Table 1 Association results for microsatellite markers

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

NS not significant, *HWE* Hardy–Weinberg equilibrium

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

[b] Fisher's exact test

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

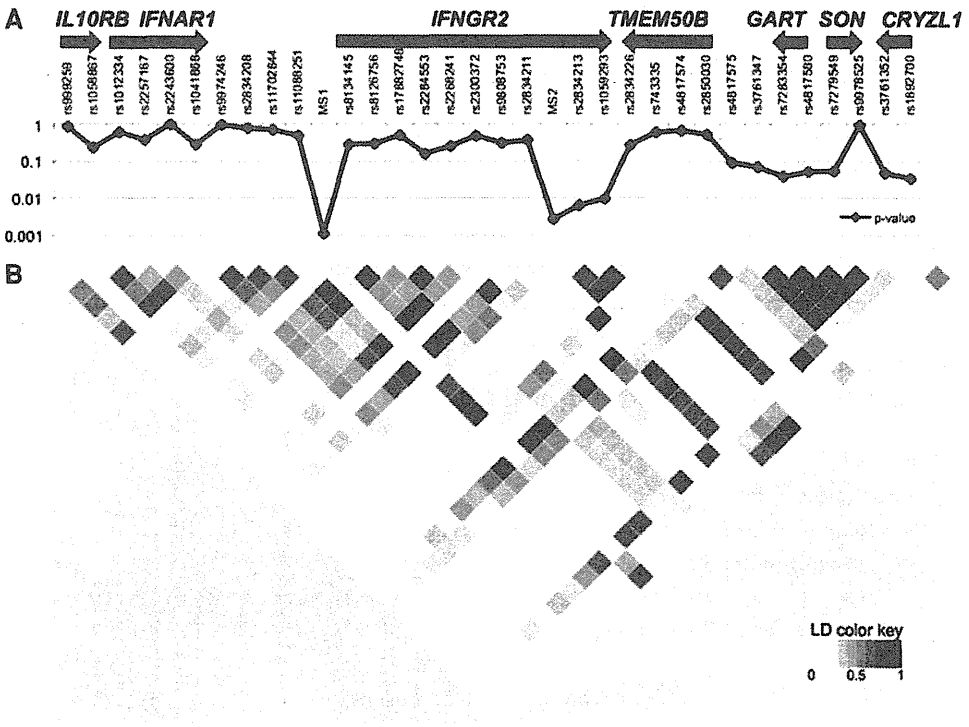

Fig. 1 Association *P* values and pairwise LD of genotyped polymorphisms around *IFNGR2* region. **a** Association results for 32 SNPs in 273 patients (panel A) and 506 controls are shown. *P* values of microsatellite markers in 98 patients and 200 controls are also included. Positions of genes are shown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

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

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

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

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

Transcription start site (TSS) of *IFNGR2*

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

Association results of TB panel B

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

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

Table 2 Association results of rs2834213 A/G SNP

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

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

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

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

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

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

Luciferase assay

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

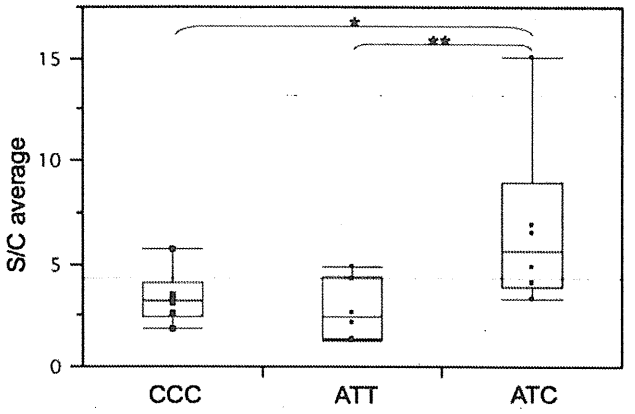

Fig. 2 Dual luciferase reporter assays. The ratios of Firefly luciferase activity (signal S) to Renilla luciferase activity (control C) are displayed using *box* and *whisker plots*. Three subcloned plasmids were prepared, and each subcloned plasmid was tested in triplicate and S/C values were averaged.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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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Circulating Levels of Adiponectin, Leptin, Fetuin-A and Retinol-Binding Protein in Patients with Tuberculosis: Markers of Metabolism and Inflammation

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Abstract

Background: Wasting is known as a prominent feature of tuberculosis (TB). To monitor the disease state, markers of metabolism and inflammation are potentially useful. We thus analyzed two major adipokines, adiponectin and leptin, and two other metabolic markers, fetuin-A and retinol-binding protein 4 (RBP4).

Methods: The plasma levels of these markers were measured using enzyme-linked immunosorbent assays in 84 apparently healthy individuals (=no-symptom group) and 46 patients with active pulmonary TB around the time of treatment, including at the midpoint evaluation (=active-disease group) and compared them with body mass index (BMI), C-reactive protein (CRP), chest radiographs and TB-antigen specific response by interferon-γ release assay (IGRA).

Results: In the no-symptom group, adiponectin and leptin showed negative and positive correlation with BMI respectively. In the active-disease group, at the time of diagnosis, leptin, fetuin-A and RBP4 levels were lower than in the no-symptom group [adjusted means 2.01 versus 4.50 ng/ml, $P<0.0001$; 185.58 versus 252.27 μg/ml, $P<0.0001$; 23.88 versus 43.79 μg/ml, $P<0.0001$, respectively]. High adiponectin and low leptin levels were associated with large infiltrates on chest radiographs even after adjustment for BMI and other covariates ($P=0.0033$ and $P=0.0020$). During treatment, adiponectin levels increased further and then decreased. Leptin levels remained low. Initial low levels of fetuin-A and RBP4 almost returned to the normal reference range in concert with reduced CRP.

