positive by IGRA (Table 1). Repeated examination by culture and PCR showed negative for MTB infection in all NTM patients. We further stratified these NTM patients into two subgroups, IGRA-positive as NTM with LTBI (NTM+) and IGRA-negative as NTM without LTBI (NTM−).

Patients in the NTM+ group had pulmonary pathology related to NTM disease based on clinical and radiographic features: consolidation and pulmonary infiltration in N2, both cavity and nodule in N5 and N9 had a combination of bronchiectasis, cavity, consolidation and nodules. Patients of the NTM− group also had typical radiographic features related to NTM infection. The patients of the NTM+ group had symptoms for longer periods (36 to 84 months), whereas the NTM patients had symptoms for variable durations (2 to 148 months), although the difference was not significant. No differences in age or clinical presentation were observed between the NTM+ and NTM− groups. Only patient N5 from the NTM+ group had a history of prior TB infection at the age of 18 that was cured by anti-TB therapy. Among all the NTM patients, only 2 NTM+ patients (N5, N9) had been receiving treatment for NTM infection because of clinical and radiological severities. Patient N5 was treated with rifampicine (RFP), clarithromycine (CAM), ciprofloxacin (CPFX) and ehtambutol (EB) for 6 months starting from July 2001. From May 2004, he was treated again with RFP and CAM, and treatment was continued until October 2005. Since then, he has been treated with different quinolone derivatives including gatifloxacin, CPFX, levofloxacin until June 2006. Finally, the patient was on combined therapy of RFP, CAM and a quinolone derivative until the time of the assay. Patient N9 was also treated with RFP, EB and CAM for 2 months before enrolling in the study. All the other patients hadn't received any drugs for NTM infection. Patients, N5 and N9 had died before the writing of this paper, though detailed information was not available.

Laboratory markers

Laboratory findings are listed in Table 2. There were no significant differences in the conventional markers between the NTM− and + groups. The levels of leptin were apparently lower in the NTM+ and TB groups than in the NTM− group, but no significant differences were found among the groups (Table 2, Fig. 2F).

Inflammatory markers

The data of inflammatory markers are shown (Fig. 2). The TBGL antibody levels are elevated in the NTM+ group and such elevations were unexpectedly not seen in the MTB group, probably because the most of the TB patients were the mild, extra-pulmonary type (Fig. 2A). Patients of the NTM+ group had increased levels of OPN (859 to 1,499 ng/ml) (normal value: < 820 ng/ml; according to Chagan-Yasutan et al. 2009) and the levels were significantly higher than those of the NTM− ($p < 0.05$) (Fig. 2B). The OPN titers in patients N5 and N9 were very high 1,150 ng/ml and 1,499 ng/ml, respectively (Fig. 2B). The CRP levels were

Table 2. Clinical characteristics of patients enrolled in the study.

Laboratory data	Ref. range	Median		
		NTM−	NTM+	TB
RBC (10^6/ul)	3.93-5.03	3.915	3.74	3.76
WBC (/ul)	3.2-9.6	5,250	5,650	4,100
Neutrophil %	31-73	62	58.5	69
Eosinophil %	0-7	1	2	1
Basophil %	0-3	0.5	0.5	0.005
Lymphocyte %	18-51	27	30.5	5
Monocyte %	1-12	8	7.5	5
Platelets (10^3/ul)	155-347	232.5	229.5	277
Hb (g/dl)	11.7-14.8	12.9	11.2	11.7
CRP (mg/dl)	0-0.2	1.2	2.45	0.5
Albumin (g/dl)	4.2-5.3	4.1	3.6	3.7
IgG (mg/dl)	748-1,694	1,320	1,542	1,564
IgM (mg/dl)	33-254	86.5	103	115.5
IgA (mg/dl)	91-391	281	336	295
ESR (30 min) (mm)	(−)	8	11	23
ESR (1 hr) (mm)	(−)	22.5	25	53
KL-6 (U/ml)	105-435	310	315.5	347.5
Leptin (pg/ml)	4,700-32,500	8,105	3,437	4,013

NTM−, IGRA negative non-tuberculosis mycobacterium patients; NTM+, IGRA positive non-tuberculosis mycobacterium patients; TB, tuberculosis patients.

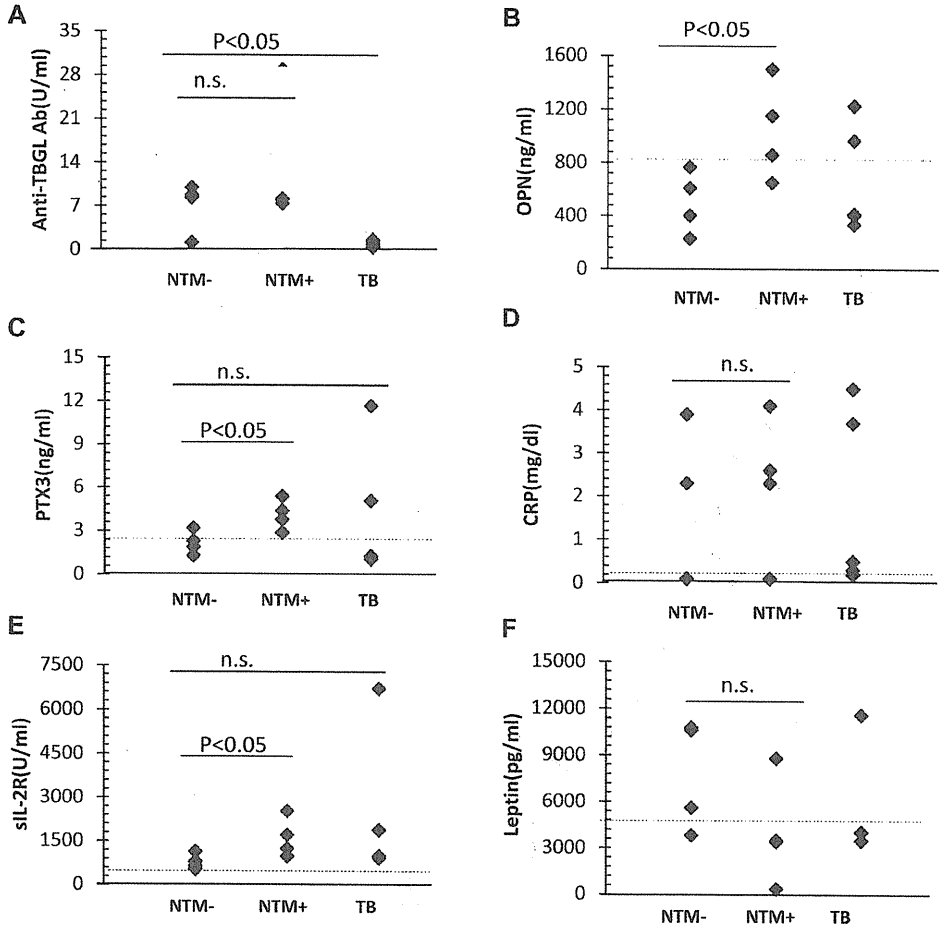

Fig. 2. Comparison of plasma inflammatory molecules.
NTM−, NTM patients without LTBI ($n = 4$); NTM+, NTM patients with LTBI ($n = 4$); TB, active TB patients ($n = 5$).

not different between the NTM+ and NTM− groups, but the PTX-3 levels (normal value: < 2.3 U/ml) and sIL-2R (normal value: 122-496 U/ml) were elevated in all NTM+ patients and the difference from the NTM− group was significant ($P < 0.05$) (Fig. 2C, D and E).

Discussion

In the current study, 4 of 8 NTM patients (50%) were found to be LTBI, and their average age was 74.5 y.o. In addition, PCR analysis showed that all of our NTM isolates lacked the RD1 region. An age-dependent increase of LTBI has already been described in Japan, where 9.8% for those aged 60-69 were IGRA ELISA positive (Mori et al. 2007). The excellence of IGRA ELISA to differentiate NTM and TB infection in children in non endemic countries was reported (Detjen et al. 2007). In Japan, only 1-8% of IGRA ELISA positive rate in Japanese patients with MAC disease was reported (Kobashi et al. 2006; Kobashi et al. 2009). However 34-49% IGRA ELISA positive cases were reported in NTM in endemic countries (Ra et al. 2011). The reasons of high rates of IGRA-positive rates in this study could be explained by IGRA ELISPOT assay employed here. It is known that IGRA ELISA tends to show false negative results among thin elderly people, presumably due to decreased immune levels, whereas IGRA ELISPOT assay might have detected LTBI more sensitively in the elderly patients with NTM disease. It was reported that the long-lasting positive IFN-γ response to antigenic challenge continues for 5 to 10 years following anti-TB therapy (Adams et al. 2008).

