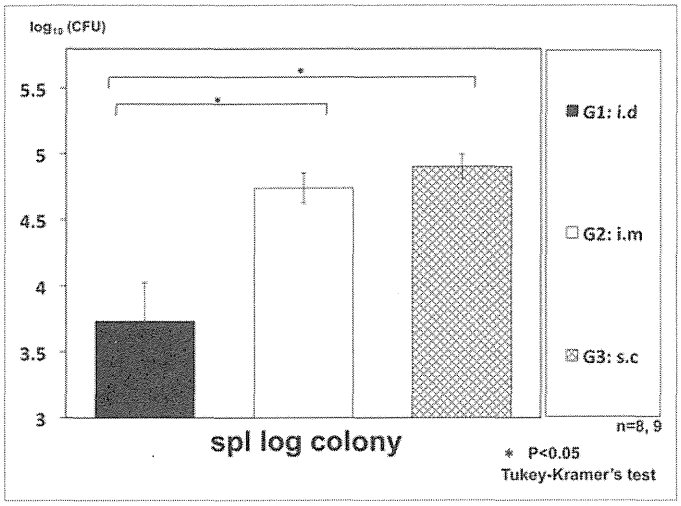

Figure 3. Therapeutic efficacy of intradermal (i.d.) vaccination of HVJ-Envelope/HSP65 DNA + IL-12 DNA, compared with intramuscular (i.m.) or subcutaneous (s.c.) vaccination using intratracheally aerosol infected DBA/1 mice. DBA/1 mice were infected with H37Rv TB by using intratracheal aerosol challenge using aerosol chamber. One week after challenge of TB, 100μg of HVJ-Envelope/HSP65 DNA + IL-12 DNA were administered 6 times for 3 weeks by i.d, i.m, or s.c administration. Four weeks after TB challenge, mice were sacrified, and CFUs of TB in the spleen were evaluated. G1 vs. G2; $p < 0.05$; G1 vs. G3; $p < 0.05$; Student's test.

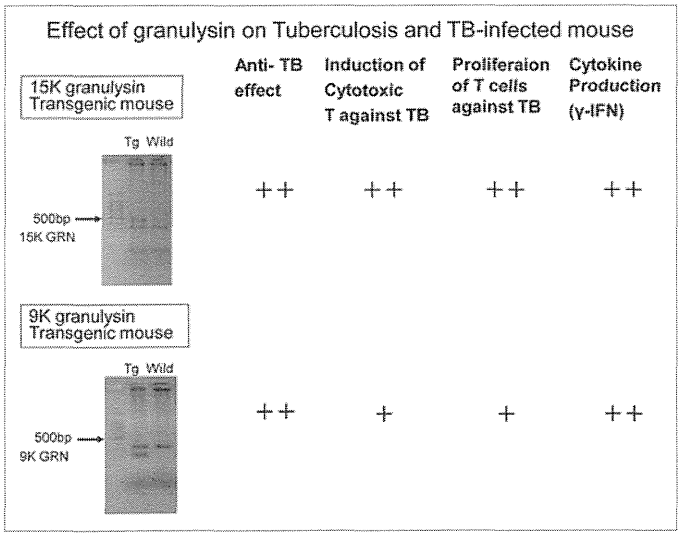

Figure 4. The establishments of 15K granulysin transgenic mice and 9K granulysin transgenic mice. The efficacies of 15K granulysin transgenic mice and 9K granulysin transgenic mice on TB infection were summarized in this Figure. An anti-TB effect, the induction of CTL against TB, the proliferation of T cells against TB and γ-IFN production were augmented in these transgenic mice, compared with wild type C57BL/6 mice.

mice. We measured CFU number of *M. tuberculosis* in the lung four weeks after intravenous injection of TB (5×10^5 /mouse). As shown in **Table 3**, reduction of CFU number was observed in 15K granulysin transgenic mice compared with the normal C57BL/6 mice, indicating the in vivo anti-TB effect of 15K granulysin. As summarized in **Figure 4**, augmentation of immune responses were also observed in 15K granulysin transgenic mice: in vivo induction of cytotoxic T cells against TB, enhanced proliferation of T cells stimulated with TB antigen and augmentation of cytokine production. Furthermore, we examined synergistic effects of the combination of two vaccines. As shown in **Figure 5**, the combination of HSP65-vaccine and granulysin-vaccine showed synergistic effects and 10 times reduction of the CFU number in the liver of TB-infected mice was observed. The number of TB in the liver was significantly reduced by the combination of two vaccines.

