker's test). When assessing changes in specific findings after 3 years, the Japanese rater detected infiltrates more frequently than the Vietnamese rater (P < 0.0001; Figure 3). Among 55 cases of infiltrates, 12 (22%) were assessed as "further spread" by the Japanese rater while 2 (8%) out of 25 cases of infiltrates were assessed as "further spread" by the Vietnamese rater (data not shown).

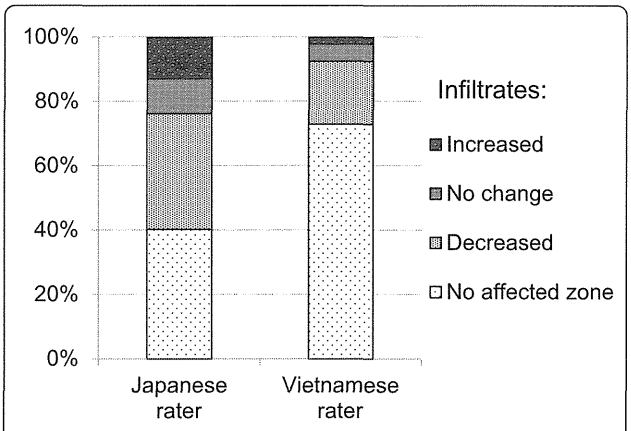

Figure 3 Infiltrates on chest X-rays after a 3-year interval. Evaluation of infiltrates on chest X-ray films between 2003 and 2006 using JVCS codes shows changes in the number of affected areas (upper, middle, and lower zones of each side of the lung). "No affected zone" indicates that the rater did not indicate the presence of infiltrates in either film. The Japanese rater detected infiltrates more commonly than did the Vietnamese rater (P < 0.0001), which corresponds with the results of a greater proportion of "increased" and a smaller proportion of "no detected zone" readings after 3 years. JVCS = Japan-Vietnam chest X-ray coding system

Discussion

Our study confirmed that the readings of CXR findings of suspected TB vary significantly among the raters. Differences in the backgrounds of the raters and different coding systems were considered potential factors affecting the levels of agreement. We found the following two patterns of marked tendency toward inconsistency in the CXR findings: 1) disagreement presumably attributed to the raters' home country and typically observed for infiltrates and secondary fibrosis/scarring and 2) disagreement observed for nodules, irrespective of the rater background. Through discussions conducted with the four raters after the trial, we identified some possible causes of this disagreement, though pre-existing problems were not disclosed when the standard films were checked prior to commencement of the study.

First, it is likely that this disagreement was partly caused by differences between countries regarding the definition of pulmonary lesions. For example, the Vietnamese raters limited the definition of infiltrates to relatively homogenous opacities greater than 10 mm in size, whereas the Japanese raters also included groups of smaller-sized scattered lesions with unclear margins in this classification. As a result, positive findings of infiltrates were more frequently reported by the Japanese raters.

Second, spontaneously cured mild TB resulting in parenchymal fibrosis or scarring, which is commonly seen in countries with high prevalence of TB, is a probable reason for the more frequent detection of these lesions by the Vietnamese raters. In addition, CT scans are compared with plain CXRs more commonly in Japan than in Viet Nam. This practice in TB diagnosis and management might affect the interpretations of the Japanese raters.

Disagreement between the raters from the two Asian countries could be attributed to many background factors, including the medical educational systems and on-the-job training imparted after graduation. In Japan, plain CXR films are read predominantly by clinicians, while in Viet Nam, radiologists also perform this role. Such differences are likely to affect the reading and should be taken into consideration in international studies. Even within a single country, inter-rater agreement depends on the experience of the raters [7,10,12] and is relatively low between raters in different centers [10].