NTM+ patients had significantly higher titers of inflammatory markers such as OPN, PTX-3 and sIL-2R, though CRP did not show significant differences (Fig. 2). We also observed sustained high levels of OPN after treatment in NTM+ patients. It is possible that anti-NTM was ineffective because two patients died. Alternatively, persistent elevations of OPN after chemotherapy were already reported by us in AIDS patients treated by anti retroviral therapy (Chagan-Yasutan et al. 2009). Additionally our recent study supports the idea of immune-modulator effect of quinolone which enhance the production of OPN in human lung epithelial cell line A549 *in vitro* (Shiratori et al. in press). We assume that the quinolone treatment may be one of the factors of persistent OPN elevation. The

increased plasma OPN in TB patients contributed to the disease pathology by activating the IL-12 mediated Th1 immunity (Koguchi et al. 2003). It was also found that OPN expression correlates with an effective inflammatory response and contributes to human resistance against MTB (Nau et al. 2000). In cattle, it was proposed that OPN is a key regulator against *M. avium* (Karcher et al. 2008). Immune responses by *M. avium complex* preferentially depend on the phase of infection in human. Early acute infection causes increased IFN-γ secretion, while the chronic phase has been reported to be associated with copious IL-10 production (Azouaou et al. 1997) with an inclination toward Th2 cytokines (Vouret-Craviari et al. 1997) that may provide protection against chronic diseases. We have already reported that the plasma levels of IFN-γ, OPN and leptins did not show any significant changes between LTBI and non-LTBI health care workers (HCW). Though only LTBI HCWs showed the association of TBGL-IgA antibody titer and serum IFN-γ (Siddiqi et al. in press). Our finding may imply that NTM co-infection with LTBI can synergistically induce large amounts of OPN. The synergistic effect could be explained by the natural resistance associated macrophage protein 1 (NRAMP1) because it was reported as host genetic factor for development of both tuberculosis and NTM, however the involvement of NRAMP1 in OPN production was not studied (Li et al. 2011; Sapkota et al. 2012).

It is also interesting that the PTX-3 levels were significantly higher in the NTM+ group while the CRP levels did not differ. It was documented that 5 of 220 TB contacts who developed active TB within 5 to 12 months of follow-up had elevated levels of PTX-3 (Azzuri et al. 2005). The PTX3 haplotype frequencies significantly differed in TB cases compared to controls, and a protective effect against MTB was found in association with a specific haplotype (Olesen et al. 2007). Hence, Th1 mediated PTX-3 production in mycobacterial infection also warrants further investigation.

In conclusion, frequent LTBI was detected in aged NTM patients, and these patients expressed higher levels of inflammatory markers than NTM without LTBI patients. The low number of patients is the main limitation of this study, but careful observation and extensive therapeutic intervention appear to be necessary in NTM with LTBI patients.

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Conflict of Interest

The authors declare no conflict of interest.

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Clinical Study

Frequent Detection of Anti-Tubercular -Glycolipid-IgG and -IgA Antibodies in Healthcare Workers with Latent Tuberculosis Infection in the Philippines

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Anti-tubercular-glycolipid-IgG (TBGL-IgG) and -IgA (TBGL-IgA) antibodies, and the QuantiFERON-TB Gold test (QFT-G) were compared in healthcare workers (HCWs, $n = 31$) and asymptomatic human immunodeficiency virus-carriers (HIV-AC, $n = 56$) in Manila. In HCWs, 48%, 51%, and 19% were positive in QFT, TBGL-IgG, and -IgA, respectively. The TBGL-IgG positivity was significantly higher ($P = 0.02$) in QFT-positive than QFT-negative HCWs. Both TBGL-IgG- and -IgA-positive cases were only found in QFT-positive HCWs (27%). The plasma IFN-γ levels positively correlated with TBGL-IgA titers ($r = 0.74$, $P = 0.005$), but not TBGL-IgG titers in this group, indicating that mucosal immunity is involved in LTBI in immunocompetent individuals. The QFT positivity in HIV-AC was 31% in those with CD4+ cell counts > 350/μl and 12.5% in low CD4 group (< 350/μl). 59 % and 29% were positive for TBGL-IgG and -IgA, respectively, in HIV-AC, but no association was found between QFT and TBGL assays. TBGL-IgG-positive rates in QFT-positive and QFT-negative HIV-AC were 61% and 58%, and those of TBGL-IgA were 23% and 30%, respectively. The titers of TBGL-IgA were associated with serum IgA ($P = 0.02$) in HIV_AC. Elevations of TBGL-IgG and IgA were related to latent tuberculosis infection in HCWs, but careful interpretation is necessary in HIV_AC.

1. Introduction

Although the incidence of tuberculosis has been falling since 2002, there were still 8.8 million incident cases of TB, 1.1 million deaths from TB, and an additional 0.35 million deaths from HIV-associated TB in 2010 [1]. The high rate of latent TB infection (LTBI) is one of the factors that make it difficult to achieve global control and eliminate TB [2]. The recent introduction of the immune-based interferon-γ release assay (IGRA) made a great impact on facilitating the diagnosis of LTBI [3] and clarified the high rate of infection in TB-high-risk populations including healthcare workers (HCWs) [4]. Attempts to detect LTBI in HIV-infected individuals were also facilitated by the development of IGRA, although their higher rates of pseudonegative IGRA response due to low CD4+ T cell counts and diminished Th1 immunity cannot be ignored [5]. Trehalose 6,6-dimycolate (TDM), which constitutes a major part of the mycobacterial cell wall, was identified as the most immunogenic glycolipid and is produced predominantly by virulent MTB as well as by atypical mycobacteria [6]. Tubercular-glycolipid antigen (TBGL) consists of TDM purified from virulent mycobacterial strain H37Rv [7, 19]. The immunoglobulin-G to tubercular-glycolipid antigen (TBGL-IgG) has been proposed to be a useful marker for the serodiagnosis of active pulmonary tuberculosis (PTB) in Japan [7]. However, frequent elevated titers (17%) were also found in healthy elderly control people (age: >40 yrs) in the same study,

and the possibility of LTBI was suggested by Maekura and colleagues [7]. Although IgA antibody to TBGL antigen (TBGL-IgA) was not evaluated earlier as a biomarker, strong association was revealed between the TBGL-IgG and-IgA titers in PTB cases [8]. Frequent positivity for TBGL-IgG (46%) and -IgA (36%) in healthy adults was also observed in our very recent study in Thailand, a TB-endemic country [9]. The TBGL-IgG-positive responses were not related to BCG vaccination [10]. Since both cellular-mediated and humoral immunity are necessary for an effective immune response against MTB, we aimed to clarify the relationship between the TBGL-IgG and -IgA responses with QuantiFERON-TB Gold In-Tube (QFT) assay system, in healthcare workers (HCWs) in a hospital of the Philippines.

Infection of human immunodeficiency virus (HIV) has substantially boosted the occurrence of tuberculosis (TB) disease worldwide [1]. The devastating association between HIV and TB is responsible for one of four TB-related deaths [11]. The East-Asian countries are predominantly TB endemic [1]. Similarly to Sub-Saharan Africa, the rapid, progressive increase of HIV infections in East-Asian countries may further accelerate TB infection in HIV/AIDS patients [12]. To clarify how HIV infection may alter immune responses in LTBI, newly diagnosed, asymptomatic, non-TB HIV-infected individuals were studied.

To understand the health condition of the individuals, we measured two TB-related biomarkers. Leptin, a cytokine-like hormone produced by bronchial epithelial cells and type II pneumocytes in addition to adipose tissue, exhibits a Th1-bias immune response [13]. Osteopontin (OPN) is a member of extracellular matrix proteins that is synthesized within the immune system by activated T cells, NK cells, dendritic cells, and macrophages. Involvement of OPN in Th1 immune responses has been reported [14]. OPN deficiency was found to be associated with the dissemination of mycobacterial disease, and its expression correlated with an effective immune and inflammatory response against mycobacteria in rodents as well as in human [15, 16]. Elevated levels of circulatory plasma OPN [17] and low levels of leptin [18] were reported to be associated with active tuberculosis; these biomarkers served as a negative evidence of active disease.

2. Materials and Methods

2.1. Study Subjects. A case-control study was conducted between March and October of 2010 in adult participants (age > 18 years) in the Philippines. Thirty-one healthy, adult healthcare workers (HCWs) without any concomitant symptoms or chest radiographic findings relevant to active TB and who had negative HIV serology were recruited from San Lazaro Hospital (SLH), Manila, Philippines. Fifty-six newly diagnosed, asymptomatic HIV carriers (HIV-AC) without any clinical symptoms relevant to tuberculosis were randomly selected from among patients receiving care at the outpatient department of the SLH. None of the subjects took any anti-HIV therapy. Subjects with AIDS-defining events, currently active tuberculosis, or any symptoms relevant to tuberculosis, other than active pulmonary diseases, underlying malignancy or metabolic disorders were excluded from the study. The exclusion criteria for active tuberculosis were based on both clinical findings and chest X-ray (CXR) findings in the HCWs. The study was approved by the ethics committee of SLH and the Tohoku University Hospital. We obtained written informed consent from all the participants. Three mL of blood was obtained directly (one mL in each tube) from each participant to perform the QFT assay. Simultaneously, plasma was separated from blood by centrifugation after treatment with EDTA and was aliquoted to CryoTubes for storage at −80°C until further utilization. All the procedures were conducted in accordance with the Helsinki declaration.

2.2. TBGL-Antibody Assay. TBGL-IgG antibody and -IgA antibodies were measured using the Determiner TBGL Antibody ELISA kit (Kyowa Medex, Tokyo, Japan), an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of anti-TBGL-IgG and -IgA in plasma. This assay employs glycolipid antigens purified from *M. tuberculosis* H37Rv (TBGL antigen) coated on a 96-well plate. The details of the assay were described in our previous study [8]. The antibody titers for TBGL-IgG and -IgA were expressed as U/mL. Positive TBGL-IgG titers were determined according to the cutoff index proposed by Kishimoto et al. [19]. The samples were classified as positive when the serum levels of anti-TBGL-IgG were ≥2 U/mL. An arbitrary cutoff value of ≥2 U/mL for TBGL-IgA was used according to the unpublished data of our previous study [8].