Conclusions: Our data and recent literature suggest that low fat store and underlying inflammation may regulate these metabolic markers in TB in a different way. Decreased leptin, increased adiponectin, or this ratio may be a promising marker for severity of the disease independent of BMI. We should further investigate pathological roles of the balance between these adipokines.

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Introduction

Tuberculosis (TB) is a major infectious cause of death around the world, with most of the 1.5 million deaths per year attributable to the disease occurring in developing countries. Negative energy balance in chronic inflammation has been recognized as a prominent feature of TB and one of the major obstacles to manage the patients [1,2]. Recent emergence of drug resistant TB is assumed to be driven by poorly implemented drug regimens, but malnutrition as well as HIV co-infection might worsen the condition: Inflammatory responses evoked by infection increase the demand for anabolic energy, leading to a synergistic vicious circle and further deterioration of the clinical condition [3].

It is generally believed that undernourishment diminishes protective immunity against *Mycobacterium tuberculosis*. [4]. A series of animal experiments, particularly aerosol-infected guinea pig models have demonstrated that chronic protein-energy malnutrition reduces secretion of T-helper 1 (Th1) cytokines [5]. It is rapidly reversed with alimentary supplement, indicating a pivotal role of nutrition, although it remains unclear what the optimal nutritional interventions are for improving the human disease in an effective manner [4].

On the other hand, in many countries today, rapid industrialization and urbanization are accompanied by changing patterns of diet and physical activity and this results in overnutrition [6]. Consequently, a combination of these two unfavor-

Table 1. Characteristics of study population.

	no-symptom group (N = 84)	active-disease group (N = 46)	*P* values
Male/Female (n)	41/43	42/4	<0.0001
Age (year)*	40.0 (28.1–48.6)	47.2 (34.7–55.0)	0.0064
BMI (kg/m^2)*	21.8 (20.0–23.7)	18.3 (17.1–19.5)	<0.0001
BCG history (yes/no/unknown)	33/28/23	10/3/33	<0.0001
positive/negative results of IGRA (n)	55/29	41/4**	0.0015

*Median and 25-to-75 percentiles in parenthesis are shown.
**One indeterminate case is not shown here.
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able conditions, a slow decline of infectious diseases associated with undernutrition and a rapid increase in obesity and diabetes are a serious double burden to public health and clinical medicine in resource limited settings [7].

Mainly in studies carried out in industrialized countries, fat-cell-derived hormones/cytokines designated as adipokines and relevant mediators have been investigated extensively and proposed as markers of obesity and diabetes [8]. Of these adipokines, adiponectin is a unique insulin sensitizer with atheroprotective role [9,10]. Plasma levels of adiponectin are inversely correlated with body weight and visceral fat mass [11,12]. Leptin is another major adipokine in proportion to fat stores [13,14] and one of the key mediators of energy metabolism [2] Even mild weight loss induced by dietary restriction is known to reduce leptin levels [11]. These markers supposedly shift towards the opposite in lean patients with wasting diseases. However, the significance of these metabolic markers in chronic infectious diseases like TB has not been fully understood [2].

We have recently conducted a proteomic research and demonstrated that plasma levels of fetuin-A and retinol-binding protein 4 (RBP4), also closely linked to the metabolic and inflammatory state, were significantly lower in patients with active pulmonary TB than in control subjects [15]. Fetuin-A, also known as α2-Heremans-Schmid glycoprotein, is an abundant plasma component of hepatic origin [16] and a negative regulator of insulin signaling [17,18]. Elevation of plasma fetuin-A is strongly associated with atherogenic lipid profile as well as fatty liver in obese patients [18]. Lipid components in the liver presumably upregulate fetuin-A expression, which may in turn repress adiponectin and impair adipocyte function [19,20]. Fetuin-A is also downregulated in acute inflammation as a negative acute-phase protein [21]. RBP4, synthesized in the liver and adipose tissue, has recently been identified as another adipokine involved in the development of insulin resistance [22]. In humans, similar to leptin, circulating RBP4 levels are high in obesity and decreased after calorie-restriction induced weight loss [11,23]. RBP4 is also known as a specific transporter protein for retinol (vitamin A) and can be used to assess the short-term fluctuation of nutritional states as a rapid turnover protein [24].

Alteration of the circulating levels of these markers should be investigated in TB, since they are expected to provide a basis of a critical link among nutritional status, metabolism and immunity of the disease, and hopefully to consider efficient nutritional interventions. In the present study, we thus measured circulating adiponectin and leptin in addition to fetuin-A and RBP4 levels in patients with active pulmonary TB versus apparently healthy individuals and compared the levels with body mass index (BMI), a simple estimate of adiposity [25] and C-reactive protein (CRP),

Table 2. Correlation of tested marker levels with BMI, CRP and IGRA values in each of the no-symptom and active-disease groups.

	no-symptom group (N = 84)			active-disease group (N = 46)		
	Pearson's *r* (*P* values)[a]			Pearson's *r* (*P* values)[a]		
Variable	by BMI (kg/m^2)	by CRP (μg/ml)	by IFN-γ (IU/ml)[b]	by BMI (kg/m^2)	by CRP (μg/ml)	by IFN-γ (IU/ml)[b]
Adiponectin (μg/ml)	−0.4530	−0.2892	−0.2254	−0.4421	0.1477	−0.1092
	(<0.0001)*	(0.0076)	(0.0393)	(0.0021)	(0.3274)	(0.4700)
Leptin (ng/ml)	0.4518	0.1694	0.1179	0.2771	−0.0918	0.3568
	(<0.0001)*	(0.1234)	(0.2855)	(0.0623)	(0.5442)	(0.0149)
Leptin/adiponectin ratio	0.5820	0.2793	0.2067	0.4901	−0.1633	0.2804
	(<0.0001)*	(0.0101)	(0.0592)	(0.0005)*	(0.2783)	(0.0591)
Fetuin-A (μg/ml)	0.0309	0.0415	0.0322	0.1243	−0.1833	0.2402
	(0.7805)	(0.7079)	(0.7714)	(0.4105)	(0.2226)	(0.1078)
RBP4 (μg/ml)	0.1605	−0.0213	0.0716	0.1535	−0.3018	−0.0916
	(0.1447)	(0.8475)	(0.5173)	(0.3085)	(0.0415)	(0.5448)