Table 3 Overall assessment of radiographic findings after 3 years

JVCS					CRRS				
JP	VN			Total	JP	VN			Total
	Better	Same	Worse			Better	Same	Worse	
Better	23	6	0	29	Better	28	6	0	34
Same	18	21	0	39	Same	16	18	1	35
Worse	7	7	7	21	Worse	4	10	5	19
Total	48	34	7	89	Total	48	34	6	88
Weighted kappa = 0.40 [0.22-0.57]					Weighted kappa = 0.47 [0.31-0.63]				

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *JP* Japanese rater, *VN* Vietnamese rater

The tested coding systems had both advantages and disadvantages in the context of our study. With CRRS, parenchymal abnormalities are classified into primary and secondary lesions, and it is not easy for raters to differentiate between the two. The Japanese raters emphasized on cavitation and presence of infiltrates as primary lesions of active TB, but the Vietnamese raters objectively judged the primary lesions on the basis of the size of lesions and proportion of the lung involved.

Although fairly reproducible, a disadvantage of JVCS is that it cannot provide any information regarding the significance of active lesions. Thus, CRRS is more informative. Activity, however, is a subjective term and the reproducibility of this description apparently worsens when included in a coding system. This implies the limitations of the plain CXR as a classic imaging tool. It may be assumed that defining necessary medical terms carefully through training and in-depth discussion prior to actual reading would minimize misunderstandings, even with a detailed coding system. However, this was not effective in our study, possibly because of language barriers, different medical backgrounds, and insufficient recognition of the problems. Collectively, our results support the concept of reproducibility of a simplified coding system [6,7,19], which may be critical when a system is shared by raters from different countries, such as even Asian countries.

On comparing CXR findings 3 years after the prevalence survey, Japanese raters detected deterioration in more cases than Vietnamese raters. The fact that the Japanese raters more frequently detected infiltrates may partly explain this discrepancy, because infiltrates generally signify active lesions, though unknown factors may also have affected their readings. This should be considered when CXRs are used for follow-up because the radiological appearance of lesions will not provide sufficient information for monitoring TB unless patient history and bacteriological examination are combined [8,10,19].

Our study has several limitations. First, caution should be exercised when extrapolating the results to describe the way CXRs are generally read in the two Asian countries. Although different medical backgrounds in the countries were obvious after reviewing and discussing the results, the raters' qualifications should also be considered. Second, in the present study, the overall sensitivity and specificity of CXR-based diagnosis of tuberculosis were not determined because the number of active TB cases detected in our cohort study was rather small (< 10%) and because these parameters would be influenced more by individual raters' skills and experiences than by the coding system used. Third, the coverage rate of the radiographic follow-up study after 3 years was not high, one of the reasons being the rapid speed of urbanization and an increasingly mobile population in Hanoi, which caused difficulties when tracing particular individuals. Nevertheless, our findings present an important point to be considered in international studies of TB using a CXR coding system.

Conclusions

In our study, CXR findings of suspected TB were inconsistent between raters with different backgrounds, presumably because of differences in medical practice and education between the two countries. Although each coding system has its advantages and disadvantages, a simplified classification system is suitable for maintaining sufficient agreement between raters from different countries. To improve the quality of future international collaborative studies, harmony could be obtained between raters of different nationalities by thorough discussion regarding the possible causes of disagreement in CXR readings, using standard films and descriptions of major findings.

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Authors' contributions

SS and NTLH participated in supervising the on-site implementation of the study, drafting the paper, and substantially revising it. ET, LDH, PTC, and NKo read the chest X-ray films. LTL and PHT participated in the conception, design, and supervision of the study. PTNB participated in on-site implementation of the study. NI supervised and performed statistical analysis. NKe participated in the conception and design of the study, analysis and interpretation of data, drafting of the paper, and substantially revising it. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Hopewell PC, Pai M, Maher D, Uplekar M, Raviglione MC: **International standards for tuberculosis care.** *Lancet Infect Dis* 2006, **6**:710-725.
2. Golub JE, Mohan CI, Comstock GW, Chaisson RE: **Active case finding of tuberculosis: historical perspective and future prospects.** *Int J Tuberc Lung Dis* 2005, **9**:1183-1203.
3. Horie T, Lien LT, Tuan LA, *et al*: **A survey of tuberculosis prevalence in Hanoi, Vietnam.** *Int J Tuberc Lung Dis* 2007, **11**:562-566.
4. Koppaka R, Bock N: **How reliable is chest radiography?** *Toman's Tuberculosis Case detection, treatment, and monitoring–questions and answers.* 2 edition. Geneva: World Health Organization; 2004, 51-60.

