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

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

Materials and methods

Study population

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

Microsatellite markers

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

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

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

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

Selection of representative SNPs around *IFNGR2* and genotyping

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

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

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

Luciferase assay

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

Statistical analysis

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

Results

Microsatellite markers

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

Screening of genetic polymorphisms in *IFNGR2*

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

Genotyping of selected SNPs around *IFNGR2*

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

Table 1 Association results for microsatellite markers

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

NS not significant, *HWE* Hardy–Weinberg equilibrium

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

[b] Fisher's exact test

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

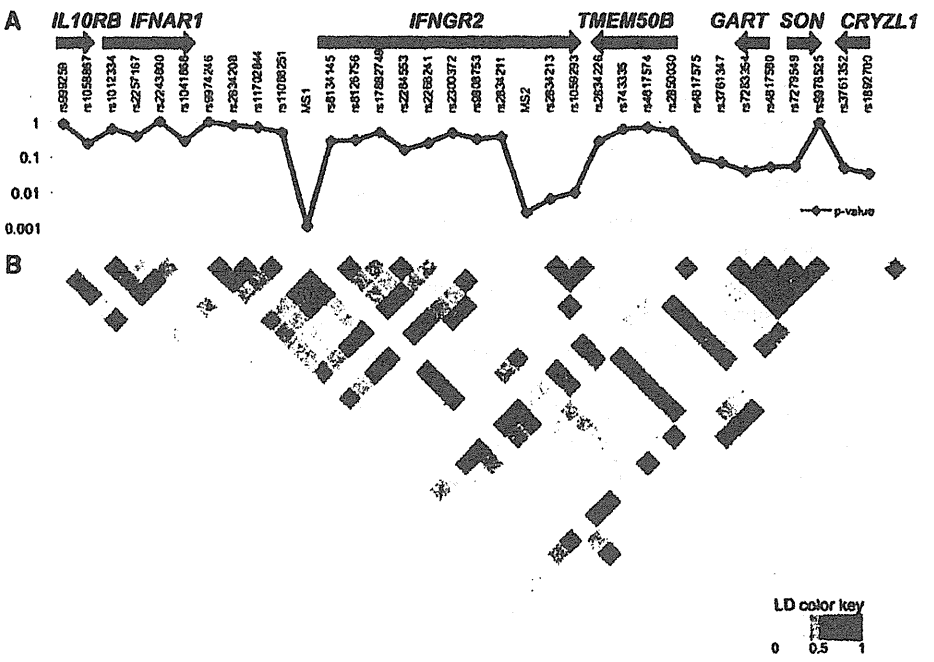

Fig. 1 Association *P* values and pairwise LD of genotyped polymorphisms around *IFNGR2* region. **a** Association results for 32 SNPs in 273 patients (panel A) and 506 controls are shown. *P* values of microsatellite markers in 98 patients and 200 controls are also included. Positions of genes are shown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

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

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

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

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

Transcription start site (TSS) of *IFNGR2*

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

Association results of TB panel B

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

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

Table 2 Association results of rs2834213 A/G SNP

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

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

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

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

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

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

Luciferase assay

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

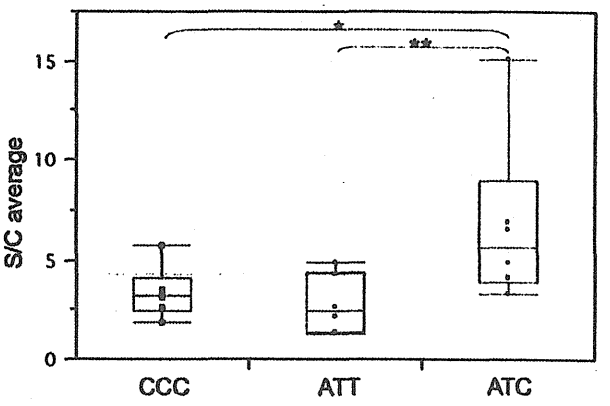

Fig. 2 Dual luciferase reporter assays. The ratios of Firefly luciferase activity (signal S) to Renilla luciferase activity (control C) are displayed using *box* and *whisker plots*. Three subcloned plasmids were prepared, and each subcloned plasmid was tested in triplicate and S/C values were averaged.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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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ASSOCIATION BETWEEN CIRCULATING FULL-LENGTH OSTEOPONTIN AND IFN-γ WITH DISEASE STATUS OF TUBERCULOSIS AND RESPONSE TO SUCCESSFUL TREATMENT

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Abstract. The T helper type 1 (Th1) immune response plays an important role in protective immunity, pathophysiology and development of tuberculosis (TB). To investigate whether osteopontin (OPN) and other Th1 response-related molecules are associated withTB disease status, including co-infection with HIV, and response to anti-TB treatment, circulating levels of full-length OPN (F-OPN), thrombin-cleaved N-terminal fragment of OPN (N-half OPN), IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10, IL-15 and C-reactive protein (CRP) were measured before and after anti-TB treatment. Patients with newly active pulmonary TB had significantly higher plasma levels of F-OPN, IFN-γ and CRP than healthy controls (HC). F-OPN, N-half OPN, IFN-γ, IP-10, IL-18 and IL-10 levels were higher in patients with extensive TB/HIV co-infection than in patients with a single disease of TB or HIV. Plasma levels of F-OPN correlated well with those of IP-10, IL-18 and N-half OPN among patients with active TB. The F-OPN, IFN-γ, IP-10 and CRP levels decreased significantly after effective anti-TB treatment. These data suggest that circulating OPN and Th1 response-related molecules, including IFN-γ, may be regulated in response to expansion of active TB and could serve as markers of disease activity before and during treatment.