2.3. QuantiFERON-TB Gold In-Tube (QFT). The QFT test was performed using fresh whole blood in accordance with the manufacturer's instruction (Cellestis, Australia). The results were interpreted using specific software provided by Cellestis. The result was scored positive if the IFN-γ concentration in the tube TB-specific antigen containing was >0.35 IU/mL after subtracting the value of the nil control (IFN-γ-nc) and at least >25% of NC value. If the net IFN-γ response (TB Ag minus nil) was <0.35 IU/mL for the antigens and the response to the mitogen-positive control was >0.5 IU/mL, the response was considered as test negative. An intermediate result was recorded if the net IFN-γ response was <0.35 IU/mL for the antigen and <0.5 IU/mL for the mitogen and/or was above 8 IU/mL for the NC.

2.4. Leptin and OPN Elisa Assay. Plasma leptin levels were determined by sandwich ELISA using Quantikine Human Leptin Immunoassay kit (R&D Systems) for the quantitative determination of the human leptin concentrations in plasma according to the manufacturer's guidelines. Plasma OPN concentrations were determined using Human OPN Elisa kit (Immuno-Biological Laboratories, Takasaki, Japan) according to the manufacturer's guidelines, and values were expressed as ng/mL.

2.5. Clinical Data. We measured different laboratory markers including complete red blood cell counts, the number of white blood cells with their differential counts, levels of

hemoglobin, and serum levels of IgG and IgA. The number of CD4+ T cell counts and HIV RNA load of HIV-AC were also determined.

2.6. Statistical Analysis. The data of quantitative variables are summarized as median and range. Categorical variables were computed as frequency and percentage. The data were analyzed using Stat Flex software, version 5 (Artech Co., Ltd: http://www.statflex.net/index.html) and Statcel 2 (OMS Publishing Inc. Saitama, Japan). The ability of each single marker to discriminate HIV from HCW by receiver operating characteristic (ROC) curve and the area under curve (AUC) was also analyzed. The percentage of overall agreement between QFT and TBGL-IgG/IgA ELISA assays was calculated, and a Cohen's Kappa coefficient was used to assess the level of agreement. The significance of association for categorical variables was estimated by Fisher's exact test, whereas correlations between continuous variables were evaluated by Spearman's rank correlation coefficient. The differences in significance between continuous variables were compared by the Mann-Whitney U test. A 2-tailed P value of <0.05 was considered significant.

3. Results

3.1. Characteristics of Study Participants. A total of 31 HCWs and 56 newly diagnosed HIV-AC were enrolled in the current study. Basic demographic and clinical characteristics of the study participants are shown in Table 1. The participating HIV-AC were relatively young ($P = 0.03$) with a significant male predominance ($P < 0.0001$) compared to the HCWs. Although lymphocyte counts were comparable between the two groups, total counts of WBC, neutrophils, and monocytes were significantly lower in HIV-AC.

3.2. QFT and TBGL-Antibody Assays in HCWs. Forty-eight percent (15/31) of the HCWs showed positive reactions in the QFT assay indicating high incidences of LTBI (Table 1). The median age of the QFT-positive responders from among the HCWs were significantly higher than those of the QFT-negative group ($P = 0.002$). TBGL-IgG and TBGL-IgA were positive in 51% and 19% of HCWs, respectively (Table 1).

Eleven of 15 (73%) QFT-positive HCWs had positive TBGL-IgG responses (categorical agreement 73%), whereas 5 of 16 (31%) QFT-negative subjects had positive TBGL-IgG responses (categorical agreement 68.7%). The overall κ value was 0.42, indicating a moderate association between the two assays (overall agreement: 71%; 95% CI: 0.10~0.73). The TBGL-IgG-positive proportions were also significantly different between QFT-positive and QFT-negative groups of HCWs ($P = 0.02$). Although the number of positive TBGL-IgA responders was small in HCWs and failed to show any significant difference ($P = 0.072$), the TBGL-IgG+IgA double-positive response was shown only by QFT-positive HCWs and none of the QFT-negative HCWs had double-positive reactions ($P = 0.043$) (Figure 1) (Table 2).

In addition, significant positive correlation was observed between the concentrations of IFN-γ-nc and TBGL-IgA

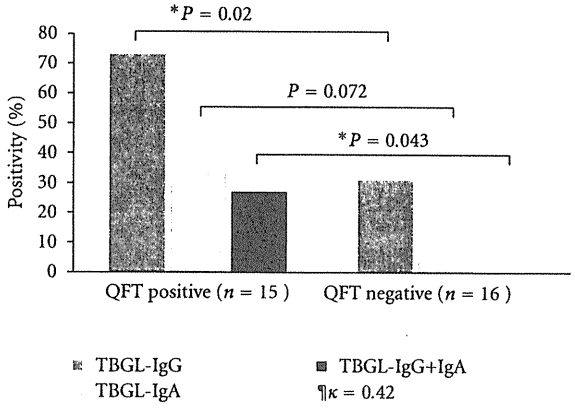

FIGURE 1: Positivity percentage of TBGL-IgG/IgA assay in QFT-positive/negative healthcare workers. The level of agreement between QFT and the TBGL-Ab assay was measured by Cohen's kappa (κ). ¶κ = 0.42; overall agreement 71%; 95% confidence interval: 0.1~0.73.* Significant difference ($P < 0.05$).

titers in the QFT-positive group ($r = 0.74$, $P = 0.005$) (Figure 2), but not in the QFT-negative group. There was no such association between IFN-γ-nc and TBGL-IgG levels in HCWs, although a tendency for a positive correlation was observed in the QFT-positive HCWs ($r = 0.43$, $P = 0.11$) (Figure 2). No association was observed in the net IFN-γ concentrations in antigen-stimulated QFT-plasma with TBGL-IgG or -IgA titers (data not shown). The plasma levels of OPN and leptin were not different between QFT-positive and QFT-negative HCWs (Table 2).

3.3. QFT and TBGL-Antibody Assays in HIV-AC. As shown in Table 1, only 13 of 56 (23%) HIV carriers showed positive reactions by QFT assay. The rate of positivity was closely associated with high median CD4+ T cell counts ($P = 0.012$) and younger age ($P = 0.036$) (Table 2). Seven of 56 (12.5%) HIV-AC who had lower mitogen responses (IFN-γ concentrations: median: 1.78 IU/mL; range: 0.38~6.73 IU/mL) than the rest (>10 U/mL) had negative responses by QFT assay. Their median CD4+ T-cell counts were 60/μL (range: 43~425/μL) (data not shown). Thirty-three of 56 (59%) and 16 of 56 (29%) HIV-AC were attributed with positive TBGL-IgG and TBGL-IgA responses, respectively (Table 1). The positive proportions of TBGL-IgG and -IgA responses were not significantly different between QFT-positive and -negative HIV-AC (Table 2). However, 6 of 7 QFT-negative low mitogen responders in HIV-AC were positive for both TBGL-IgG and -IgA assay (data not shown). The TBGL-IgA titers were significantly higher in the TBGL-IgG-positive HIV-AC ($P = 0.041$) (Table 3). In addition, TBGL-IgA-positive HIV-AC had significantly elevated titers of TBGL-IgG ($P = 0.042$), serum IgA ($P = 0.015$), and OPN ($P = 0.03$), (Table 3). Interestingly, the TBGL-IgA-positive proportion was inversely correlated with the CD4+ T-cell counts ($P = 0.018$), and the titers were significantly higher in the HIV-AC with CD4+ T-cell count < 350/μL

Table 1: Demographic and clinical data of study participants.

Variables	HCWs ($n = 31$)	HIV-AC ($n = 56$)	P
Demographic data			
Gender: male; n (%)	16 (51.6)	55 (96.5)	<0.0001*
Age year; median (range)	35 (19~62)	28 (19~48)	0.03*
Laboratory findings†			
Hemoglobin (g/dL)	13.2 ± 2.6	13 ± 1.49	0.36
RBC (million/μL)	4.96 ± 1.6	4.43 ± .55	0.069
WBC ($10^3/\mu$L)	7.5 ± 2.5	5.9 ± 1.9	0.01*
Neutrophil ($10^3/\mu$L)	4.4 ± 2.2	3.3 ± 1.2	0.048*
Lymphocyte ($10^3/\mu$L)	2.4 ± 0.6	2.2 ± 0.9	0.82
Monocyte (/μL)	562 ± 237	338 ± 182	<0.001*
CD4+ T-cell count (/μL)	ND	443 ± 286	NA
QFT assay positive; n (%)	15 (48)	13 (23)	0.03*
TBGL-IgG positive; n (%)	16 (51)	33 (59)	0.9
TBGL-IgA positive; n (%)	6 (19)	16 (29)	0.87
IFN-γ-nc (IU/mL)	0.42 ± 0.96	0.13 ± 0.11	<0.001*
TBGL-IgG (U/mL)	3.12 ± 3.36	3.94 ± 6.63	0.14
TBGL-IgA (U/mL)	1.68 ± 2.56	3.1 ± 6.64	0.012*
Serum IgG (mg/dL)	1409 ± 212	1391 ± 224	0.49
Serum IgA (mg/dL)	246 ± 92	319 ± 138	0.058
OPN (ng/mL)	14.4 ± 11	159 ± 191	<0.00001*
Leptin (ng/mL)	18.6 ± 13.9	7.2 ± 5.4	<0.001*

Abbreviations: HCWs, healthcare workers; HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin; ND, not determined; NA, not applicable.
† values were presented as mean ± SD unless indicated otherwise; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P values for statistical differences between HCW and HIV-AC; * significant differences ($P < 0.05$).