Efficacy of granulysin-vaccine in monkey models. We examined the efficacy of granulysin-vaccine in the therapeutic model of TB. The survival rate of granulysin-vaccine (HVJ-Envelope/15K granulysin DNA vaccine)-treated group was 25% (1/4) at 1 y after TB infection (**Fig. 6**). In contrast, all monkeys in saline group were died within 200 d after TB challenge. Thus, survival rate at one year after TB infection was 0% (0/4).

The proliferation of PBL from the monkeys treated with granulysin-vaccine was augmented compared with that of control (saline treated) monkeys (**Fig. 7**). These results indicated the efficacy of granulysin-vaccine in therapeutic models using monkey. Thus, granulysin-vaccine is effective in the monkey as well as the mouse model of TB.

Efficacy of Ksp37-vaccine in therapeutic models. Ksp37 protein is produced from CTL, TypeI helper T cell, γ/δ T cell and NK cell. Ksp37 is composed of 223 amino acids. We analyzed the concentration of Ksp37 in the serum of patients with TB by ELISA. The level of Ksp37 protein in the serum of patients with TB ($n = 31$) was significantly lower than that of healthy volunteers ($n = 60$) ($p < 0.05$) (**Fig. 8**). This is first report suggesting the relation between the serum level of Ksp37 and TB disease (**Fig. 8**).

Therefore, we tried to elucidate the in vivo function of Ksp37 protein, especially function as an anti-TB agent in vivo.

In the first step, we have established a Ksp transgenic mouse for the analysis of function in vivo. We measured the CFU number of M. tuberculosis in the lung 3 weeks after TB aerosol infection. In Ksp transgenic mice, the CFU number of M. tuberculosis was decreased compared with that of wild type control mice (**Fig. 9**). This result indicated the anti-TB effect of Ksp37 in vivo.

These finding suggested that Ksp37 produced from CTL and NK cell functions as an important anti- TB factor in humans and mice.

Discussion

In the present study, we evaluated the potential of three kinds of novel therapeutic vaccines (HSP65-, granulysin- and Ksp37-vaccines) in mouse and monkey models of TB. All of the vaccines showed anti-TB effects in therapeutic models. It is noteworthy

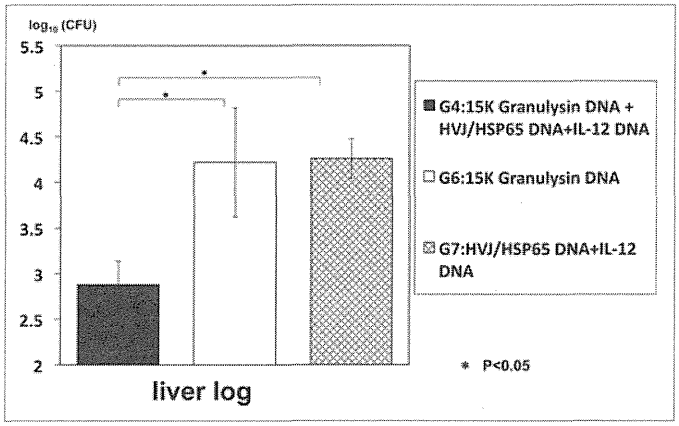

Figure 5. Therapeutic effect of granulysin DNA vaccine on TB-infected (DBA/1) mice DBA/1 mice were infected with H37Rv TB using intratracheal aerosol challenge. One week after challenge of TB, 100μg of HVJ-Envelope/HSP65 DNA + IL-12 DNA and/or 100 μg of HVJ-Envelope/granulysin DNA were injected i.m. into mice 6 times for 3 weeks. Four weeks after TB challenge, mice were sacrified, and CFUs of TB in the liver were evaluated. G1 vs. G2; $p < 0.05$; G1 vs. G3; $p < 0.05$; Student's test.