Keywords: osteopontin, IFN-γ, CRP, tuberculosis, HIV/TB

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INTRODUCTION

Tuberculosis (TB) is one of the most important infectious causes of death worldwide (WHO, 2009). Despite its long historical interaction with humans, our understanding of host response to the TB

pathogen remains incomplete. Investigation of the molecular differences in host immune status between patients with active TB, co-infected with HIV and control subjects may provide a clue to understand the disease process.

In response to *M. tuberculosis*, activated macrophages and $CD4^+$ T lymphocytes produce Th1 cytokines, including IFN-γ, IL-12 and IL-18 (Schluger and Rom, 1998; van Crevel *et al*, 2002). IFN-γ triggers initiation of the major effector mechanism for the Th1 immune response (Flynn *et al*, 1993). IL-12 induction is observed following uptake of *M. tuberculosis* by dendritic cells and macrophages, which drives the production of IFN-γ in NK and T cells (van Crevel *et al*, 2002). Similarly, IL-18 exhibits strong IFN-γ inducing activity synergistically with IL-12 (Dinarello and Fantuzzi, 2003). The expression of IL-10 mRNA has been demonstrated in lymph nodes of TB patients, particularly in those with HIV/TB co-infection (Lin *et al*, 1996). Although IL-10 may down-regulate the immune response to mycobacterial infection (van Crevel *et al*, 2002), the exact role of IL-10 in TB remains controversial. IP-10, an IFN-γ inducible chemokine, is also predominant in active TB lymph nodes and the lung (Ferrero *et al*, 2003). Elevated circulating IP-10 levels have been reported in patients with active TB (Juffermans *et al*, 1999; Azzurri *et al*, 2005; Djoba Siawaya *et al*, 2009) and HIV/TB co-infection (Juffermans *et al*, 1999).

Osteopontin (OPN), a phosphorylated acidic glycoprotein associated with inflammation and tissue repair, is abundantly produced in the early stage of macrophage and T cell activation in granulomatous inflammation (O'Regan and Berman, 2000). OPN may polarize early Th1 cytokine responses through induction of IL-12 and suppression of IL-10 in macrophages (Ashkar *et al*, 2000). In a mouse model, a protective role of OPN in mycobacterial infection has clearly been demonstrated through experiments using OPN-null mice in which clearance of *M. bovis* BCG was reduced (Nau *et al*, 1999). In humans, OPN accumulates in well-formed granulomas with local mycobacterial infection, whereas OPN is absent or low in histologically ill-defined granulomas with disseminated infection (Nau *et al*, 2000). OPN is considered to play an active role in effective granuloma formation, inducing a Th1 response at an early stage of mycobacterial infection. Circulating OPN has also been measured in patients with active TB and their levels are generally high initially and decrease after anti-TB treatment (Koguchi *et al*, 2003; Inomata *et al*, 2005). Although these OPN levels have been reported to be correlated with Th1 cytokines, IFN-γ and IL-18 (Yamada *et al*, 2000; Inomata *et al*, 2005), the results of measuring circulating Th1 cytokine levels in human TB patients have often been inconsistent or unclear (Yamada *et al*, 2000; Morosini *et al*, 2003; Deveci *et al*, 2005; Inomata *et al*, 2005; Aktas *et al*, 2009). Immune reconstitution syndrome occurs after commencement of highly active antiretroviral therapy (HAART), at a stage when the *M. tuberculosis*-specific Th1 response is partially restored (Lawn *et al*, 2005). In HIV infected individuals, elevated OPN levels are found in cerebrospinal fluid and plasma and correlate with neurocognitive abnormalities (Burdo *et al*, 2008). OPN is the only pro-inflammatory cytokine found to increase after 1 month of HAART in lymph nodes (Li *et al*, 2004) and persists for 6 months of HAART (Chagan-Yasutan *et al*, 2009). OPN is susceptible to proteolytic fragmentation and a thrombin-cleaved N-terminal fragment of OPN (N-half OPN) is known to

affect its biological activity (O'Regan and Berman, 2000).

In this study, we attempted to address three questions unsolved by previous studies: 1) is OPN associated with TB even with HIV co-infection ($CD4^+$ T cell-depletion) in which granulomatous formation is generally poor? 2) is the N-half form, presumably cleaved by thrombin at the site of disease, more accurately connected with parameters of disease activity? 3) Do a variety of Th1-related molecules all coordinate with OPN levels? We investigated the concentrations of both full-length and N-half OPN, cytokines and a chemokine, including IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10 and IL-15, in the plasma of patients with newly active pulmonary TB, HIV/TB co-infection, HIV single infection and healthy controls and their levels within and between groups were compared. OPN and Th1 response-related molecules in patients with newly active pulmonary TB were also evaluated before and after anti-TB treatment. C-reactive protein (CRP) was simultaneously measured as a marker to monitor response to anti-TB treatment and an indicator of inflammation (Sahiratmadja *et al*, 2007; Peresi *et al*, 2008).