Table 2: Comparison between QFT-positive and QFT-negative HCWs and HIV-AC.

Variables	HCWs			HIV-AC		
	QFT+ ($n = 15$)	QFT− ($n = 16$)	P	QFT+ ($n = 13$)	QFT− ($n = 43$)	P
Age; median (range)	45 (21~62)	23.5 (19~48)	0.002*	25 (19~45)	31 (21~35)	0.036*
Gender: male; n (%)	7 (46.6)	9 (47.4)	0.43	12 (92.3)	42 (97.67)	0.43
Work duration->10 yrs; n (%)	11(73.3)	6 (37.5)	0.098	NA	NA	NA
CD4+ count (/μL); median (range)	ND	ND	NA	611 (148~1466)	356 (13~1125)	0.012*
TBGL-IgG positive; n (%)	11 (73)	5 (31)	0.02*	8 (61.5)	25 (58.13)	0.545
TBGL-IgA positive; n (%)	5 (33)	1 (6)	0.072	3 (23)	13 (30)	0.415
TBGL-IgG+IgA positive; n (%)	4 (27)	0 (0)	0.043*	2(15.4)	10 (23.3)	0.42
IFN-γ-nc (IU/mL)†	0.3 ± 0.4	0.2 ± 0.13	0.9	0.21 ± 0.17	0.1 ± 0.07	0.0087*
Serum IgG (mg/dL)†	1450 ± 188	1368 ± 235	0.2	1306 ± 207	1414 ± 249	0.5
Serum IgA (mg/dL)†	268 ± 81	225 ± 101	0.32	330 ± 130	312 ± 138	0.68
OPN (ng/mL)†	14.5 ± 11.2	14.2 ± 11.2	0.87	115.4 ± 130	173.2 ± 203	0.43
Leptin (ng/mL)†	21.3 ± 13.3	15.9 ± 14.3	0.25	6.46 ± 4.12	7.448 ± 5.68	0.24

Abbreviations: HCWs, healthcare workers; HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin; ND, not determined; NA, not applicable.
†mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P values for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

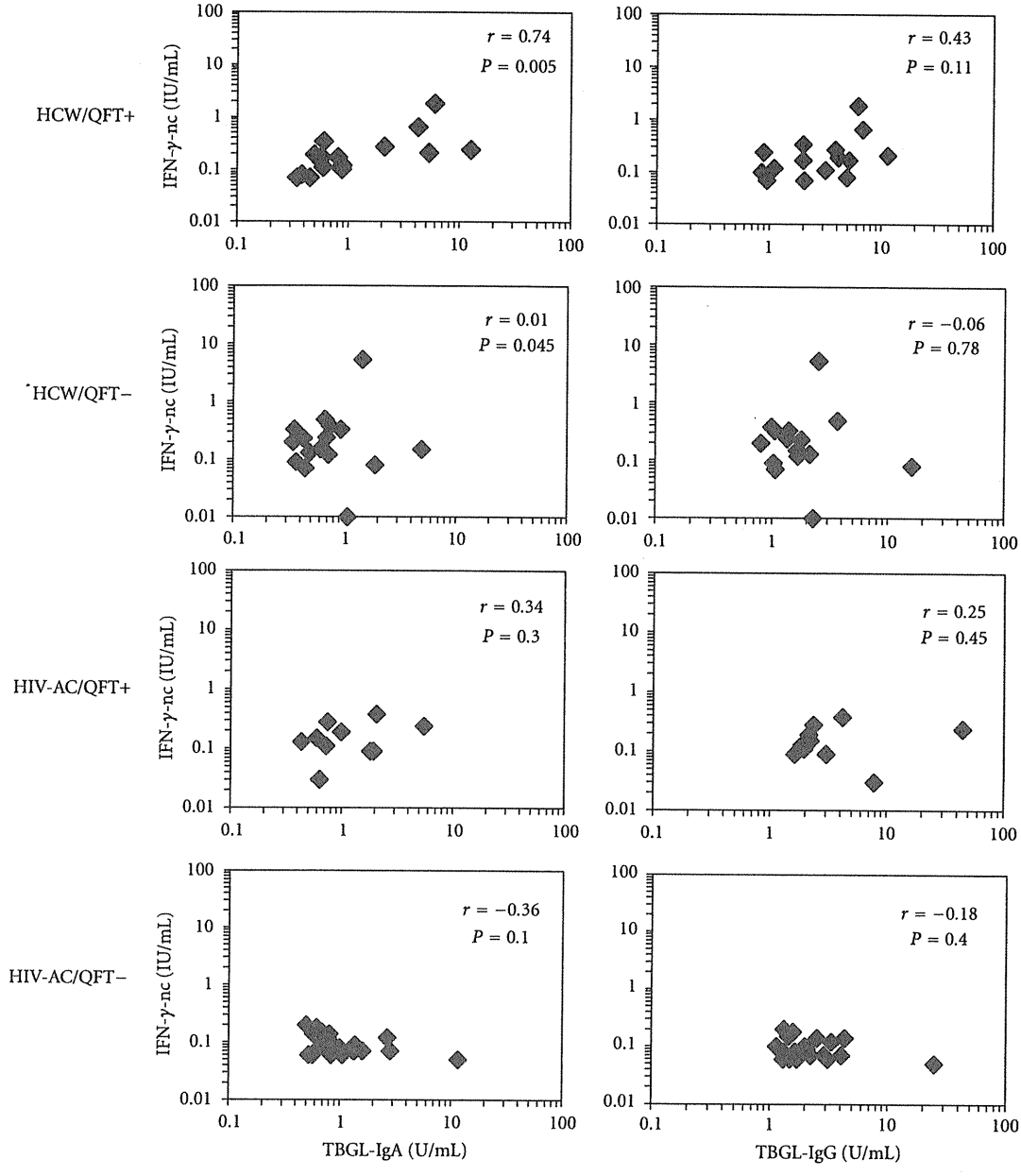

FIGURE 2: Correlations between TBGL-IgA or TBGL-IgG titers and IFN-γ concentrations measured in nonstimulated QFT-plasma samples (IFN-γ-nc) in QFT-positive/QTF-negative healthcare workers (HCWs) and asymptomatic HIV carriers (HIV-AC). The only significant positive correlation was observed between the IFN-γ-nc concentrations and TBGL-IgA titers in the QFT-positive HCW group ($r = 0.74$, $P = 0.005$).

(HIV-LCD) ($P = 0.048$) (Table 4). Furthermore, in the HIV-AC, a relatively higher proportion of double positive (TBGL-IgG+IgA) responders was found in the HIV-LCD group (29%) than in the HIV-HCD group (CD4+ count $\geq$ 350/μL) (16%), although the difference was not statistically significant ($P = 0.32$) (Table 4).

Moreover, the IFN-γ-nc concentrations were significantly lower in the QFT-negative HIV-AC ($P = 0.008$) (Table 2). No association was observed between the IFN-γ-nc concentrations and TBGL-IgG or -IgA titers in any group of HIV-AC (Figure 2). The plasma levels of OPN and leptin were not different between QFT-positive and QTF-negative HIV-AC (Table 2).

3.4. Comparison between the Serum Antibodies and TBGL Antibodies. The TBGL-IgG and -IgA had no correlation with the serum IgG and IgA in HCW and HIV-AC except for the

Table 3: Comparison between TBGL-IgG or TBGL-IgA-positive and -negative HIV-AC.

Variables	TBGL-IgG			TBGL-IgA		
	Positive ($n = 33$)	Negative ($n = 23$)	P	Positive ($n = 16$)	Negative ($n = 40$)	P
Age; median (range)	28 (19~48)	30 (19~41)	0.18	31.5 (19~48)	28 (19~45)	0.038*
Gender: male; n (%)	33 (100)	21 (91.3)	0.43	16 (100)	38 (95)	1
CD4 count (/μL); mean (range)	436 (13~1466)	450 (60~851)	0.45	346 (46~1125)	480 (13~1466)	0.06
QFT positive; n (%)	8 (24.2)	5 (21.7)	0.545	3 (19)	10 (25)	0.45
TBGL-IgA positive; n (%)	12 (36.4)	4 (17.4)	0.1	—	—	—
TBGL-IgG positive; n (%)	—	—	—	12 (75)	21 (52.5)	0.14
IFN-γ-nc (IU/mL)†	0.13 ± 0.09	0.1 ± 0.05	0.4	0.12 ± 0.09	0.12 ± 0.07	0.9
TBGL-IgA (U/mL)†	4.36 ± 8.4	1.28 ± 1.21	0.041*	—	—	
TBGL-IgG (U/mL)†	—	—		7.5 ± 11.6	2.5 ± 1.5	0.042*
Serum IgG (mg/dL)†	1439 ± 277	1515 ± 677	0.5	1615 ± 404	1355 ± 135	0.46
Serum IgA (mg/dL)†	277 ± 95	279 ± 74	0.37	410 ± 165	313 ± 138	0.015*
OPN (ng/mL)†	176.3 ± 199.9	136 ± 172.5	0.67	280 ± 275	115 ± 109.7	0.03*
Leptin (ng/mL)†	7.33 ± 6.16	7.18 ± 4.12	0.68	7.33 ± 6.16	7.18 ± 4.12	0.07

Abbreviations: HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin.
†mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

Table 4: Comparison between HIV-AC with high§ and low‡ CD4+ T-cell count.