5. Den Boon S, Bateman ED, Enarson DA, *et al*: **Development and evaluation of a new chest radiograph reading and recording system for epidemiological surveys of tuberculosis and lung disease.** *Int J Tuberc Lung Dis* 2005, **9**:1088-1096.
6. Graham S, Das GK, Hidvegi RJ, *et al*: **Chest radiograph abnormalities associated with tuberculosis: reproducibility and yield of active cases.** *Int J Tuberc Lung Dis* 2002, **6**:137-142.
7. Zellweger JP, Heinzer R, Touray M, Vidondo B, Altpeter E: **Intra-observer and overall agreement in the radiological assessment of tuberculosis.** *Int J Tuberc Lung Dis* 2006, **10**:1123-1126.
8. Linh NN, Marks GB, Crawford AB: **Radiographic predictors of subsequent reactivation of tuberculosis.** *Int J Tuberc Lung Dis* 2007, **11**:136-1142.
9. Ralph AP, Ardian M, Wiguna A, *et al*: **A simple, valid, numerical score for grading chest x-ray severity in adult smear-positive pulmonary tuberculosis.** *Thorax* 2010, **65**:863-869.
10. Balabanova Y, Coker R, Fedorin I, *et al*: **Variability in interpretation of chest radiographs among Russian clinicians and implications for screening programmes: observational study.** *BMJ* 2005, **331**:379-382.
11. Brealey S, Westwood M: **Are you reading what we are reading? The effect of who interprets medical images on estimates of diagnostic test accuracy in systematic reviews.** *Br J Radiol* 2007, **80**:674-677.
12. Abubakar I, Story A, Lipman M, *et al*: **Diagnostic accuracy of digital chest radiography for pulmonary tuberculosis in a UK urban population.** *Eur Respir J* 2010, **35**:689-692.
13. **Global tuberculosis control–surveillance, planning, financing. WHO Report 2005.** [http://www.who.int/tb/publications/global_report/2005/en/index.html], WHO/HTM/TB/2005.349.
14. Kantor IN, Kim SJ, Frieden T, *et al*: **Laboratory Service in Tuberculosis Control Part II: Microscopy.** *WHO/TB/98.258* 1998.
15. Kantor IN, Kim SJ, Frieden T, *et al*: **Laboratory Service in Tuberculosis Control Part III: Culture.** 1998, WHO/TB/98.258.
16. Landis JR, Koch GG: **The measurement of observer agreement for categorical data.** *Biometrics.* 1977, **33**:159-174.
17. Kundel HL, Polansky M: **Measurement of observer agreement.** *Radiology* 2003, **228**:303-308.
18. Taplin SH, Rutter CM, Elmore JG, Seger D, White D, Brenner RJ: **Accuracy of screening mammography using single versus independent double interpretation.** *AJR Am J Roentgenol* 2000, **174**:1257-1262.
19. Van Cleeff MR, Kivihya-Ndugga LE, Meme H, Odhiambo JA, Klatser PR: **The role and performance of chest X-ray for the diagnosis of tuberculosis: a cost-effectiveness analysis in Nairobi, Kenya.** *BMC Infect Dis* 2005, **5**:111.

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Association of *SLC11A1* (*NRAMP1*) polymorphisms with pulmonary *Mycobacterium avium* complex infection

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ABSTRACT

Although genetic variants in *SLC11A1* (*NRAMP1*) have been associated with mycobacterial diseases, these findings have not been extensively validated in pulmonary *Mycobacterium avium* complex (MAC) infection. This study investigated the genomic structure of *SLC11A1* and its association with MAC infection. Nineteen polymorphic loci were genotyped in European descendents and the Japanese population. Linkage disequilibrium (LD) structures and frequencies of major haplotypes differed between these 2 populations. Tag single nucleotide polymorphisms (SNPs) were chosen from the data set, and 6 polymorphic sites were genotyped in 122 pulmonary MAC cases and 211 controls from Japan. We observed that the T allele of rs2279014 in the 3′ untranslated region was associated with protection from MAC disease when comparing allele frequencies with an odds ratio of 0.582 (95% confidence interval 0.379–0.894, $p = 0.013$). The frequencies of haplotypes constructed with the above 6 variants did not differ between cases and controls. Allele-specific expression imbalance of *SLC11A1* mRNA was evaluated in peripheral blood cells from heterozygous individuals, but no difference was observed among haplotypes. Although the significance was modest, rs2279014 is in strong LD with nearby SNPs and further studies are required for conclusive validation.