MATERIALS AND METHODS

Subjects

Twenty-three patients with pulmonary TB and 6 HIV/TB co-infected patients without highly active antiretroviral therapy (HAART) (HIV+TB+HAART) were recruited from the outpatient and inpatient clinics of Mae Chan and Chiang Rai hospitals, Chiang Rai Province, northern Thailand. HAART was defined as the regular use of two nucleoside reverse transcriptase inhibitors, NRTI [Stavudine (d4T) and Lamivudine (3TC)] plus a non-nucleoside reverse transcriptase inhibitor, NNRTI [Nevirapine (NVP) or Efavirenz (EFV)]. The patients with TB and HIV+TB+HAART-(HAART-) were all newly diaganosed pulmonary TB patients with sputum smears positive for acid-fast bacilli and confirmed by positive cultures for *M. tuberculosis* and abnormal chest radiographic findings. The patients had never received anti-TB treatment or had taken anti-TB drugs for less than 7 days at the time of enrollment. They had never received any immune-suppressive drugs or other immunomodulators. None of them had diabetes mellitus or other acute infections. On enrollment, the HIV/TB co-infected patients had not previously received antiretroviral therapy but were positive for HIV antibodies detected by particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Abbott Park, Ill) followed by a confirmation test using enzyme-linked immunosorbent assay (ELISA) (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

Ten HIV patients not taking HAART (HIV+HAART-) and 17 HIV patients receiving HAART (HIV+HAART+) were recruited from the HIV Care and Treatment Project (Daycare clinic), Mae Chan Hospital. These patients had no previous history of TB. One patient who was HIV+HAART+ was taking isoniazid preventive therapy (IPT) for active TB on enrollment. Their sputum smears were negative for acid-fast bacilli and the cultures were negative for *M. tuberculosis*. They were negative (induration<5 mm) for tuberculin skin test and had no concomitant active AIDS-related opportunistic infections during the 30 days prior to enrollment. None had diabetes mellitus or

Table 1
Baseline characteristics of study subjects.

Characteristics	HC	TB	HIV+ HAART-	HIV+ HAART+	HIV+TB+ HAART-
	n=25	*n*=23	*n*=10	*n*=17	*n*=6
Age, median (range), years	35.0 (21-52)	46.0 (18-64)	37.5 (31-53)	39.0 (27-52)	43.0 (30-47)
Sex, number of males/females	15/10	15/8	6/4	8/9	5/1
WBC x 10^3, median	6.80	9.60	5.21	5.48	8.62
(range), cells/μl	(3.64-11.20)	(3.10-15.80)	(3.31-6.06)	(2.82-9.11)	(5.70-12.80)
CD4' T cell count, median	1,050	564	274	437	146
(range), cells/μl	(451-1,580)	(226-1,081)	(30-789)	(104-843)	(19-344)
≤200, No. (%)			3 (30.0)	4 (23.5)	4 (66.7)
201-500, No. (%)	1 (4.0)	10 (43.5)	5 (50.0)	7 (41.2)	2 (33.3)
>500, No. (%)	24 (96.0)	13 (56.5)	2 (20.0)	6 (35.3)	
CXR findings, No. (%)					
Normal	23 (92.0)		9 (90.0)	17 (100.0)	
Infiltrate /non-cavitary		20 (87.0)	1 (10.0)		5 (83.3)
Cavitary		3 (13.0)			1 (16.7)
No definite infiltration	2 (8.0)				
Site of TB infection by member					
Pulmonary		22			3
Extra-pulmonary					
Both		1			3

HAART, highly active antiretroviral therapy; HC, healthy control; TB, patients with tuberculosis; HIV+HAART-, HIV patients without HAART; HIV+HAART+, HIV patients with HAART; HIV+TB+HAART-, HIV/TB co-infected patients without HAART.

was receiving immune-suppressive drugs or other immunomodulators during the 90 days prior to enrollment.

Twenty-five Thai healthy controls (HC) were recruited through the blood bank at Mae Chan Hospital and served as controls. They had no previous history of TB or risk factors for TB. Their chest radiographs were normal. They had no latent TB infection detected by interferon-γ release assays [QuantiFERON®-TB Gold In-Tube (QFT), Cellestis, Victoria, Australia]. None of them had diabetes mellitus. All were negative for hepatitis B surface antigen, hepatitis C antigen and HIV antibodies using particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or ELISA (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

The baseline characteristics of this patients and healthy controls are summarized in Table 1. Patients with TB had significantly higher white blood cell (WBC) counts ($p<0.05$) than HC; patients with HIV+TB+HAART- tended to have higher WBC counts. Patients with HIV+HAART- had significantly lower WBC counts than HC ($p<0.01$). The $CD4^+$ cell counts in TB patients were significantly higher than in HIV+HAART- patients ($p<0.01$), but were not significantly different from those with

Table 2
Clinical characteristics of six patients with HIV/TB co-infection.