Variables	CD4+ high§ ($n = 32$)	CD4+ low‡ ($n = 24$)	P value¶
Age; mean (range)	25.5 (19~45)	25 (22~48)	0.018*
Gender: male; n (%)	31 (97)	23(98)	1.0
CD4+ count (/μL); median (range)	618 (356~1466)	201 (13~349)	<0.001*
QFT-positive; n (%)	10(31)	3(12.5)	0.12
TBGL-IgG positive; n (%)	16 (50)	16 (67)	0.27
TBGL-IgA positive; n (%)	5(16)	11(46)	0.018*
TBGL-IgG+ IgA positive; n (%)	5(16)	7(29)	0.32
IFN-γ-nc (IU/mL)	0.14 ± 0.12	0.13 ± 0.09	0.9
TBGL-IgG (U/mL)†	4.6 ± 8.4	3 ± 2.8	0.59
TBGL-IgA (U/mL)†	1.55 ± 2	5.16 ± 9.6	0.048*
Serum IgG (mg/dL)†	1352 ± 185	1549 ± 380	0.5
Serum IgA (mg/dL)†	265 ± 89	423 ± 149	<0.001*
OPN (ng/mL)†	119 ± 126	214 ± 246	0.19
Leptin (ng/mL)†	7.7 ± 6	6.6 ± 4.9	0.5

Abbreviation: HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN: osteopontin.
§High: CD4+ T cell count ≥350/μL; ‡low: CD4+ T-cell count <350/μL; †mean±SD; IFN-γ-nc: levels of IFN-γ, measured in the non-stimulated QFT-plasma samples; P values for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

association between the serum IgA levels and the TBGL-IgA titers in HIV-AC ($P = 0.02$) (data not shown).

3.5. Comparison of Biomarkers between HCW and HIV-AC. The levels of IFN-γ-nc ($P < 0.001$) were significantly higher in HCWs than in HIV-AC. However, the titers of TBGL-IgA ($P = 0.012$), but not -IgG, were significantly higher in HIV-AC than in HCWs. Similarly, the serum IgA levels were also higher ($P = 0.058$). The OPN levels were significantly higher ($P < 0.0001$), and the leptin levels were considerably lower ($P < 0.001$) in the HIV-AC compared to the HCWs (Table 1).

ROC curve analysis was used to discriminate HIV from HCW groups using the net IFN-γ, leptin, and plasma levels of OPN (log) as biomarkers. As shown in Figure 3, the plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCWs based on the AUC (0.883), followed by leptin (0.763) and net IFN-γ (0.648). However,

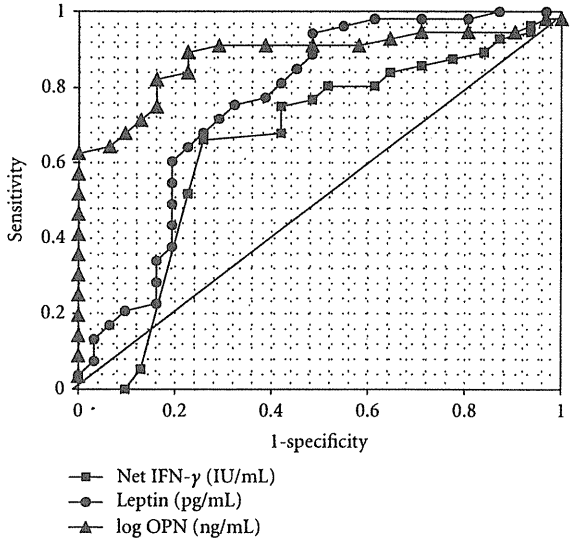

FIGURE 3: Receiver operating characteristic analysis for comparison of biomarkers between healthcare workers and asymptomatic HIV carriers. The result showed that the OPN plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCW based on the AUC (0.883), followed by leptin (0.763) and net IFN-γ (0.648).

QFT assay as well as TBGL-IgA and IgG did not show such profiles (data not shown).

4. Discussion

In our data, the application of QFT assay to HCWs in the Philippines demonstrated a high incidence (48%) of LTBI, which was comparable to other already published data in HCWs in TB-endemic developing countries [4]. The increased risk of LTBI among HCWs was confirmed by the recent introduction of IGRA [20, 21]. In our country, a higher incidence of LTBI in HCWs was reported in high-risk groups for TB, such as homeless areas [22], compared to other areas [23].

We aimed to clarify the relationship between the TBGL-IgG and -IgA responses and that of IFN-γ in the QFT assay in LTBI. The rate of TBGL-IgG positivity was significantly higher in the QFT-positive than QTF-negative group of HCWs. The significant association between the two assay systems indicated by the κ value in HCWs demonstrated the TBGL-IgG in LTBI. However, about 30% of QFT-positive populations from among the HCWs lacked TBGL-IgG, and 30% of those of the QFT-negative group have elevated TBGL IgG antibody, and the discordant cases were higher in TBGL-IgA. However, the reasons for such discordances between the two systems in HCWs are not clear. It is possible that the generation of antibody requires larger amounts of antigens than does the generation of T-cell responses. Although associated immunosuppressive conditions were found as risk factors for false-negative QFT responses [24], such cases were excluded from HCWs in our study.

The mechanism of the synthesis of anti-TDM antibody is not clear, though TDM is known to bind to Mincle (macrophage-inducible C-type lectin) that is present on macrophages [25], and upon the activation, on T cells [26]. It was found that Mincle is specific for the ester linkage of a fatty acid to the trehalose, which explains the strong binding of TDM, but not trehalase-treated TDM, soluble trehalose, or purified mycolate [26]. The conversion of TDM into glucose monomycolate (GMM) upon mycobacterial infection might be the mechanism by which mycobacteria escape from the Mincle-mediated immunity. However, the immune system possesses other tools to monitor and eliminate live mycobacteria through CD1 molecules expressed on the activated macrophages and dendritic cells, which are different from MHC I, II molecules. Recently, GMM but not TDM was demonstrated to interact with CD1b and may induce adaptive immunity [27]. Although it is not known whether the adaptive immune system leads to antibody synthesis, the generated antibody may recognize both TDM and GMM because the two molecules are structurally very similar.

Interestingly, the IFN-γ-nc levels that were observed to have a significant association with the TBGL-IgA titers in LTBI of HCWs. IgA is a typical marker of the mucosal immune response. An elevated serum IgA has been proposed to have a protective role in IFN-γ-positive immunocompetent LTBI individuals [28]. Frequent exposure to tubercle bacilli can possibly stimulate the mucosal immune system in TB-endemic countries. It is also known that commensal bacteria on the mucosal surface induce IgA in an NO-dependent manner [29], although it is not known whether MTB in LTBI has a similar effect in lung mucosa. Circulating glycolipid immune complexes might lead to nonspecific stimulation of T cells, but a component of TBGL, TDM, could also enhance the in vivo production of IL-12p40 and IFN-γ in mouse model [30]. IgA antibody and IFN-γ induce TNF-α and NO production, which mediated the inhibitory mechanism for *M. tuberculosis* infection in mouse model [28]. Furthermore, there is strong evidence of a synergic effect between IgA and IFN-γ in bactericidal activities against MTB infection [31]. Therefore, the association between anti-TBGL-IgA and IFN-γ may indicate protective, mucosal immune activities in LTBI in HCWs.

In HIV carriers, the QFT-positive responses were significantly lower than in HCWs and were greatly dependent on the high CD4+ T-cell counts in the present study. Much evidence suggests that the baseline CD4+ T-cell count is a determining factor for a positive QFT response in HIV infection [32]. Since HIV infection is a disease of immune deficiency, immune deprivation may be less prominent in relatively young QFT-positive cases because IFN-γ could be synthesized properly by stimulation with the appropriate signals. In contrast, the response could be altered in advance immune-deficiency state, as indicated by low CD4+ T-cell counts. Therefore, it is expected that significant numbers of false-negative reactions are present in QFT-negative HIV carriers. The relatively low IFN-γ levels by mitogen stimulation in some of the QFT-negative responders also support this possibility. Therefore, for TB diagnosis

in advanced immunosuppression, the ratio of the IFN-γ response/CD4+ T-cell count Elispot assay was suggested to improve the sensitivity of the assay [33].

It is not clear why HIV infection does not diminish the TBGL antibody titers. It is known that the CD-1 presentation pathway persists in patients with HIV, but antiglycolipid antibodies were found to have no relationship with the TST results [34] or bacillary yield [35]. Similarly, we did not find any correlation between the QFT result and anti-TBGL antibodies. It is also possible that concomitant non-TB mycobacterium infection may stimulate the TBGL antibody synthesis in HIV-AC [7]. Significant numbers of HIV carriers have antibodies to TBGL, but we could not confirm if they indicate LTBI or not.

The increases of serum IgA in advanced HIV infection and of IgG in the early stage were already reported [36]. Although specific antibody titers in HIV infection are decreased by some infectious agents including hepatitis B virus but not in hepatitis A virus, probably because of alterations in the immune systems in advanced HIV infection [37], it is not known whether nonfunctional or functional IgA was synthesized in our cases. The main limitation of the current study is the small number of study subjects and the lack of a follow-up study for estimating the risk of developing active tuberculosis.