[a]Pearson's correlation coefficients with *P* values were calculated. Plasma concentrations were analyzed after logarithmic transformation.
[b]TB-antigen stimulated IFN-γ response
*Statistically significant when the significance level is set as $P<0.002$ based on the Bonferroni correction.
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Table 3. BMI, CRP and tested marker levels in IGRA-positive and -negative subgroups in the no-symptom group.

	IGRA-negative (N = 29)		IGRA-positive (N = 55)		
marker	**adjusted mean[a]**	**(95%CI)**	**adjusted mean[a]**	**(95%CI)**	***P* values (ANCOVA)**
BMI (kg/m^2)	21.52	(20.58–22.46)	21.48	(20.74–22.22)	0.9392
CRP (μg/ml)	1.12	(0.60–2.08)	1.30	(0.80–2.12)	0.6663
Adiponectin (μg/ml)	7.19	(5.67–9.11)	6.39	(5.30–7.70)	0.3792
Leptin (ng/ml)	4.50	(3.34–6.05)	4.38	(3.47–5.54)	0.8783
Leptin/adiponectin ratio	0.63	(0.40–0.97)	0.69	(0.49–0.97)	0.7080
Fetuin-A (μg/ml)	234.22	(212.40–258.29)	263.88	(244.26–285.06)	0.0333
RBP4 (μg/ml)	39.64	(32.28–48.69)	42.88	(36.45–50.43)	0.4997

[a]Estimated means of plasma concentrations were compared after logarithmic transformation, being adjusted for gender and age as covariates. The data shown are transformed back to the original unit.
No *P* values were statistically significant when the significance level is set as $P<0.007$ based on the Bonferrroni correction.
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a representative positive acute phase protein [26]. We further characterized their relationship with disease severity and alterations during the course of treatment.

Methods

Study design

We randomly selected and used plasma samples and demographic information in 46 patients with active pulmonary TB (= active-disease group) without treatment history as a biomarker sub-study of a large cohort study [27]. All patients entered the study from July 2007 to March 2009. Diagnosis of active pulmonary TB was made clinically and radiologically and confirmed bacteriologically in Hanoi Lung Hospital. A sputum smear test showed positive results in all of the patients in the active disease group and all of them completed anti-TB treatment following the national standard regimen, 2 months of streptomycin, isoniazid, rifampicin, and pyrazinamide followed by 6 months of isoniazid and ethambutol (2SHRZ/6HE).

Chest radiographs were taken at the time of diagnosis and interpreted by two readers independently in a blind manner. The presence of cavitary lesions and the number of lung zones (zero to six corresponding to the upper, middle, and lower fields on the right and left sides of the lung) affected by infiltrates were recorded [28]. HIV status was examined before starting anti-TB treatment. The proportion of HIV co-infection is less than 10% in this study area and those with HIV positive were excluded from the drawing up of this sub-study.

As a reference, we also measured plasma samples derived from 84 apparently healthy men and women who may have chances of direct or indirect contacts with TB patients as health care staff (= no-symptom group). All participants were tested for TB-antigen specific interferon-γ response by the commercially available enzyme-linked immunosorbent assay (ELISA)-based interferon-γ release assay (IGRA), QuantiFERON-TB Gold In-Tube™ (Cellestis, Victoria, Australia). In the no-symptom group, IGRA-positive individuals suspected of latent TB infection were recommended to take chest radiography and to confirm there were no active pulmonary lesions. Subsequently a chance of receiving isoniazid prophylactic therapy was given. The protocol was approved by ethical committees of the Ministry of Health, Viet Nam and National Center for Global Health and Medicine, Japan respectively and written informed consent was obtained from each participant.

Table 4. BMI, CRP and tested marker levels in the no-symptom and active-disease groups after adjustment for gender and age.

	no-symptom group (N = 84)		active-disease group (N = 46)		
marker	**adjusted mean[a]**	**(95%CI)**	**adjusted mean[a]**	**(95%CI)**	***P* values (ANCOVA)**
BMI (kg/m^2)	21.68	(21.06–22.30)	17.65	(16.66–18.65)	<0.0001*
CRP (μg/ml)	1.22	(0.86–1.74)	36.88	(20.94–64.94)	<0.0001*
Adiponectin (μg/ml)	6.82	(5.73–8.12)	9.29	(7.02–12.30)	0.0136
Leptin (ng/ml)	4.50	(3.78–5.35)	2.01	(1.52–2.66)	<0.0001*
Leptin/adiponectin ratio	0.66	(0.50–0.88)	0.22	(0.14–0.34)	<0.0001*
Fetuin-A (μg/ml)	252.27	(234.55–271.33)	185.58	(165.07–208.64)	<0.0001*
RBP4 (μg/ml)	43.79	(38.09–50.34)	23.88	(19.08–29.88)	<0.0001*

[a]Estimated means of plasma concentrations were compared after logarithmic transformation, being adjusted for gender and age as covariates. The data shown are transformed back to the original unit.
*Statistically significant when the significance level is set as $P<0.007$ based on the Bonferrroni correction.
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Table 5. CRP and tested marker levels in the no-symptom and active-disease groups after adjustment for gender, age and BMI.