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age in years/sex	42/Male	47/Male	37/Male	46/Female	30/Male	44/Male
CD4+ T cell count at TB diagnosis, cells/μl	46	198	19	321	94	344
CXR findings at TB diagnosis	Non-cavitary	Non-cavitary, infiltrates, pleural effusion	Non-cavitary, pleural effusion	Non-cavitary	Non-cavitary	Cavitary
Site of TB	PTB	PTB + EPTB (meningeal)	PTB	PTB + EPTB (colitis)	PTB + EPTB (lymphatic)	PTB
Treatment regimen for TB	2HRZE/4HR	2HRZE/4HR	2HEOS/18HE	2HRZE/4HR	2HRZE/4HR	2HRZE/4HR
HAART initiation during study period[a] (regimen)	Yes (d4T,3TC,NVP)	No	Yes (d4T,3TC,EFV)	No	No	No
Outcomes after 6-9 mo of anti-TB treatment	Cure	Cure	On treatment	Died[b]	Died[c]	Cure

PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis; 2HRZE/4HR, treatment regimen consisted of the 2-months (mo) initial phase of isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E) followed by the 4-months continuation phase consisted of isoniazid and rifampicin; 2HEOS/18HE, treatment regimen consisted of the 2-months initial phase of isoniazid, ethambutol, ofloxacin (O) and streptomycin (S) followed by the 18-months continuation phase consisted of isoniazid and ethambutol; d4T, Stavudine; 3TC, Lamivudine; NVP, Nevirapine; EFV, Efavirenz; HAART, highly active antiretroviral therapy.
[a]HAART initiated 2 months after starting anti-TB treatment.
[b]After 87 days of anti-TB treatment.
[c]After 4 days of anti-TB treatment.

HIV+HAART+ (p=0.07). Among patients with HIV+HAART+, the median time interval between initiation of HAART and enrollment was 35 months (range 14-56 months). The baseline and follow-up characteristics of the 6 patients with HIV+TB+HAART- are shown in Table 2. Of these 6 patients, 3 had pulmonary TB and 3 had both pulmonary and extra-pulmonary TB, 2 of them died during anti-TB treatment with a principal diagnosis of disseminated TB. Among the remain patients, 3 were considered to be cured and 1 patient was still undergoing TB treatment after 6-9 months based on National Tuberculosis Program (NTP) guidelines. Of the 3 patients that could be followed-up, 1 patient with a baseline $CD4^+$ cell count<200 cells/μl had started HAART 2 months after anti-TB treatment. Twelve patients with TB and 3 patients with HIV+TB+HAART- were able to be followed-up after 6-9 months of anti-TB treatment and were considered as cured according to the standard criteria.

This study was approved by the Ethical Review Committee for Research on Human Subjects, Ministry of Public Health, Thailand (Reference number 15/2550) and the National Center for

Global Health and Medicine, Japan (Reference number 415). Written informed consent was obtained from all subjects prior to enrollment.

Blood samples

Blood samples were collected in ethylene diaminetetraacetic acid (EDTA) vacutainer tubes from patients and healthy controls at the time of enrollment and after 6-9 months of anti-TB treatment when they were considered as cured. After centrifugation at 1,000*g* for 10 minutes at room temperature, the plasmas were collected and kept at -80°C until used.

Determination of full-length and N-half OPN by ELISA

The levels for full-length (F-OPN) and N-terminal fragment OPN (N-half OPN) were determined with a sandwich ELISA kit according to the manufacturer's instructions (IBL, Gunma, Japan). The tests were done in duplicate and the concentrations of F-OPN/N-half OPN were calculated from a linear equation for each standard curve developed with recombinant human F-OPN/N-half OPN. The subtracted absorbance below zero was considered as zero. The lower detection limits of the F-OPN and N-half OPN assay kits were 3.3 ng/ml and 92.7 pg/ml, respectively.

Determination of cytokines, a chemokine and CRP

IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10 and IL-15 levels in plasma were determined using sandwich ELISA kits according to the manufacturer's instructions. The tests were done in duplicate and the concentrations of cytokines/chemokines were calculated from a linear equation for each standard curve. The subtracted absorbance below zero was considered as zero. The lower detection limits of the assays were 4.7 pg/ml for IFN-γ (BD Biosciences Pharmingen, San Diego, CA), 7.8 pg/ml for IP-10 (BD Biosciences Pharmingen), 12.5 pg/ml for IL-18 (MBL, Nagoya, Japan), 62.5 pg/ml for IL-12/IL-23 (p40) (BioLegend, San Diego, CA), 3.9 pg/ml for IL-10 (BioLegend) and 4.0 pg/ml for IL-15 (BioLegend).

Highly sensitive C-reactive protein (CRP) levels in plasma were measured by means of particle enhanced immunonephelometry using the BN system (*Cardio*Phase® *hs*CRP, Dade Behring, Newark, DE). The lower detection limit was 148 ng/ml. Values below this level were considered equal to 148 ng/ml. A level of 3,000 ng/ml in the serum was considered as the upper limit of normal.

Statistical analysis

Statistical analysis was performed using SPSS software version 17.0. The data were expressed as medians and ranges. Since not all the parameters exhibited normal distribution, comparison between two independent groups was performed using the nonparametric Mann-Whitney *U* test, and comparison between the two dependent groups was performed using the nonparametric Wilcoxon signed-ranks test. The correlations among the F-OPN, N-half OPN and T cell response-associated molecules were analyzed using a Spearman's rank correlation test. A p-value <0.05 was considered significant.