Finally, to determine the correlations between biomarkers in infected states, we evaluated data by ROC curve analysis (Figure 3). In this study, the plasma levels of OPN were most specific to HIV and the levels were not elevated in LTBI HCWs (Figure 3, Table 2). Therefore the levels can be a good marker for active TB in non-HIV individuals, because the OPN is known as a marker of active TB [17]. In HIV-AC, the OPN plasma levels are already elevated as described here, and it was already reported that the levels further increase when they developed active TB [38, 39]. It is also known that interferon-inducible protein-10 (IP-10) and IL-18 were elevated in HIV/TB patients than in HIV patients and suggested to be helpful in monitoring the treatment for patients [38]. All these biomarkers were mainly produced by macrophages, and it was also reported that OPN is synthesized by macrophages as well as CD4+ T cells in HTLV-1-induced lymphoma [40, 41].

In this study we noted elevations of anti-TBGL antibody in LTBI in HCWs, but no link between the elevations with LTBI in HIV-AC was confirmed, probably due to the inflammatory conditions in HIV.

5. Conclusion

We have found the elevation of TBGL-IgG titers in LTBI in HCWs. In addition, the association between TBGL-IgA and IFN-γ in HCWs was found, and it was hypothesized that the mucosal immunity is involved in LTBI in HCWs. We could not find any relationships between QFT and TBGL in HIV-AC. Low CD4 cell count was associated with inflammatory conditions as represented by high OPN in HIV-AC, which may be the reason for ambiguous results.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Both authors contributed equally to this work.

Acknowledgments

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Composition Comments

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RESEARCH ARTICLE Open Access

Identification of tuberculosis-associated proteins in whole blood supernatant

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Abstract

Background: Biological parameters are useful tools for understanding and monitoring complicated disease processes. In this study, we attempted to identify proteins associated with active pulmonary tuberculosis (TB) using a proteomic approach.

Methods: To assess TB-associated changes in the composition of human proteins, whole blood supernatants were collected from patients with active TB and healthy control subjects. Two-dimensional difference gel electrophoresis (2D-DIGE) was performed to analyze proteins with high molecular weights (approximately >20 kDa). Baseline protein levels were initially compared between patients with active TB and control subjects. Possible changes of protein patterns in active TB were also compared *ex vivo* between whole blood samples incubated with *Mycobacterium tuberculosis* (*Mtb*)-specific antigens (stimulated condition) and under unstimulated conditions. Immunoblot and enzyme-linked immunosorbent assays (ELISA) were performed to confirm differences in identified proteins.

Results: Under the baseline condition, we found that the levels of retinol-binding protein 4 (RBP4), fetuin-A (also called α-HS-glycoprotein), and vitamin D-binding protein differed between patients with active TB and control subjects on 2D gels. Immunoblotting results confirmed differential expression of RBP4 and fetuin-A. ELISA results further confirmed significantly lower levels of these two proteins in samples from patients with active TB than in control subjects ($P < 0.0001$). *Mtb*-specific antigen stimulation *ex vivo* altered clusterin expression in whole blood samples collected from patients with active TB.

Conclusions: We identified TB-associated proteins in whole blood supernatants. The dynamics of protein expression during disease progression may improve our understanding of the pathogenesis of TB.

Background

Tuberculosis (TB) is one of the most important infectious causes of death worldwide [1]. Despite its long historical interaction with humans, our understanding of host response to the TB pathogen remains incomplete. Investigation of the molecular basis of differences in the host immune status and metabolism between patients with active TB and control subjects may provide a clue to understand the disease process, and thus contribute to future strategies for TB prevention and treatment.

Recent advances in comprehensive analytical techniques, such as transcriptomics and proteomics, have enabled us to identify proteins associated with active TB in humans. As a pioneering approach, Jacobsen et al. compared the gene expression profiles of peripheral blood mononuclear cells from patients with TB and *Mtb*-infected healthy donors by microarray analysis [2], and Mistry et al. analyzed gene expression patterns in whole blood in an attempt to find a candidate biomarker for discriminating cured patients from those with a risk of relapse [3].

Agranoff et al. [4] identified amyloid A and transthyretin in human serum as potential indicators for distinguishing patients with TB from those with non-TB

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inflammatory conditions. They also reported that a combination of four protein markers, including amyloid A and transthyretin, achieved a diagnostic accuracy of up to 78%. Chegou et al. [5] reported that EGF, VEGF, TGF-α, and sCD40L in supernatants obtained from interferon-gamma (IFN-γ)-release assays (IGRAs) are informative markers for differentiating active disease from latent infection. Although the above studies are promising, such comprehensive analytical techniques are still in the developmental stages and further investigations are required before they can be applied clinically.

IGRA detects TB infection by measuring the *Mtb*-specific immune response with high specificity [6]. IFN-γ is released by reactivation of *Mtb*-specific effector memory T cells in whole blood. Despite its advantages, IGRA is not a perfect tool for use in most developing countries. In countries with a high TB burden, patients with active TB, and not those with latent TB infection, need to be immediately identified and treated in order to prevent disease transmission. However, IGRA is not capable of distinguishing active TB from latent infection. Also, cytokine measurements to be performed for IGRA are rather expensive in a resource-limited setting and difficult to distribute. Thus, from a practical as well as a research standpoint, development of new markers for TB is desired.

In the present study, by high-resolution two-dimensional difference gel electrophoresis (2D-DIGE) followed by liquid chromatography-mass spectrometry (LC-MS), we analyzed the expression profiles of high molecular weight proteins (approximately >20 kDa) that have not been studied fully among components of residual whole blood supernatants after performing IGRA.

We used two comparative frameworks. One was the direct comparison of plasma supernatants collected from patients with active TB and healthy control subjects. This comparison aimed to identify proteins that are markedly upregulated or downregulated in the disease state; even if such proteins are not disease specific, they might act as useful markers for monitoring the disease before, during, and after treatment. The other comparative framework was more TB specific since whole blood samples from patients were stimulated with *Mtb*-specific antigens or left unstimulated, and the results were compared.

Methods

Patients and control subjects

In this study, whole blood samples collected from Japanese and Vietnamese individuals were used. The study was approved by the ethical review committees of the National Center for Global Health and Medicine (formerly the International Medical Center of Japan), Tokyo, Japan, and the Ministry of Health, Vietnam. Written informed consent was obtained from each participant. Blood samples were collected from patients with active TB immediately before (Vietnamese patient samples) or within 7 days (Japanese patient samples) of treatment initiation. Patients with potential complications attributable to malignancies, autoimmune diseases, or HIV coinfection were excluded from the study.

At the initial screening and confirmation stage, blood samples were collected from 14 Japanese patients with bacteriologically confirmed active pulmonary TB (9 men and 5 women; median age 50 years, range 22-75 years) and 13 age- and gender-matched healthy Japanese patients (8 men and 5 women; median age 48 years, range 24-64 years). We could not completely rule out the possibility of latent TB infection in 2 of the 13 control subjects, according to the results of a commercially available IGRA (QuantiFERON®-TB Gold in Tube; Cellestis, Victoria, Australia). However, we analyzed all samples together at the initial stage to identify proteins associated with active TB disease. The tuberculin skin test was not useful for detecting latent TB infection in our study since most individuals in the tested populations had received BCG vaccination after birth. Blood samples from 4 patients with active TB and 4 healthy individuals were chosen for screening by 2D-DIGE and immunoblotting. The stability of proteins measured by the enzyme-linked immunosorbent assay (ELISA) was investigated by comparing a set of plasma samples directly separated from EDTA-containing peripheral blood and another set of plasma supernatants obtained from heparinized blood after 18 h of incubation (under the same conditions as the IGRA negative control).

At the next verification stage, we utilized samples from 25 Vietnamese patients with sputum smear-positive active pulmonary TB (13 men and 12 women; median age 35 years, range 20-55 years) and 50 age- and gender-matched Vietnamese healthy control subjects (26 men and 24 women; median age 36 years, range 21-54 years) of which 25 were IGRA positive and 25 were IGRA negative. None of the IGRA-positive individuals had any signs or symptoms of active TB but at least some were reasonably suspected to have latent TB infections because the prevalence of TB in the population is high. Following IGRA, the remaining unstimulated plasma supernatants were used for ELISA.

Sample collection and preparation

Whole blood was separately collected in heparin-containing tubes precoated with mitogen as a positive control or cocktails of ESAT-6, CFP-10, and TB7.7 (p4) peptides as *Mtb*-specific antigens (QuantiFERON®-TB Gold in Tube; Cellestis); the negative control tubes had no precoat. After 18 h of incubation at 37°C, each sample was centrifuged and the plasma supernatants were

harvested and stored at -80°C until use in subsequent assays. For proteomic analysis, four sample sets that were either unstimulated or stimulated with *Mtb*-specific antigens or mitogen from patients with active pulmonary TB and four corresponding sets from control subjects were used to screen for candidate proteins by 2D-DIGE. To increase resolution, 14 human major plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha 2-macroglobulin, alpha 1-acid glycoprotein, IgM, apolipoprotein Al, apolipoprotein All, complement C3, and transthyretin) were removed prior to electrophoresis using a Multiple Affinity Removal LC Column-Human 14 (Agilent Technologies, Santa Clara, CA, USA). The samples were then concentrated by ultrafiltration (Agilent Technologies, Concentrators Spin 5 kDa MWCO, 4 ml) followed by acetone precipitation in preparation for subsequent electrophoresis.