	no-symptom group (N = 84)		active-disease group (N = 46)		
marker	**adjusted mean[a]**	**(95%CI)**	**adjusted mean[a]**	**(95%CI)**	***P* values (ANCOVA)**
CRP (μg/ml)	1.11	(0.77–1.60)	47.80	(25.36–90.09)	<0.0001*
Adiponectin (μg/ml)	7.80	(6.63–9.19)	6.39	(4.81–8.49)	0.1671
Leptin (ng/ml)	3.77	(3.26–4.37)	3.28	(2.54–4.24)	0.2790
Leptin/adiponectin ratio	0.48	(0.38–0.61)	0.51	(0.35–0.76)	0.7704
Fetuin-A (μg/ml)	248.04	(229.95–267.57)	194.46	(170.48–221.80)	0.0004*
RBP4 (μg/ml)	42.90	(37.08–49.63)	25.27	(19.62–32.55)	0.0001*

[a]Estimated means of plasma concentrations were compared after logarithmic transformation, being adjusted for gender, age and BMI as covariates. The data shown are transformed back to the original unit.
*Statistically significant when the significance level is set as $P<0.008$ based on the Bonferroni correction.
doi:10.1371/journal.pone.0038703.t005

Measurements of markers of metabolism and inflammation

Immediately after making the diagnosis of active TB disease, heparinized blood samples were drawn for IGRA before starting anti-TB treatment (0 month) and the remaining plasma without mixing any stimulants was reserved in a −80°C freezer until measurement. Samples were collected twice again, after the initial phase of treatment (2 months) and at the end of treatment (7 months) in the active disease group. This study was originally intended to identify a variety of biomarkers associated with TB phenotypes [15] and the participants were not obliged to keep fasting. The blood was collected in the daytime between 8 am and 4 pm at the outpatient clinic to avoid interference in dosing schedule of anti-TB drugs.

The AssayMax Human C-Reactive Protein ELISA kit was used for detection of human c-reactive protein (CRP) in plasma (Assaypro LLC. St. Charles, MO, USA). The minimum detectable dose was less than 0.25 ng/ml. The Quantikine® Human Total Adiponectin/Acrp30 Immunoassay kit was used to detect total (low, middle and high molecular weight) human adiponectin in plasma (R&D Systems, Inc.; Minneapolis, MN, USA). The mean minimum detectable dose was 0.246 ng/ml. The Quantikine® Human Leptin Immunoassay kit was used to detect human leptin in plasma (R&D Systems, Inc.). The mean minimum detectable dose was 7.8 pg/ml. The AHSG ELISA kit was used to detect fetuin-A in plasma (BioVender Laboratory Medicine Inc.; Modrice, Czech Republic). The detection limit was 0.35 ng/ml. A competitive ELISA for quantitative determination of RBP4 in human plasma was also applied (AdipoGen Inc.; Seoul, Korea) and the detection limit was 1 ng/ml. All were performed according to the manufacturer's instructions. Differences in measured concentrations between EDTA plasma samples as reference and these heparin samples were within a range of variation generally accepted in ELISA (coefficient of variance <15%) (data not shown)

Statistical analysis

Plasma protein levels were served for subsequent statistical analysis after logarithmic transformation of the measurements to minimize distortion of the data distribution. Means of demographic data between two groups were compared by analysis of variance (ANOVA) after testing for equal variances and

Table 6. BMI, CRP and tested marker levels in patients with small and large infiltrates on chest radiographs after adjustment for gender and age.

	small infiltrates[a] (N = 22)		large infiltrates[a] (N = 23)		
marker	**adjusted mean[b]**	**(95%CI)**	**adjusted mean[b]**	**(95%CI)**	***P* values (ANCOVA)**
BMI (kg/m^2)	18.73	(16.74–20.71)	18.11	(15.95–20.27)	0.3065
CRP (μg/ml)	26.14	(12.63–54.10)	35.92	(16.29–79.21)	0.1520
Adiponectin (μg/ml)	10.28	(5.38–19.66)	18.83	(9.31–38.11)	0.0033*
Leptin (ng/ml)	2.42	(1.64–3.57)	1.65	(1.08–2.52)	0.0020*
Leptin/adiponectin ratio	0.24	(0.11–0.52)	0.09	(0.04–0.21)	0.0002*
Fetuin-A (μg/ml)	201.97	(149.87–272.18)	184.68	(133.52–255.46)	0.3222
RBP4 (μg/ml)	36.14	(21.76–60.03)	31.56	(18.17–54.79)	0.3770
IFN-γ (IU/ml)[c]	11.04	(2.13–57.16)	5.80	(0.97–34.82)	0.2039

[a]Small infiltrates = less than 3 of 6 zones in the lung affected, large infiltrates = 3 or more than 3 of 6 zones affected
[b]Estimated means of plasma concentrations were compared after logarithmic transformation, being adjusted for gender and age as covariates. The data shown are transformed back to the original unit.
[c]TB-antigen stimulated IFN-γ response
*Statistically significant when the significance level is set as $P<0.006$ based on the Bonferroni correction.
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Table 7. CRP and tested marker levels in patients with small and large infiltrates on chest radiographs after adjustment for gender, age and BMI.