1. Introduction

Nontuberculous mycobacteria–associated disease has often been detected with human immunodeficiency virus (HIV) infection but non-HIV pulmonary *Mycobacterium avium* complex (MAC) disease has been recognized with increasing frequency [1]. Middle-age to elderly women who have no apparent risk factors are reported to contract the pulmonary MAC infection [2]. Although detailed worldwide epidemiological information is lacking, the incidence rate of MAC disease is <5 cases per 100,000 and 60–80% of nontuberculous mycobacteria infection is caused by MAC in Japan [3].

Several lines of evidence including twin studies, linkage studies, candidate gene studies, and recent genome-wide association studies indicate that host genetic factors are important in determining susceptibility to mycobacteria, including *Mycobacterium tuberculosis* and *Mycobacterium leprae* [4–6]. However, host genetic factors for MAC infection have not been extensively studied, and only a few reports have demonstrated an association with genetic polymorphisms within *HLA* [7–9], *SLC11A1* [10,11], *MICA* [12], and *TLR2* [13].

Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (*SLC11A1*) gene, also known as natural resistance–associated macrophage protein 1 (*NRAMP1*), is located in chromosome 2q35, which spans a genomic length of 14 kbp and is composed of 15 exons and 1 alternatively spliced exon present within intron 4 [14]. The SLC11A1 protein functions as a divalent transition metal transporter; however, its precise biochemical function remains unclear [15,16]. *SLC11A1* is a promising candidate gene to interpret sensitivity and resistance to intracellular pathogens including mycobacteria, because the murine *Nramp1* gene was originally identified by positional cloning in this way [17,18].

In most association studies for human mycobacterial infection, 4 *SLC11A1* polymorphisms, rs17235416 in the 3′ untranslated re-

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gion (UTR), rs17235409 (D543N), rs3731865 in INT4, and rs34448891 (5′(GT)n), have been mainly analyzed. In the case of tuberculosis, which has been most extensively analyzed for association with *SLC11A1* polymorphisms, the results were not always consistent [19–24], but in recent meta-analyses, significant associations were reported as a whole [25,26]. Discrepancies in the results among studies may be attributed to the presence of an unknown causal variant, differences in phenotypes across studies, differences in the genetic or environmental background of the studied populations, or the lack of adequately powered sample size.

In this study, we extended our previous study of the 4 polymorphisms of *SLC11A1* [10] to compare genomic structural differences within the *SLC11A1* gene in European and Japanese populations and analyzed the association of this gene with pulmonary MAC infection in the Japanese population. Allele-specific expression levels of mRNA among haplotypes were also investigated.

2. Subjects and methods

2.1. Human subjects

The study protocol was approved by the ethical committee of the National Center for Global Health and Medicine, Japan, and written informed consent was obtained from each individual. A total of 122 Japanese cases of pulmonary MAC infection were recruited for the genetic study. Their patient characteristics, including comorbidity and age at onset, have been described elsewhere [10,12]. Briefly, patients with pulmonary MAC infection comprised 29 (23.8%) males and 93 (76.2%) females in the age range of 21 to 91 years. The mean age at onset of disease was 60.5 ± 13.0 years old. Of these, 63 (51.6%) were without comorbidity, whereas 59 (48.4%) were with comorbidity with previous lung disease or other potential predisposing conditions. In this study, all cases and controls were recruited from central Tokyo, which is considered a homogeneous population with respect to genetic background [27,28]. Our recruited subjects consisted of a single ethnicity and the cases and controls were unrelated. The first control panel (n = 211) was sex unmatched and age unknown, and the second control panel (n = 300) for testing reproducibility was sex matched and age unmatched. To adjust for sex and age, logistic regression models were used. All recruited controls were healthy individuals with no previous history of MAC infection, *M. tuberculosis*, or *M. leprae*. Overall, in all cases, approximately 90% of patients were infected with *M. avium* and the remaining had *Mycobacterium intracellulare*. Diagnosis of MAC infection was based on American Thoracic Society criteria [29]. All patients had clinical manifestations, small pulmonary nodules with or without bronchiectasis on computed tomography images, and positive smears or cultures of bacteria from at least 3 sputum samples. Patients with obvious immunodeficiency were excluded from this study. Differentiation of cultured mycobacterial species was determined by polymerase chain reaction (PCR; AMPLICOR Mycobacterium tests; Roche Diagnostics, Basel, Switzerland).