RESULTS

Circulating F-OPN levels in TB

The plasma F-OPN levels from patients with TB (251.9-959.9 ng/ml) and HIV+TB+HAART-(853.2-4,005.4 ng/ml) were significantly higher than in patients with HIV+HAART- (209.5-450.8 ng/ml) ($p<0.01$, $p<0.01$, respectively), HIV+HAART+ (141.2-655.1 ng/ml) ($p<0.01$, $p<0.001$, respectively) and HC

(37.3-517.8 ng/ml) ($p<0.000001$, $p<0.001$, respectively) (Fig 1a). The plasma F-OPN levels in patients with HIV+TB+HAART- were significantly higher than in patients with TB ($p<0.001$). Although the N-half OPN levels were below the detection sensitivity (92.7 pg/ml) in many study subjects (Fig 1b), the N-half OPN levels in patients with TB tended to be higher than in patients with HIV+HAART- and HIV+HAART+ and HC. Half of patients with HIV+TB+HAART- had even higher N-half OPN levels than patients with TB ($p<0.01$).

Changes in circulating IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), CRP and IL-10 in TB

Before anti-TB treatment, the plasma levels of IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40) and CRP in patients with TB were significantly higher than in HC ($p<0.0000001$, $p<0.01$, $p<0.0001$, $p<0.00001$, and $p<0.0000001$, respectively), whereas IL-10 levels in patients with TB were significantly lower than in HC ($p<0.01$) (Fig 1c-1h). Patients with TB had significantly higher plasma IFN-γ, IP-10, IL-18 and CRP levels than patients with HIV+HAART+ ($p<0.001$, $p<0.0001$, $p<0.01$ and $p<0.000001$, respectively), and they had significantly higher IFN-γ and CRP levels than patients with HIV+HAART- ($p<0.01$ and $p<0.0001$, respectively).Patients with TB had significantly lower IL-12/IL-23 (p40) levels than patients with HIV+HAART- ($p<0.001$). Similarly, the plasma IFN-γ, IP-10, IL-18, IL-10 and CRP levels in patients with HIV+TB+HAART- were significantly higher than in HC ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$, respectively), patients with HIV+HAART- ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$ and $p<0.01$, respectively) and patients with HIV+HAART+ ($p<0.01$, $p<0.001$, $p<0.01$, $p<0.01$ and $p<0.01$, respectively). The plasma IP-10, IL-18, IL-12/IL-23 (p40) and IL-10, but not IFN-γ and CRP levels in patients with HIV+TB+HAART- were significantly higher than in patients with TB ($p<0.001$, $p<0.01$, $p<0.05$ and $p<0.01$, respectively). The circulating levels of IL-15 were below the detection sensitivity of 4.0 pg/ml in almost all studied subjects, causing no significant differences (data not shown).

Correlations among circulating F-OPN, N-half OPN, IFN-γ, IP-10, IL-18, CRP and clinical parameters in tuberculosis cases

Correlations among plasma F-OPN, N-half OPN, IFN-γ, IP-10, IL-18, IL-12/IL-23 (p40), IL-10, IL-15 and CRP levels before anti-TB treatment were analyzed in patients with TB. Plasma F-OPN correlated significantly with N-half OPN ($r=0.508$, $p<0.05$), IP-10 ($r=0.500$, $p<0.05$) and IL-18 ($r=0.568$, $p<0.01$); whereas plasma F-OPN did not correlate with IFN-γ, IL-12/IL-23 (p40), IL-10, IL-15 or CRP. Positive correlations were also found between plasma levels of IP-10 and IFN-γ ($r=0.525$, $p<0.05$), IP-10 and IL-18 ($r=0.527$, $p<0.05$) and IL-18 and CRP ($r=0.519$, $p<0.05$). In patients with HIV+TB+HAART-, plasma F-OPN levels correlated significantly with IP-10 and IL-18 levels ($r=0.943$, $p<0.01$ and $r=0.829$, $p<0.05$, respectively).

The correlations between T cell response-associated molecules and the number of WBCs, lymphocytes, monocytes, $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ ratio were analyzed in patients with TB. There were significant positive correlations between plasma F-OPN levels and WBC counts ($r=0.508$, $p<0.05$), CRP and WBC counts ($r=0.651$, $p<0.01$) and negative correlations between IFN-γ and $CD4^+/CD8^+$ ratios ($r=-0.474$, $p<0.05$), IP-10 and $CD4^+/CD8^+$ ratios ($r=-0.69$, $p<0.001$).