Quantitative analyses by 2D-DIGE

Protein samples were labeled with Cy3 and Cy5 (DIGE Fluors Minimal Labeling Dyes; GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The samples (50 μg of total protein per gel) were applied to Immobiline DryStrips (18 cm long, pH 4-7 linear; Amersham Biosciences, Pittsburgh, PA, USA), and isoelectric focusing (IEF) was performed using an Ettan IPGphor IEF system (Amersham Biosciences) according to the manufacturer's instructions. Next, SDS-PAGE was performed using a 10-18% linear gradient gel from DRC Co., Ltd. (Tokyo, Japan). The fluorescence intensity of each protein spot was digitally recorded using a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA, USA) with Quantity One software (Bio-Rad Laboratories), and differential protein expression was quantitatively analyzed using the PDQuest software (Bio-Rad Laboratories). The same gel included a reference sample that had been labeled with Cy2 and was used for spot matching, image analysis, and volume normalization. Initially, all spots were roughly matched using an automated tool in the PDQuest software suite. This estimate was followed by a more detailed manual curation to correct any inappropriately matched pairs of protein spots.

Sample preparation for mass spectrometry

A mixture of all samples (400 μg of total protein per gel) was subjected to 2D-DIGE under the same conditions as described above to isolate selected spots. To visualize individual protein spots, the gels were stained with SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR, USA) for 3 h. The fluorescence intensity of each protein spot was digitally measured using the Molecular Imager FX system with Quantity One software. Mass spectrometric analysis was performed according to the method reported by Toda *et al.* [7], with slight modification. Briefly, each protein spot on SYPRO Ruby stained gels was picked using a spot picker (Amersham Biosciences). In-gel digestion of proteins was performed according to the method reported by Saeki *et al.* [8].

Mass spectrometric analysis

An ESI ion-trap mass spectrometer (LCQ Deca XP Plus, Thermo Electron) was used for peptide detection. Mass spectrometric analysis was performed as described previously [8]. Protein identification was performed using the Mascot server (Matrix Science, Boston, MA, USA) and Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA, USA). We selected the SWISS-PROT *Homo sapiens* database and used the following parameters: peptide tolerance 1.0 Da and one missed cleavage. Carbamidomethyl modification of cysteine, acetylation of the NH2-terminal ends of lysine, and phosphorylation of serine, threonine, or tyrosine were considered in this analysis.

Immunoblotting

Immunoblotting to detect the proteins identified as described above was performed using anti-human retinol-binding protein 4 (RBP4) rabbit polyclonal IgG (A-0040; Dako; Glostrup, Denmark), anti-human fetuin-A (AHSG) goat polyclonal IgG (G-20; Santa Cruz Biotechnology; Santa Cruz, CA, USA), anti-human vitamin D-binding protein (VDBP) (Gc-Globulin) rabbit polyclonal IgG (Dako), anti-human clusterin-α mouse monoclonal IgG1 (B-5; Santa Cruz Biotechnology), or anti-human clusterin-β rabbit polyclonal IgG (N-18; Santa Cruz Biotechnology).

Total protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). To detect clusterin-α and -β, mixed protein samples (20 μg) were applied to 2D PAGE with 1D IEF using the Immobiline DryStrip (pH 3-5.6 nonlinear). Proteins were then transferred to PVDF membranes. The membranes were probed with polyclonal antibodies, anti-clusterin-α, and anti-clusterin-β. To detect other proteins, each sample (10 μg) was subjected to conventional SDS-PAGE. Membranes were probed with anti-VDBP, anti-fetuin-A, or anti-RBP4 polyclonal antibodies. Anti-mouse and anti-rabbit (GE Healthcare) as well as anti-goat (Santa Cruz Biotechnology) HRP-conjugated secondary antibodies were prepared. Protein bands were detected using the ECL plus detection reagent (GE Healthcare). Band intensities were calculated using the Quantity One software.

ELISA

A competitive ELISA for quantitative determination of RBP4 in human plasma was performed according to the manufacturer's instructions (AdipoGen Inc.; Seoul, Korea). The detection limit was 1 ng/ml. An AHSG ELISA kit was used to detect fetuin-A in plasma (Bio-Vender Laboratory Medicine Inc.; Modrice, Czech Republic). The detection limit was 0.35 ng/ml. A Quantikine® Human Vitamin D-Binding Protein Immunoassay kit was used to detect VDBP in plasma (R&D Systems, Inc.; Minneapolis, MN, USA). The mean minimum detectable VDBP level was 0.65 ng/ml. Distribution of levels was represented using the median and interquartile range (IQR).

Statistical analysis

Proteins showing differential expression between two conditions were first determined with P values using the Student's t-test preinstalled in the PDQuest software suite. To select candidate proteins with expression levels that differed between unstimulated samples from patients with active TB and healthy control subjects, a significance level of $P < 0.05$ was selected. To select candidate proteins showing differential expression in *Mtb*-specific antigen-stimulated and unstimulated plasma samples, a less stringent cut-off value of $P < 0.10$ was applied. Assuming an alpha error of 0.1 and a standardized effect size of 2.0, the power to detect a difference was calculated as 0.8 given our sample size. When a normal distribution of measurements was not predicted, the Wilcoxon rank sum test (Mann-Whitney U test) was applied for confirmation using the JMP software (version 7.0.1; SAS Institute, Cary, NC, USA).

Results

Quantitative analyses by 2D-DIGE

In a preliminary experiment, we used an immobilized linear pH gel strip with a broad pH range (pH 3-10 linear) for 1D IEF. Although more than 500 protein spots were visualized in fresh plasma with SYPRO Ruby staining, the number of spots after incubation of whole blood with stimuli decreased, and detectable spots were primarily located in the pH range 4-7 (data not shown). Therefore, we performed subsequent analyses using an immobilized linear pH gel strip with a narrower range (pH 4-7 linear) to obtain a finer resolution. We used two comparative frameworks in our analyses, and the corresponding spot patterns are schematically depicted in Figure 1.

Differential gel images were acquired and displayed using the PDQuest 2D gel analysis software (Figure 2A, B). In our comparison of the protein expression profiles of patients with active TB and control subjects, red indicates proteins increased in the supernatants collected from the patients and green indicates proteins decreased in the patients compared with the control subjects. Yellow indicates no significant differences (Figure 2A). In 2D gel profiles comparing the antigen-stimulated and unstimulated samples collected from patients with active TB, red indicates proteins increased in the supernatants after *Mtb*-specific antigen stimulation, and green indicates proteins decreased after stimulation. Yellow indicates no significant changes (Figure 2B). From 367 spots compared between patients with active TB and control subjects, and 293 spots generated with samples collected from patients with active TB that were either stimulated with *Mtb*-specific antigens or left unstimulated, we selected several candidates for subsequent mass spectrometric analysis (Table 1) according to the criteria described in the Materials and Methods section.

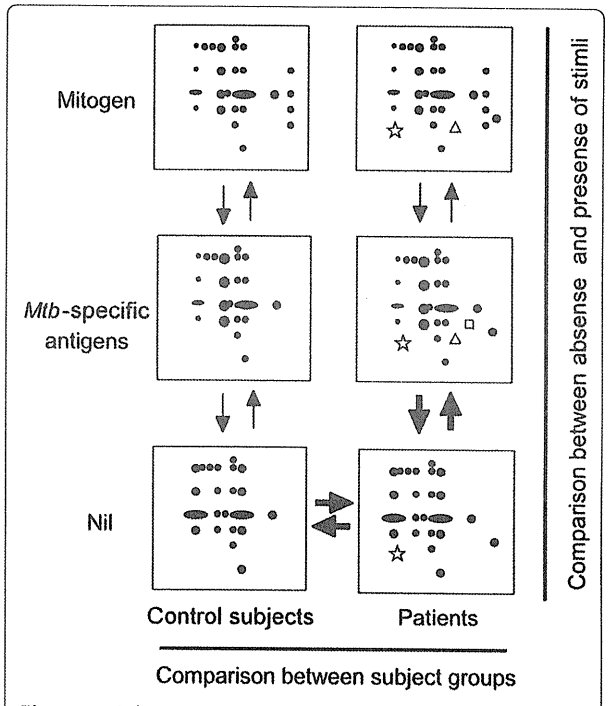

Figure 1 Schematic representation of protein expression profiles. Comparison between subjects, in particular, spot patterns showing differences in plasma proteomes between patients and control subjects. Comparison between the presence and absence of stimuli, especially *ex vivo* spot patterns of *Mtb*-specific antigen-stimulated plasma samples and unstimulated samples from patients. Two major comparative frameworks in our study are shown (bold arrows).