	small infiltrates[a] (N = 22)		large infiltrates[a] (N = 23)		
marker	**adjusted mean[b]**	**(95%CI)**	**adjusted mean[b]**	**(95%CI)**	***P* values (ANCOVA)**
CRP (μg/ml)	26.59	(12.78–55.28)	35.50	(16.02–78.63)	0.1991
Adiponectin (μg/ml)	10.84	(6.01–19.53)	18.15	(9.57–34.40)	0.0061*
Leptin (ng/ml)	2.37	(1.63–3.47)	1.67	(1.11–2.52)	0.0040*
Leptin/adiponectin ratio	0.22	(0.11–0.44)	0.09	(0.04–0.20)	0.0002*
Fetuin-A (μg/ml)	200.77	(148.59–271.28)	185.46	(133.74–257.18)	0.3886
RBP4 (μg/ml)	35.69	(21.43–59.46)	31.83	(18.29–55.42)	0.4626
IFN-γ (IU/ml)[c]	11.41	(2.17–59.90)	5.68	(0.94–34.53)	0.1760

[a]Small infiltrates = less than 3 of 6 zones in the lung affected, large infiltrates = 3 or more than 3 of 6 zones affected
[b]Estimated means of plasma concentrations were compared after logarithmic transformation, being adjusted for gender, age and BMI as covariates. The data shown are transformed back to the original unit.
[c]TB-antigen stimulated IFN-γ response
*Statistically significant when the significance level is set as $P<0.007$ based on the Bonferrroni correction.
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proportions between two groups were compared by the chi-squared test. Since it is well known that levels of adipokines such as leptin are influenced by gender and age, measurements of protein markers in any two groups were compared by analysis of covariance (ANCOVA) to allow for the covariates. The relationship between markers and other parameters were assessed by Pearson's correlation coefficients. Overall alterations of the measurements at three time points were initially analyzed by repeated-measures ANOVA and only when statistically significant, post-hoc comparisons were proceeded to: Difference of values between two time points was assessed by the paired-T test, under normal approximation based on the central limit theorem. *P* values<0.05 were considered to be statistically significant in general. When the Bonferroni correction was applied, however, a level of statistical significance was set as 0.05/n (n = the number of comparisons). Statistical analysis was performed using Stata version 11 (StataCorp, College Station, TX, USA).

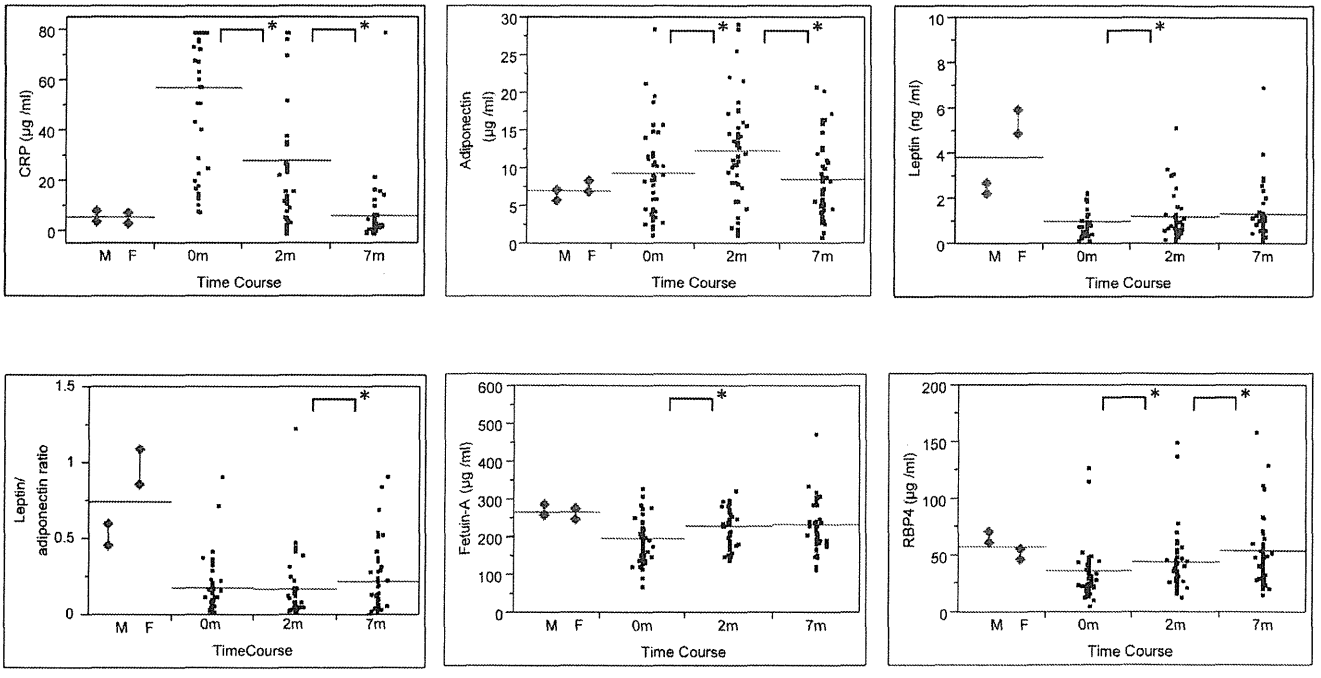

Figure 1. CRP and tested marker levels in patients with active TB before (0 month), during (2 months) and at the end (7 months) of anti-TB treatment (N = 46). Vertical bars with diamonds on the left side (M and F) indicate reference values, means ± SEM of the values in men (N = 41) and women (N = 43) of the no-symptom group. A horizontal bar indicates the grand mean of the values in each condition. * indicates $P<0.05$ by paired comparison between 0 month and 2 months. When significant, 2 months and 7 months were also compared.
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Results

Characteristics of study population

The no-symptom group consisted of 84 apparently healthy individuals, whose blood samples were used to obtain the standard values of markers in the study population. This group includes an approximately equal number of men and women with median age of 40, and more than half of the individuals had latent TB infection diagnosed by the IGRA method (Table 1). The active-disease group members were 46 patients with smear-positive active pulmonary TB. The majority of the patients were male with low body mass index (BMI<18.5 kg/m^2) and the median age was 47, slightly older than in the non-symptom group.