Circulating OPN, IFN-γ, IP-10 and CRP levels after anti-TB treatment

Plasma F-OPN, IFN-γ, IP-10, IL-18,

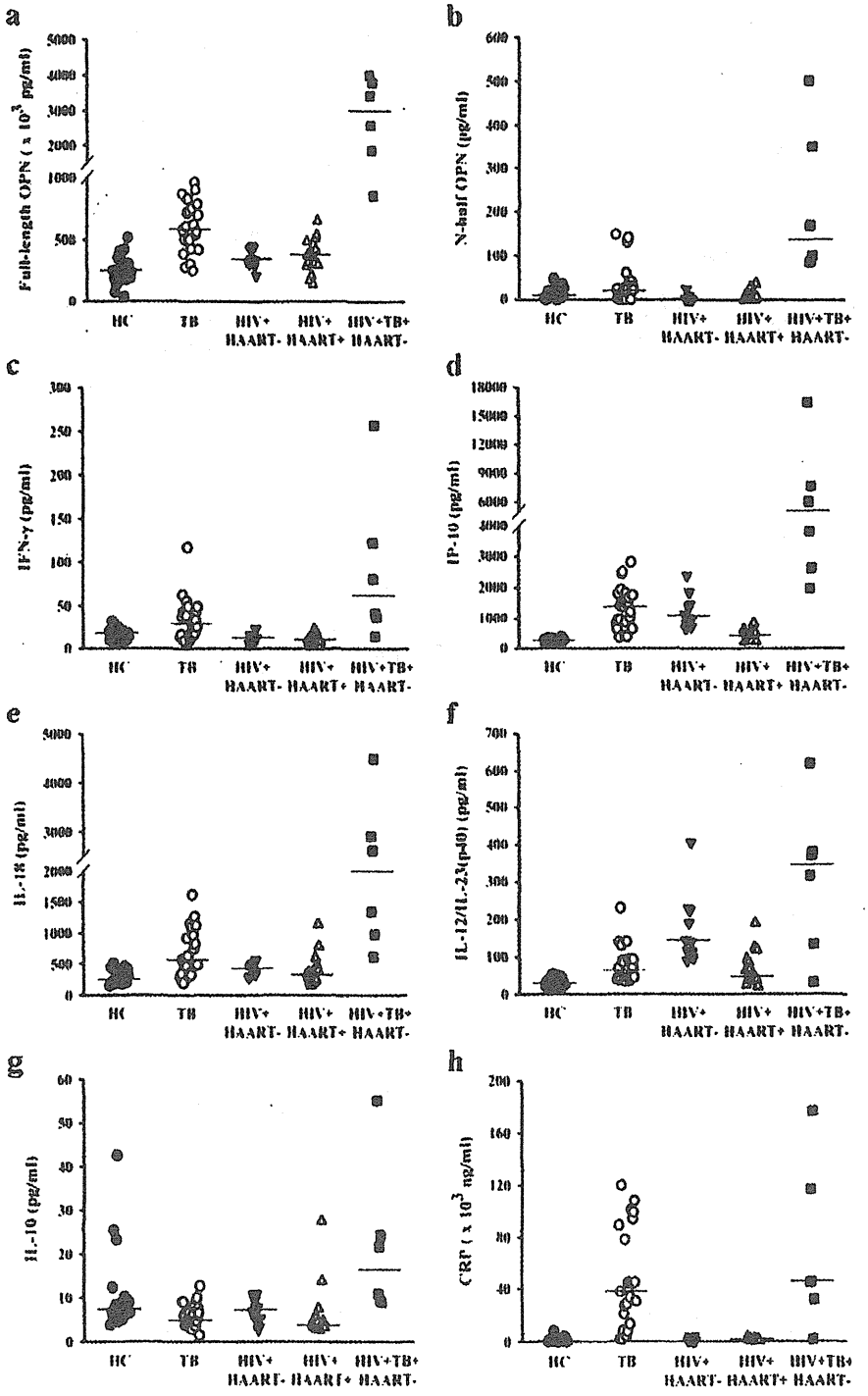

Fig 1–Circulating full-length OPN (a), N-half OPN (b), IFN-γ (c), IP-10 (d), IL-18 (e), IL-12/IL-23 (p40) (f), IL-10 (g) and CRP (h) levels in patients with tuberculosis (TB) and HIV/TB co-infection without HAART (HIV+TB+HAART-). HIV patients without HAART (HIV+HAART-) and with HAART (HIV+HAART+) were tested in comparison. Healthy individuals (HC) were used as controls. Bars represent the median values. The horizontal lines represent the lower limits of each measurement.