Fifty DNA samples for European descendents were obtained from the NIGMS Human Variation Panel (HD50CAU of Caucasian individuals, Coriell Institute for Medical Research, Camden, NJ), and 42 Japanese individuals were also recruited for the allele-specific expression assay and screening of polymorphisms.

2.2. Selection of polymorphic loci for genotypic analysis

A total of 19 polymorphic loci, including 1 insertion/deletion and 1 microsatellite, were extracted from the entire genomic region of the *SLC11A1* gene spanning from the 5′ flanking region at position −3,890 to 14,425 with reference to the transcription start site (NM_000578.3) [30] (Table 1). Nine single nucleotide polymorphisms (SNPs) were selected from the HapMap database (http://hapmap.ncbi.nlm.nih.gov) and the remaining variants were selected from the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP), the JSNP database (http://snp.ims.u-tokyo.ac.jp), or the published literature including 4 frequently reported polymorphisms: rs17235416 in the 3′ UTR, rs17235409 (D543N), rs3731865 in INT4, and rs34448891 (5′(GT)n) [19,31,32].

Genotype data of these 19 polymorphisms were obtained from DNA samples of 100 healthy individuals (50 Japanese and 50 European descendents) and linkage disequilibrium (LD) blocks were determined by confidence interval method using Haploview 4.1 (http://www.broadinstitute.org). The variants having minor allele frequency (MAF) >0.01 were subjected to compute pairwise LD statistics and haplotype frequencies (PHASE program, v. 2.1; http://www.stat.washington.edu). For association analysis, 6 tag SNPs (rs2276631, rs2695343, rs17235409, rs17235416, rs1059823, and rs2279014) were chosen from the data set of Japanese individuals

Table 1
Positions of the genetic markers and allele frequencies in the present study

SN	Position as of TSS	rs No.	Location	Allele major/minor	JP MAF (n = 50)	HWP	ED MAF (n = 50)	HWP	Reference
1	−3890	rs4674301	5′ near	T/C	0.15	1	0.34	0.2455	HapMap
2	−127	rs34448891	Promoter	3>2>7					[19]
3	−45	rs7573065	Promoter	C/T	0.02	1	0.06	1	dbSNP
	1		TSS						
	341		ATG						
4	1896	rs2276632	Intron_2	C/T	0.01	1	0	1	JSNP
5	2262	rs2276631	Exon_3	C/T	0.13	1	0.28	0.6172	HapMap, [32]
6	3252	rs3731865	Intron_4	G/C	0.13	1	0.3	0.9276	[19,31,32]
7	5114	rs3731864	Intron_5	G/A	0.02	1	0	1	JSNP, [32]
8	5457	rs3731863	Intron_6	C/T	0.05	1	0.07	1	HapMap
9	5626	rs2290708	Intron_7	C/T	0.06	1	0.27	0.9957	HapMap
10	5879	rs17221959	Exon_8	C/T	0.06	1	0.03	1	[32]
11	7871	rs2695342	Exon_9	G/A	0	1	0	1	[32]
12	8091	rs3816560	Intron_9	T/C	0.14	1	0.29	1	HapMap
13	8672	rs2695343	Intron_9	G/A	0.28	0.6172	0.42	1	HapMap
14	12105	rs17215556	Exon_13	T/C	0	1	0.01	1	[32]
15	12519	rs2279015	Intron_13	G/A	0.27	0.9957	0.41	1	Hapmap, [32]
16	12981	rs17235409	Exon_15	G/A	0.07	1	0.04	1	[19,31,32]
17	13063	rs17235416	3′ UTR	Ins/Del	0.08	1	0.04	1	[19,31,32]
18	13093	rs1059823	3′ UTR	A/G	0.29	1	0.44	1	HapMap
19	14425	rs2279014	3′ UTR	C/T	0.23	0.4022	0.4	0.7106	HapMap