Correlation of adiponectin, leptin, fetuin-A and RBP4 levels with BMI, CRP and IGRA values in the no-symptom and active-disease groups

Correlation coefficients (r) were calculated in the no-symptom and active-disease groups respectively (Table 2). Adiponectin and leptin showed negative and positive correlations with BMI respectively in the no-symptom group ($r=-0.4530$, $P<0.0001$; $r=0.4518$, $P<0.0001$). Leptin/adiponectin ratio showed a positive correlation with BMI in the active-disease group ($r=0.4901$, $P=0.0005$) as well as in the no-symptom group ($r=0.5820$, $P<0.0001$). These correlations were statistically significant even after Bonferroni correction for multiple comparisons. The other possible correlations including a pair of leptin and TB-antigen stimulated IFN-γ response did not reach significant levels in this study, when Bonferroni correction was applied.

Pairwise correlations between four tested markers

Pairwise correlation coefficients (r) between four tested metabolic markers were further calculated in the no-symptom and active-disease groups respectively (Table S1). A significant correlation was found only between fetuin-A and RBP4 levels ($r=0.4007$, $P=0.0058$) in the active disease group.

Adiponectin, leptin, fetuin-A and RBP4 levels with IGRA-positive and -negative subgroups in the no-symptom group

IGRA-positive values higher than the cutoff value, 0.35 IU/ml are regarded as latent TB infection after active disease is ruled out. We thus categorized the no-symptom group into IGRA-positive and -negative subgroups and compared plasma concentrations of the above markers. However, none of the marker levels including fetuin-A were significantly different between IGRA-positive and -negative subgroups after adjustment for gender and age, when considering the number of comparisons (Table 3).

Adiponectin, leptin, fetuin-A and RBP4 levels in the no-symptom and active-disease groups

The active-disease group had significantly low BMI and very high CRP levels at the time of diagnosis, when assessed by using ANCOVA with adjusted means (Table 4). In the disease group, leptin, leptin/adiponectin ratio, fetuin-A and RBP4 levels were remarkably lower than in the no-symptom group ($P<0.0001$ respectively) after adjustment for gender and age and these differences were statistically significant even after Bonferroni correction (Table 4).

Since BMI was strongly correlated with some of the adipokine values as shown in Table 2, we further analyzed levels of the four markers after adjustment for BMI as well as gender and age. Consequently, adiponectin and leptin levels were not significantly different between the two groups any more, whereas fetuin-A and RBP4 levels remained significant ($P=0.0004$ and $P=0.0001$) (Table 5).

Adiponectin, leptin, fetuin-A and RBP4 levels in patients with mild and severe disease

At the time of diagnosis, severity of the disease was assessed by spread of infiltrates on chest radiographs (Table 6). Small infiltrates affecting less than 3 of the 6 lung zones and large ones affecting more, categorized the patients into two subgroups (= mild and severe disease) half-and-half.

After adjustment for gender and age, adiponectin levels were higher and leptin levels were lower in patients with large infiltrates than in those with small infiltrates ($P=0.0033$ and $P=0.0020$). Interestingly, differences in the levels of these two adipokines between small and large infiltrates were significant respectively ($P=0.0061$ and $P=0.0040$), even after adjustment for BMI as well as gender and age (Table 7). Leptin/adiponectin ratio was lower, or adiponectin/leptin ratio was higher, in patients with large infiltrates than in those with small infiltrates independent of BMI ($P=0.0002$). None of the markers were associated with the presence of cavity on the chest radiographs (data not shown).

Adiponectin, leptin, fetuin-A and RBP4 levels in patients with active TB before, during and at the end of anti-TB treatment

Figure 1 shows plasma values at the time points before (0 month), during (2 months) and at the end (7 months) of anti-TB treatment. Mean values in men (N = 41) and women (N = 43) of the no-symptom group are shown as a reference, in which gender difference was observed in leptin levels and leptin/adiponectin ratio ($P<0.0001$).

Overall differences of the measurements during anti-TB treatment in all of these four markers were statistically significant by repeated-measures ANOVA ($P<0.01$). Post-hoc analysis showed that adiponectin levels increased transiently ($P=0.0004$; 0 month vs. 2 months) and then decreased close to the reference range by the end of treatment ($P<0.0001$; 2 months vs. 7 months). Leptin levels remained low throughout the treatment course, though gradually elevated ($P=0.0226$; 0 month vs. 2 months). Initial low levels of fetuin-A and RBP4 significantly improved during treatment ($P=0.0001$ and $P=0.0016$; 0 month vs. 2 months), almost reaching the reference range by the end in concert with reduced CRP levels.

Discussion

We assessed the clinical significance of four metabolic markers, adiponectin, leptin, fetuin-A and RBP4 in patients with active TB, analyzing them in relation to classical nutritional and inflammatory parameters, BMI and CRP, severity of disease and treatment course. BMI is known to be lower in patients with active TB than in control subjects [1,2]. After effective treatment, weight often increases but patients may remain underweight [11].

Plasma levels of adiponectin were inversely correlated with BMI in concordance with previous results [11,12]. The adiponectin levels tended to be elevated in the active-disease group characterized by low BMI, though it did not reach significant levels, which was also shown by others [29]. Interestingly in our study, adiponectin levels were significantly higher in severe disease with extensive pulmonary lesions than in mild disease, even after adjustment for BMI. Adiponectin as a modulator of inflammation in a variety of diseases has recently been highlighted [30]. For instance, in critically ill patients, adiponectin levels appear to be

transiently suppressed at the initial phase and then gradually elevated at the recovery phase [31,32]. The plasma concentrations in patients with active TB were further increased after starting treatment and then decreased close to the reference range by the end of treatment. Elevated adiponectin levels in chronic inflammatory diseases may be explained by compensatory response to the underlying disease as well as concomitant low body fat mass, which is postulated by others [33,34]. A study designed to measure alteration of adiponectin and BMI simultaneously throughout the treatment period would be able to characterize it further.