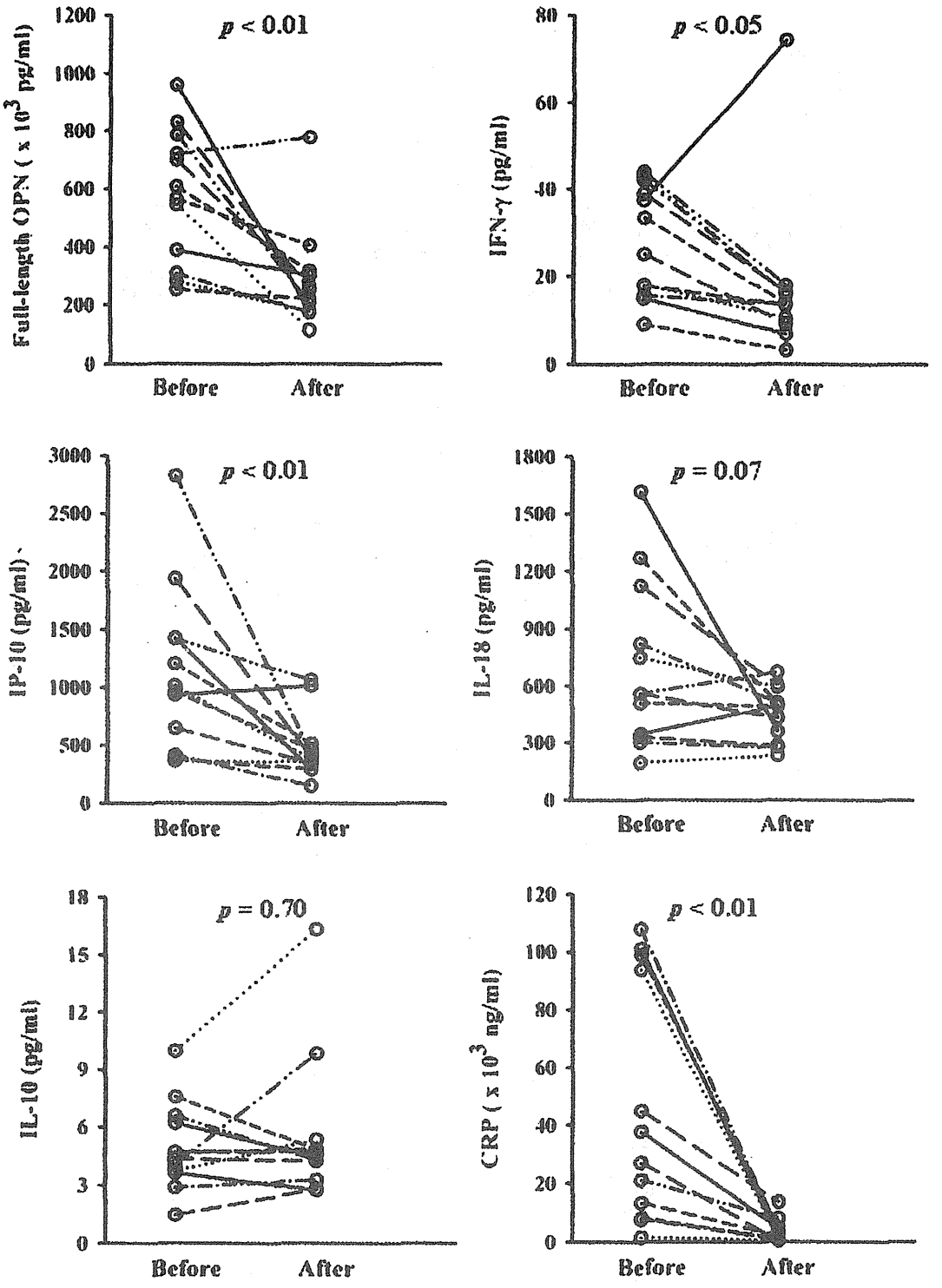

Fig 2–Circulating full-length OPN, IFN-γ, IP-10, IL-18, IL-10 and CRP levels among patients with active pulmonary TB before and after anti-TB treatment.

IL-12/IL-23 (p40), IL-10, IL-15 and CRP levels before and after 6-9 months of anti-TB treatment in the 12 patients with TB and in the 3 patients with HIV+TB+HAART- were evaluated. Significant decreases in plasma F-OPN, IFN-γ, IP-10 and CRP levels were seen in patients with TB after treatment ($p<0.01$, $p<0.05$, $p<0.01$ and $p<0.01$, respectively) (Fig 2). Although plasma IL-18 levels decreased in some TB patients after treatment, the change was not significant.

Plasma F-OPN, IFN-γ and CRP levels in patients with HIV+TB+HAART- tended to decrease after anti-TB treatment. After treatment, clinical improvement, negative sputum microscopy examinations and normal chest radiographs were observed.

DISCUSSION

To address the role of OPN in patients with TB, circulating F-OPN, N-half OPN and other cytokines and chemokine levels were evaluated along with clinical parameters in Thai patients with active pulmonary TB and HIV/TB co-infection. Circulating F-OPN, IFN-γ and CRP levels were significantly elevated in patients with active pulmonary TB and the levels decreased after effective anti-TB treatment. High concentrations of F-OPN, N-half OPN, IFN-γ, IP-10, IL-18 and IL-10 found in the plasma of patients with HIV/TB co-infection were unexpected, although this was a small-scale study. Levels of N-half OPN were much lower than those of F-OPN in all groups. Plasma levels of F-OPN correlated well with IP-10, IL-18 and N-half OPN levels among patients with active TB.

The high F-OPN levels in TB patients suggested a role for circulating F-OPN in disease activity among TB patients. Elevated circulating F-OPN levels in pulmonary TB patients is consistent with previous studies (Koguchi *et al*, 2003; Inomata *et al*, 2005). This may be partly due to leakage from granuloma sites evidenced by accumulation of OPN proteins in lung tissue sections from TB patients (Nau *et al*, 1997) and by abundant OPN expression in lymph nodes with well-formed granulomas (Nau *et al*, 2000). However, elevated circulating F-OPN and N-half OPN in patients with HIV/TB co-infection was not expected. HIV/TB co-infection is known to be associated with failure of granuloma formation and failure to control *M. tuberculosis* infection, thereby leading to mycobacterial dissemination (Corbett *et al*, 2003). The contribution of HIV infection to elevated circulating F-OPN is known and these levels correlate with HIV-induced CNS dysfunction, particularly in HIV-associated dementia, a severe neurocognitive abnormality that commonly occurs during the late stages of HIV infection (Burdo *et al*, 2008). Without receiving HAART, HIV infection chronically activates the host immune system to maintain a defense that only partially controls infection (Fauci, 1996), but chronic activation and replication, as well as storage of virus, leads to pathological consequences that may stimulate the production of various mediators of immune activation, including OPN. Collectively, prominent levels of circulating F-OPN in HIV/TB co-infection may not indicate disease status of effective granuloma formation but rather reflect spread of active TB lesions, large numbers of pathogens in the body or synergistic immune activation due to HIV/TB co-infection. F-OPN levels may not be equivalent to TB-associated inflammation simply measured by CRP because F-OPN levels did not correlate with CRP levels in the TB group.