SN, serial number; MAF, minor allele frequency; TSS, transcription start site; JP, Japanese population; ED, European descendents; JSNP, Japanese SNP database; HWP, Hardy–Weinberg equilibrium analyzed with a cutoff value of $p < 0.05$; UTR, untranslated region.
Position and location are based on NM_000578.3.

with an LD threshold of $r^2 > 0.8$ and MAF > 0.10 using the ldSelect program (v. 1.0) (http://pga.gs.washington.edu/).

2.3. Genotyping of polymorphisms

Genomic DNA was purified from whole blood using the QIAamp blood kit (Qiagen, Hilden, Germany). PCR restriction fragment length polymorphism (RFLP) analysis was carried out. The set of primers and parameters is presented in Suppl. Table 1. PCR-based genotyping of GT-repeat polymorphisms of the promoter region of *SLC11A1* was performed as described in Ref. [10], and their corresponding sequences were also determined by sequence analysis. Allele names of the GT-repeat polymorphisms were designated as described by Kojima et al. [33].

2.4. Screening of genetic polymorphisms in strong LD with the associated tag SNP

Full-length coding sequence of *SLC11A1* complementary DNA (cDNA) was amplified in 2 overlapping reverse transcription (RT)-PCR products using primer pairs 5′- GTA CCT GAA GTC GGC ATT TCA ATG AC -3′ (sense) and 5′- GTT GGC TTC TCT GAT GTC TGC TCG -3′ (antisense) and 5′- GTG GGC ATT GTT GGC GCC ATC ATC -3′ (sense) and 5′- GGT GTG GGC CTA GCC AGA GGT CTC -3′ (antisense). The promoter region of *SLC11A1* was amplified with primers 5′- GTG AGC CCC TCA GTT AAT AGA AGA -3′ and 5′- GGG CAC TGG TGC AAG TAA GTG TGC -3′, and the 3′ UTR was amplified with primers 5′- GCA TCT CCC CAA TTC ATG GT -3′ and 5′- ATT TGG GCC TCA GAG GCA CAG -3′ from genomic DNA. RT-PCR and PCR products from 35 Japanese were directly sequenced using appropriate inner primers following the standard protocol using a genetic analyzer (ABI prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA).

2.5. Allele-specific mRNA expression imbalance

Allele-specific mRNA expression was assessed by the method detecting allelic expression imbalance [12]. The primer set was designed to encompass 3 SNPs, rs17235409, rs17235416, and rs1059823, located in exon 15. Genomic DNA was obtained from whole blood in an EDTA tube and RNA was extracted from 2.5 mL of blood dispensed to the PAXgene RNA tube containing RNA-stabilizing reagent (PreAnalytiX, Hombrechtikon, Switzerland). Pairwise haplotypes were constructed for 7 markers (rs34448891, rs2276631, rs2695343, rs17235409, rs17235416, rs1059823, and rs2279014) by the PHASE program (v. 2.1) to identify the informative heterozygous individuals. A total of 12 representative heterozygote samples were selected for further analysis.