In most recent reports, leptin levels are low in TB [29,35–38], though other earlier or smaller studies have shown conflicting results [39–42]. In the present study, using a commercial ELISA, significantly lower levels of leptin were demonstrated in patients with active TB, which could be mostly explained by marked undernutrition in our disease population. Within the active-disease group, however, correlation between leptin and BMI was less clear. BMI-independent regulation of plasma leptin concentrations should also be taken into consideration in TB at least in part [13,37]. This idea is also supported by an *ex vivo* study by others demonstrating that continuous exposure of IL-1 or TNF-α provides a signal to downregulate leptin in human adipose tissue [43], though acute inflammation such as sepsis may rather upregulate circulating leptin levels transiently [44–46]. In addition to relatively high levels of adiponectin, low levels of leptin were observed in patients with large infiltrates, even after adjustment for BMI. This is concordant with a recent study showing that leptin levels were low in severe TB disease [29]. We have further demonstrated that low leptin/adiponectin ratio, or high adiponectin/leptin ratio is characteristic to severe TB disease in this study. This ratio was originally proposed as an atherogenic index indicating a balance between the two markers bearing apparently opposite functions in inflammation [47]. Our findings support the idea that suppressed production of leptin may be detrimental to host defense against TB by virtue of impairment of Th1 cell-mediated immunity [13,29,48]. After starting treatment, leptin levels were slightly elevated, but remained low during the treatment period. This is also compatible with reports made by others [37,38], although the mechanism remains unknown. Long-lasting low levels of leptin may be attributed to individual predisposition to TB or delayed recovery from wasting disease.

In our study, fetuin-A levels were considerably low in TB even after adjustment for BMI. Soon after starting treatment, the levels were increased in inverse proportion to the decrease in CRP. In TB, fetuin-A may be downregulated by at least dual mechanisms, strongly mediated by underlying inflammation [21] and partly controlled by depleted liver fat due to wasting or malnutrition [18]. Low fetuin-A levels may also result in impairment of macrophage function to kill the pathogen and ectopic calcification possibly in TB lesions [49,50].

RBP4 levels were also low in TB even after adjustment for BMI. Throughout the treatment course, the levels were gradually elevated close to the reference range inversely with the decrease in CRP. These findings are supported by a recent report demonstrating that RBP4 rapidly decreases during acute inflammation, possibly acting as a negative acute phase reactant, similar to fetuin-A, albumin and prealbumin [21,51,52]. This may partly explain a close positive correlation with fetuin-A demonstrated in the active-disease group. In addition to dual regulation of RBP4 by underlying inflammation and low body fat mass, reduced renal function is also known to cause retention of the circulating levels, such that further caution is needed to interpret RBP4 measurement in disease state [53].

Our study has several limitations. Firstly, many types of nutrients including micronutrients are essential to the human body but the potential interplay between each component of nutrients was not within our scope at that time. Secondly, since change of BMI was not measured during treatment, direct comparison of improved BMI with the corresponding marker levels was not possible. Thirdly, blood was collected during the daytime without enforced fasting. Although, of course, this increases the variance of measurements, it can be inferred that daytime variations on circulating adipokines and leptin [54] are not as large as to seriously affect conclusive results of comparisons within and between groups in this study. Finally, computer tomography, which has advantages over chest radiography as an imaging tool, was not available in our setting.

Overall, our data and recent literature would suggest that all of the four markers tested are controlled partly by low fat store and partly by inflammation in TB but their regulatory mechanisms are more or less different and interactions with other relevant factors including insulin sensitivity and cellular immunity are worth further investigation. In particular, leptin, adiponectin and their ratio may be promising markers for severity of the wasting disease. Since nutritional intervention has a potential to improve prognosis of intractable TB such as HIV co-infection and MDR-TB, large-scale prospective studies using selected biomarkers to investigate metabolic contributors to disease phenotype are desired. The more fully we understand the mechanisms linking diet, health, and disease, the more effective will be our ability to design optimal interventions.

Supporting Information

Table S1 Pairwise correlations between four tested markers.
(DOC)

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Author Contributions

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

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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