The introduction of HAART among

HIV-infected patients usually results in the gradual reconstitution of the immune system (Weiss *et al*, 1999). HAART induced changes in the expression of many pro-inflammatory cytokines, including OPN in lymph nodes of HIV infected individuals 1 month after initiation (Li *et al*, 2004) but persistently elevated levels of circulating F-OPN during 6 months of HAART were observed (Chagan-Yasutan *et al*, 2009). In line with the latter findings, in this study, no differences in circulating F-OPN levels between HIV patients with or without HAART were found, despite a possible alteration in immune status with HAART. Different results are possibly due to differences in disease stage, regimen and duration of HAART.

Levels of circulating N-half OPN were much lower than those of F-OPN among all groups, and may not be helpful for monitoring disease activity. N-half OPN is generally more potent in causing cell migration and adhesions at the site of disease than in the uncleaved full-length form (Senger *et al*, 1994). In the synovial fluid of patients with rheumatoid arthritis (RA), N-half OPN has been detected at lower levels than F-OPN (Hasegawa *et al*, 2009). This indicates that N-half OPN exists at lower levels than its full form even at the site of inflammation. N-half OPN was detected in urine but not plasma from patients with RA at much lower levels than F-OPN (Shio *et al*, 2010). N-half OPN may not be stable in body fluids, including plasma, or is barely produced in tissues through strict regulation of thrombin/anti-thrombin balance. Thus, investigation regarding the functional form of OPN in TB and HIV/TB co-infection is further necessary when a more sensitive assay system is developed.

Elevation of circulating F-OPN, IFN-γ, IP-10 and IL-18 levels was documented in patients with active pulmonary TB. The results of circulating F-OPN, IFN-γ and IL-18 levels in patients with TB are consistent with other studies (Verbon *et al*, 1999; Morosini *et al*, 2003; Inomata *et al*, 2005). The finding of lower circulating IL-10 levels among TB patients than healthy controls is in contrast to some other studies (Verbon *et al*, 1999; Morosini *et al*, 2003; Deveci *et al*, 2005). This variability may result from a different status of healthy controls, in that all were negative on the interferon-gamma release assay (IGRA) in our study, whereas other studies consisted of controls with both positive and negative tuberculin skin tests (TST) (Morosini *et al*, 2003; Inomata *et al*, 2005). IL-10 levels in healthy controls in this study may have been affected by simultaneous infection with helminthes or tropical diseases, as is often seen in developing countries (Borkow and Bentwich, 2004). TB patients have different clinical characteristics, but only pulmonary TB patients with sputum smears positive for acid-fast bacilli (AFB) were recruited into this study, whereas another study included patients with both pulmonary and extra-pulmonary TB (Verbon *et al*, 1999).

The present results showed elevated IFN-γ and IP-10 levles were found in TB patients similar to previous studies (Juffermans *et al*, 1999; Azzurri *et al*, 2005; Djoba Siawaya *et al*, 2009). The present study demonstrated, for the first time, positive correlations between levels of F-OPN and IP-10, between IP-10 and IL-18 and between IP-10 and IFN-γ in patient with TB. Our findings of no correlations between circulating F-OPN and IFN-γ, between F-OPN and IL-12 and between IFN-γ and IL-12 are in contrast with some previous studies (Inomata *et al*, 2005; Pokkali and Das, 2009). Further studies are needed. OPN was found to be elevated

along other Th1-related molecules in patients with active TB.

In patients with TB, a significant decrease in circulating F-OPN, IFN-γ, IP-10, CRP levels and a trend toward a decrease in IL-18 levels were observed 6 to 9 months after anti-TB treatment. Furthermore, a decrease in circulating F-OPN, IFN-γ and CRP in 3 HIV/TB co-infected patients after completing treatment suggests these molecules may be useful for evaluating TB disease activity and monitoring response to treatment, as has been shown in previous studies (Koguchi *et al*, 2003; Inomata *et al*, 2005). However, discrepancies may occur (Verbon *et al*, 1999; Inomata *et al*, 2005; Djoba Siawaya *et al*, 2009) and caution is needed to interpret the results.

In conclusion, the present study confirmed the possible contribution of OPN for evaluating pulmonary TB disease activity, particularly in HIV/TB co-infected patients in association with Th1 response-related molecules. Clinically, the elevated OPN, IFN-γ and CRP levels and their decline after successful anti-TB treatment suggests circulating levels of F-OPN and Th1 response-related molecules, including IFN-γ, may be useful to determine expansion of active TB lesions and/or pathogens and may serve as markers of disease activity before and during treatment.

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