Here, 0.5 μg of total RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The amplified cDNA was used as a template for PCR amplification with AmpliTaq Gold DNA polymerase (Applied Biosystems) using the primer sets NRAMP1c_ex14_15_s1, 5′-TCA GCA CCT ACC TGG TCT GGA CCT-3′ labeled with FAM, and NRAMP1c_ex_15_as1, 5′-CAT GGC TGC GCT AGG AAA CAG CAG-3′. Then, single-strand conformation polymorphism (SSCP) analysis was employed to separate the 2 alleles and the relative expression of allele-specific mRNA was quantified on the basis of the difference in band intensities as described [12]. Briefly, 1 μL of each PCR product was mixed with 7 μL of deionized formamide, denatured at 94°C for 2 minutes immediately before loading, and electrophoresed on a nondenaturing 10% polyacrylamide gel with an acrylamide:bisacrylamide ratio of 49:1 in 45 mM Tris–borate, 1 mM EDTA, and 5% formamide. Electrophoresis was carried out under constant voltage of 300 V/gel at 10°C for 3 hours. Separated PCR fragments were captured by a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA) and fluorescence intensity was analyzed using Quantity One 1-D software (Bio-Rad Laboratories).

The RT-PCR-RFLP system using a single SNP, rs17235409, was designed with a reverse primer labeled with fluorescent dye (HEX, NRAMP1c_ex15_as1b: 5′- ACA TGC CAC TCC CAG CCA GG -3′); the same forward primer NRAMP1c_ex14_15_s1 was used to amplify the target sequence, and the PCR product was digested by *Ava*II. The digested products were run on a nondenaturing 8% polyacrylamide gel with an acrylamide:bisacrylamide ratio of 49:1 in 45 mM Tris–borate and 1 mM EDTA.

2.6. Statistics

Associations of the polymorphisms with clinical phenotypes of MAC disease were evaluated in allelic, genotypic, recessive, and dominant models. The χ^2 test was used to compare the frequency distribution in case and control groups. When any expected number in the 2 × 2 contingency table was less than 5, Fisher's exact test was employed using the R program (http://www.r-project.org). For all comparisons, odds ratios (OR) with 95% confidence intervals (CI) were calculated. A 2-tailed test was used to evaluate statistical significance. The tendency of having a resistant allele in MAC disease was assessed by OR and p values adjusted for sex in the first and second panels and age at diagnosis in the second panel using a logistic model. The Hardy–Weinberg equilibrium test for control was assessed using the χ^2 method. We calculated the power ($1-\beta$) to detect an association with 122 cases and 211 controls (assuming $\alpha = 0.05$ and $D' = 1$). In general, there was adequate power (>0.80) to detect an OR ≥2 for polymorphisms present at frequency ≥0.1. For polymorphisms at ≤0.05 frequency or for OR of ≤1.5, the power was not adequate (<0.80) for detecting associations.

Haplotypes were constructed using a Bayesian algorithm by the PHASE program (v. 2.1). The permutation test (Haploview 4.1) was also employed for single marker and haplotype association, and p values less than 0.05 from 100,000 permutations were considered significant.

3. Results

3.1. LD structure and haplotype frequencies within the SLC11A1 gene in European descendents and the Japanese population

Of the 19 marker variants analyzed within *SLC11A1* in the Japanese population and European descendents, only 17 and 16 polymorphic loci were observed in total, respectively. The allele frequencies of the analyzed markers are summarized in Table 1. The pairwise LD (r^2) for both the Japanese and European descendents is presented in Fig. 1. A unique allele 7 of rs3444889, the GT repeat in the promoter region, was observed only in the Japanese population but not in European descendents. Interestingly, the MAF of SNP rs2290708, located in intron 7, was 0.27 in European descendents, whereas it was only 0.06 in the Japanese population. The extent of LD differed between the populations, and the 5′ LD block was larger in European descendents than in Japanese and spanned from −3,890 to rs2695343 in intron 7, whereas the 3′ LD block was larger in Japanese than in European descendents and covered from rs3816560 (intron 9) to rs2279014 (3′ UTR), as illustrated in Fig. 1.

We next compared the haplotype frequency between the 2 populations. One major haplotype accounted for 66.7% in the Japanese population, whereas 2 major haplotypes with frequencies of 51 and 21% were observed in European descendents (Table 2). Interestingly, the minor allele (T) of rs2290708 in intron 7, which was infrequent in Japanese, was carried by the second major haplotype (21%) in European descendents. Likewise, the 4th major haplotype in the Japanese population was observed with allele 7 of the (GT)n microsatellite of the promoter region of the gene, which was not observed in the European descendents.