Mass spectrometric analysis

Following the above criteria for selecting candidates of differentially expressed proteins between two conditions, a total of 41 spots were isolated from the corresponding 2D gels on the basis that they showed sufficiently strong

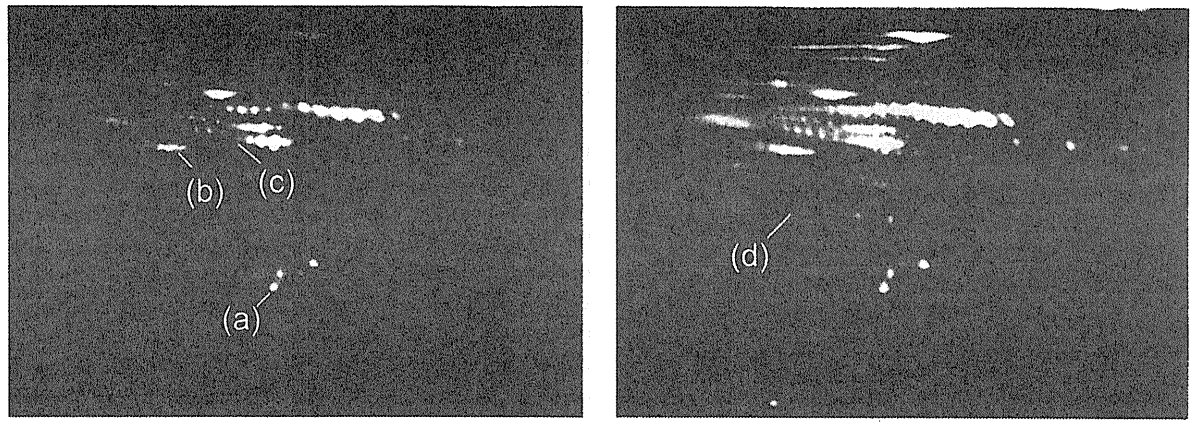

Figure 2 2D-DIGE-based protein expression analysis. Pseudo-colored images were generated using the Multi-Channel Viewer function of the PDQuest software suite. Four sample sets that were either unstimulated or stimulated with the *Mtb*-specific antigen or mitogen from patients with active pulmonary TB and four corresponding sets from control subjects were used to screen for candidate proteins by 2D-DIGE. A: Analysis based on differences in levels in samples from patients and control subjects. Red indicates proteins whose levels increased and green indicates proteins whose levels decreased in unstimulated supernatants from patients with active TB compared with control subjects. Spot (a): HT6102, Spot (b): HT2406, and Spot (c): HT5401 correspond to the PDQuest identification numbers shown in Table 2. B: Analysis based on differences in levels after stimulation. Red indicates proteins whose levels increased in whole blood supernatants from patients with active TB after incubation with *Mtb*-specific antigens. Spots (d): T3107, T4203, and T4208, correspond to Table 2.

signals. Trypsin digestion of each isolated spot was followed by LC-MS analysis. The proteins corresponding to 14 of these spots were successfully identified (Figure 3).

Of the 14 proteins in Table 2, 7 (serial numbers 1 to 7) were obtained as a result of comparisons between patients with active TB and control subjects; number 1 (spot HT6102) was identified as RBP4, number 2 (HT2406) as fetuin-A, and number 3 (HT5401) as VDBP. Four (numbers 8 to 11) were obtained as a result of comparisons between nonspecific mitogen-stimulated and unstimulated samples collected from patients with active TB (not analyzed in this study). The last 3 proteins (numbers 12 to 14) were obtained as a result of comparisons between *Mtb*-specific antigen-stimulated and unstimulated samples collected from patients with active TB; numbers 12 to 14 (T4203, T3107, and T4208) were all identified as clusterin. In Table 2, *P* values indicating a significant difference between the means of the two conditions examined, the SWISS-PROT accession numbers of the identified proteins as well as their molecular weights and theoretical pI values are indicated. We also used the *Homo sapiens* database of expressed sequence tags (ESTs) to identify clusterin in spot T4208.

Mascot search scores (indices of protein matches) were 47, 50, 98, 75, and 72 for spots T4203 (clusterin), T3107(clusterin), HT5401 (VDBP), HT2406 (fetuin-A), and HT6102 (RBP4), respectively, (Table 2), suggesting that identification of these proteins using peak lists of MS/MS spectra obtained from the LC-MS/MS system are fairly reliable since all these scores were significant

Table 1 The number of spots that may show differential expression

A: Comparison between patients with active TB and control subjects				
		***P* < 0.02**	**0.02≤*P* < *0.05***	**0.05≤*P* < 0.10**
Patients versus control subjects		18	12	24
B: Comparison between stimulated and unstimulated conditions				
		P < 0.02	**0.02≤P < 0.05**	**0.05≤P < 0.10**
Patients	*Mtb* antigens versus no stimuli	0	2	2
	Mitogen versus no stimuli	3	5	11
Control subjects	*Mtb* antigens versus no stimuli	0	1	13
	Mitogen versus no stimuli	2	83	8

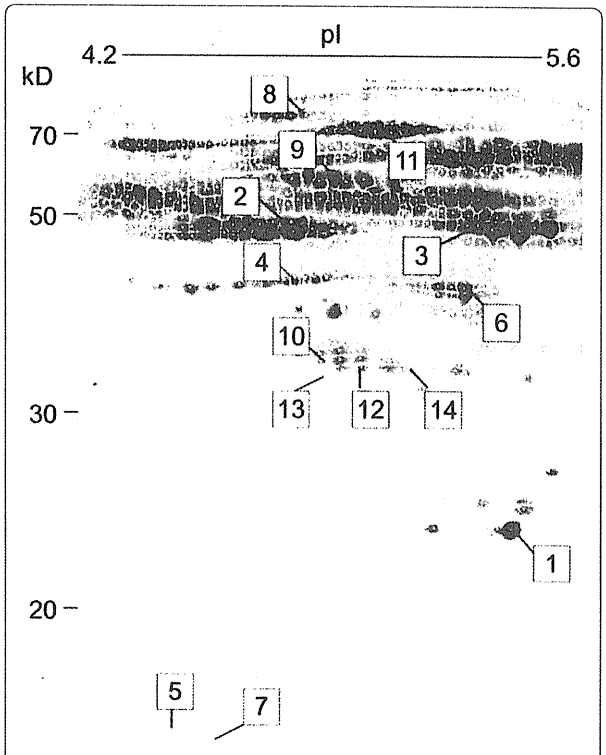

Figure 3 Representative 2D gel image obtained from pooled protein samples. Differentially expressed proteins successfully identified by LC-MS were represented on the gel using the PDQuest software suite. See Table 2 for additional information. Numbers in the figure correspond to serial numbers in Table 2.

above the 5% confidence threshold and no other proteins with comparable scores were detected for each gel spot (See Additional file 1: for supporting information). These proteins were interesting because of their potential biological significance, and we therefore analyzed them further.

Confirmation of differentially expressed proteins by immunoblotting

Immunoblot analysis was used to confirm differential expression of three proteins identified in patients with active TB compared with control subjects (Figure 4A). We measured band densities using the same samples prepared for protein confirmation (Figure 4B). The band density of RBP4 in patients with active TB (64,283 arbitrary units ± 3,861) was lower than that in control subjects (445,894 ± 16,590), and fetuin-A expression in the patients was also lower (42,710 ± 7,580) than that in control subjects (343,617 ± 58,923). These results are consistent with those of 2D gel analysis. Moreover, the band density of VDBP tended to be higher in samples from patients with active TB than from control subjects, which is similar to that observed above; however, the protein levels were widely distributed and the differences in these levels did not reach significance in the control subjects compared with patients with active TB (33,251 ± 2,572 versus 38,971 ± 11,001). Because the three clusterin spots altered after *Mtb*-specific antigen stimulation were not clearly distinguished by immunoblotting, we did not attempt any further demonstration of changes in these signals in our study. Instead, pooled samples were run on a 2D gel and followed by immunoblotting with anti-clusterin-α and anti-clusterin-β antibodies (because clusterin consists of clusterin-α and -β subunits) (Figure 4C). Based on immunoreactivity and pI values, the spots detected were confirmed to be clusterin-α. More specifically, the three spots comprised a subset of possible modified forms of clusterin-α that may be detected.

Detection of differentially expressed proteins by ELISA

Because RBP4 and fetuin-A levels determined by immunoblotting were significantly different between samples from patients with active TB and control subjects, we performed further quantitative ELISA to extend the measurements to plasma samples from 14 Japanese patients with active TB and 13 age-, gender-, and ethnicity-matched control subjects. Plasma RBP4 levels in patients with active TB (median = 23.6 μg/ml; IQR = 18.4-37.9) were significantly lower than those from control subjects (median = 44.6 μg/ml; IQR = 34.6-53.8; P = 0.0033; Figure 5A). Plasma fetuin-A levels in patients (median = 147.9 μg/ml; IQR = 115.8-159.6) were also significantly lower than those in control subjects (median = 211.0 μg/ml; IQR = 186.7-264.6; P = 0.0002; Figure 5B). No significant difference were observed in plasma VDBP levels between patients (median = 110.0 μg/ml; IQR = 85.2-151.3) and control subjects (median = 105.0 μg/ml; IQR = 88.1-215.6; P = 0.5441; Figure not shown).

We simultaneously compared the protein levels in plasma immediately separated from EDTA-containing blood with those in plasma supernatants obtained from heparinized blood as a negative control for IGRA after 18 h of incubation without stimulants. We found that the differences between the two types of plasma samples were small (coefficient of variance (CV) = 10.5% for RBP4; CV = 5.0% for fetuin-A; CV = 6.6% for VDBP) and was in a range of variation generally accepted in ELISA (CV < 15%), indicating that the measurements obtained under the latter condition can be substituted for those obtained under the former condition. Indeed, plasma RBP4 and fetuin-A levels in samples from Japanese patients with active TB were significantly lower than those from control subjects, irrespective of plasma conditions (data not shown).