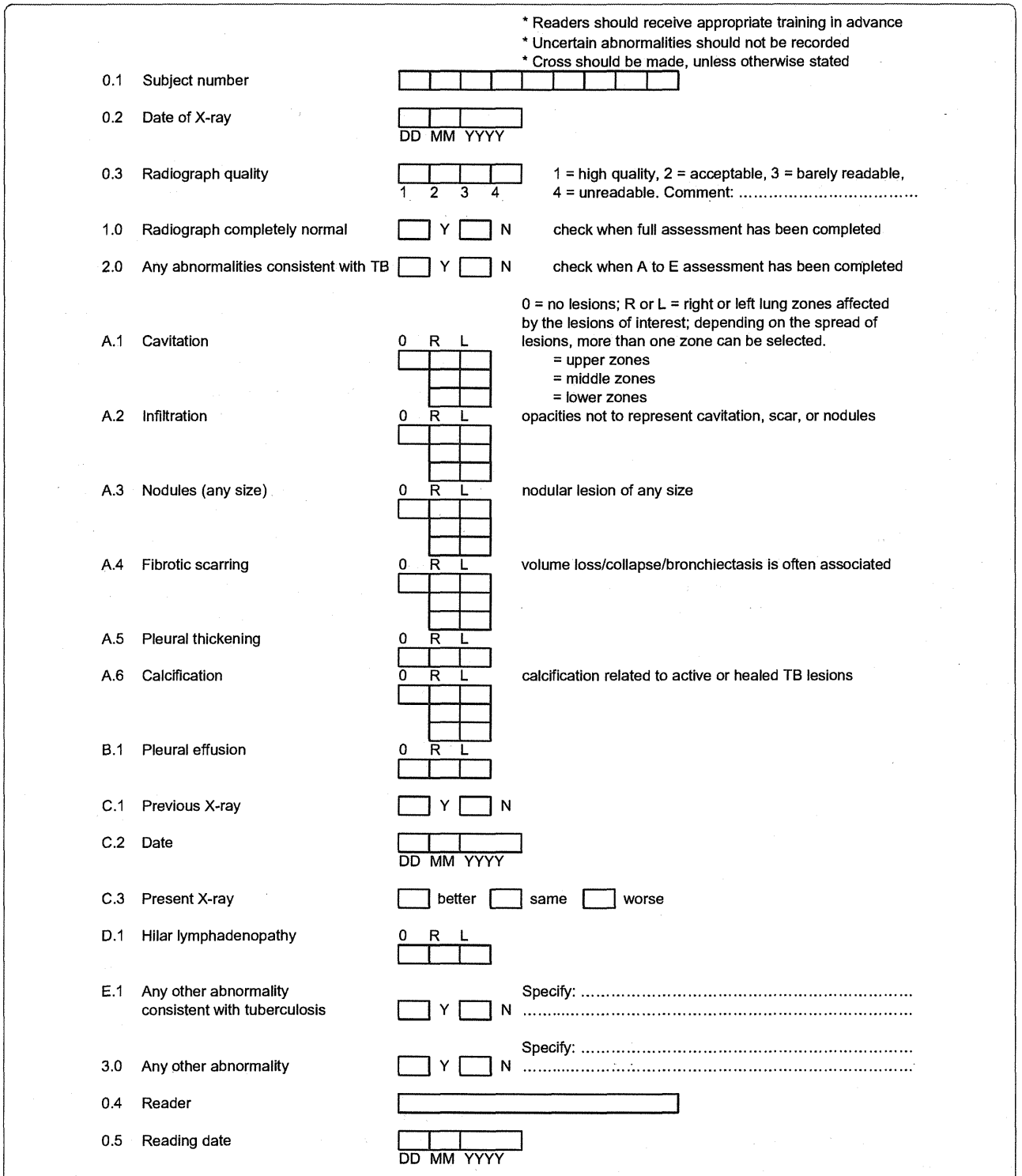

Figure 1 Chest X-ray coding: JVCS. JVCS = Japan-Vietnam chest X-ray coding system; DD = date in two digits; MM = month in two digits; YYYY = year in four digits; Y = yes; N = no; R = right; L = left.

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.

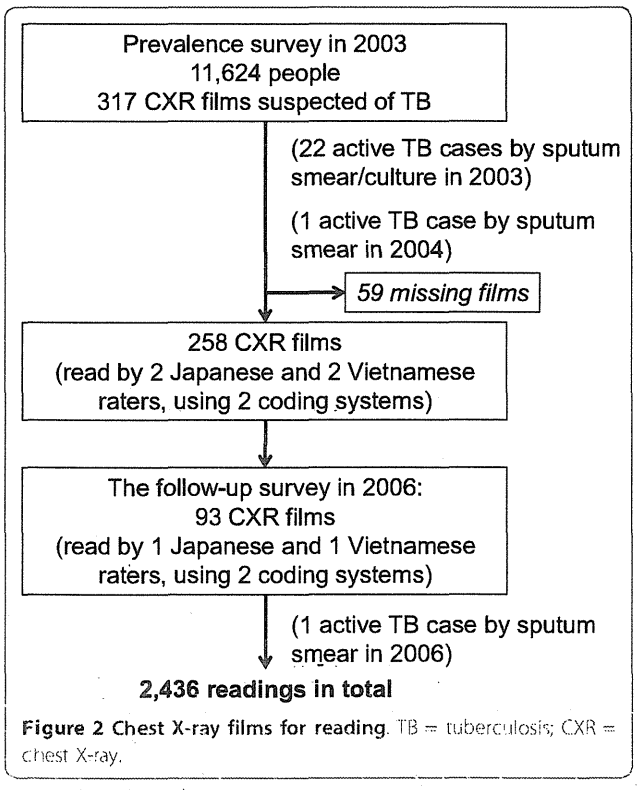

Figure 2 Chest X-ray films for reading. TB = tuberculosis; CXR = chest X-ray.

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).

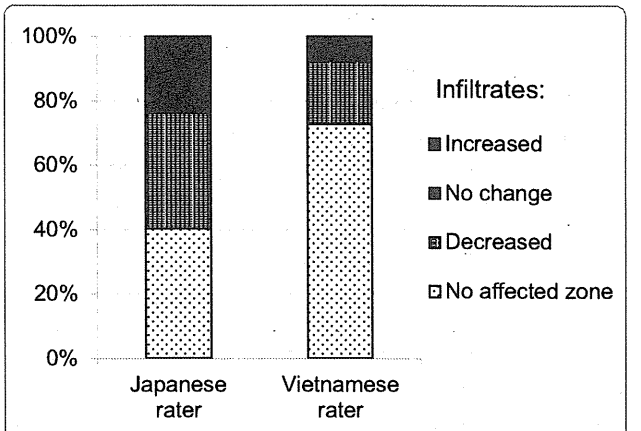

Figure 3 Infiltrates on chest X-rays after a 3-year interval. Evaluation of infiltrates on chest X-ray films between 2003 and 2006 using JVCS codes shows changes in the number of affected areas (upper, middle, and lower zones of each side of the lung). "No affected zone" indicates that the rater did 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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