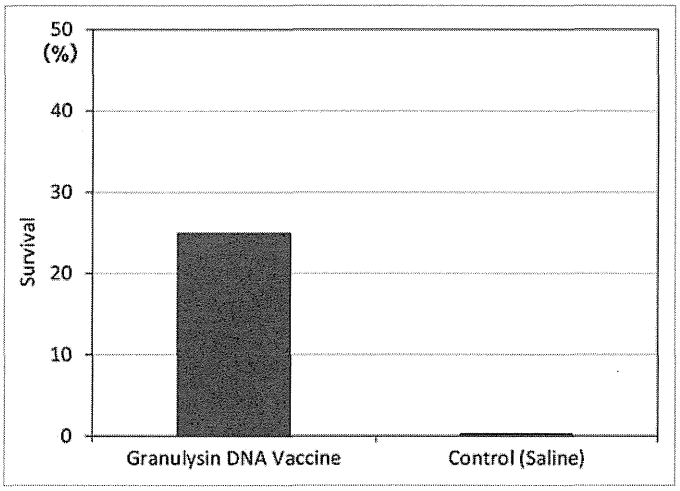

Figure 6. Therapeutic efficacy (survival) of HVJ-Envelope/15K granulysin DNA vaccine, 365 d after TB infection using cynomolgus monkey models. Five × 10^2 M. tuberculosis (Erdman strain) were intratracheally into cynomolgus monkeys as described in Materials and Methods. Four weeks after challenge of TB, 400μg of HVJ-Envelope/15K granulysin were injected i.m. Six times every two weeks. Survival of monkeys treated with this vaccine were evaluated for 1 y (365 d).

that efficacy of novel therapeutic vaccines were demonstrated in monkey models as well as murine models. Thus, this is the leading report of new vaccine against TB. According to our knowledge, only a few therapeutic vaccines against TB have been reported.[14,15] HSP65-vaccine as well as 15K granulysin-vaccine delivered by HVJ-Envelope vector prolonged the survival and augmented the immune responses in the cynomolgus monkey model which closely mimics human TB disease. Thus, we are taking advantage of the availability of multiple animal models and are accumulating essential data on the DNA vaccine/HVJ-envelope in anticipation of initiating a phase I clinical trial.

It is very important to evaluate the long-term survival in a monkey model, as human TB is a chronic infection disease. Thus, it is necessary for the development of effective vaccine to evaluate the long-term survival of monkey.[2,3,7-9] In this study, increase in the survival rate was also observed in HVJ-Envelope/15K granulysin vaccine-treated group, compared with saline-treated group (control group). In addition, it is noteworthy that histopathological improvement was observed in the lung of vaccine-treated monkey (365 d after TB infection). A lot of granulomatous lesions were observed in lung of survived monkey, while a little or no such lesions were observed in lung of saline-treated monkey, which had died of TB within 200 d after TB challenge. Histology of granulomatous lesions observed in this experiment was very similar to human lung TB granuloma by histopathological examinations.

Efficacy of 15K granulysin-vaccine was studied in murine models of TB. We used therapeutic models in this experiment. Furthermore, we examined the synergistic effect of two vaccines (the combination of HSP65- and granulysin-vaccines) in the same therapeutic model. The results indicated the synergistic effect of the combinational vaccination. Therefore, the combination of these therapeutic vaccines might be useful for the development of vaccines against human TB infectious disease. In summary, it was demonstrated that granulysin-vaccine had a therapeutic effect against TB in the mouse and monkey models of TB.

We also elucidated the in vivo function of Ksp37. Ksp37 is expressed in cytotoxic lymphocytes, selectively in the effector subset of $CD8^+$ T cells, $CD16^+$ NK cells and γ/δT cells.[13] Expression of Ksp37 mRNA was closely correlated with good prognosis in ovarian cancer cells and gliomas.[16,17] However,

Table 2. Therapeutic efficacy and Immune responses using IL-2 receptor γ-chain gene disrupted SCID-PBL/hu models

(A)	**Therapeutic efficacy**	
	Treated	CFU of TB (log)
	(-)	6.03 ± 0.06
	HSP65 DNA + IL-12 DNA vaccine	5.40 ± 0.97
(B)	**Immune reponses**	**human CTL and T cell proliferation**
	IL-2 R γ-chain(-/-) SCID PBL-hu	human CTL (+++) human T cell proliferation (+++)
	CB17-SCID PBL-hu	human CTL (+) human Tcell proliferation (+)

Therapeutic efficacy of HVJ-envelope / HSP65DNA + IL-12DNA, using in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice (SCID-PBL/hu). Groups of animals were treated with 3 times with HVJ-envelope / HSP65DNA + IL-12DNA (50ug i.m.) . Ten days after the third vaccination, mice were sacrificed and CFU of TB in the liver of mice were accessed as described in Materials and Methods. One × 10^7 PBL from a healthy human volunteer were injected i.p. into IL-2 receptorγ-chain disrupted NOD-SCID mice. Twenty one days after injection of PBL, mice were challenged with 5 × 10^5 H37Rv i.v. and then treated with vaccine. *Student's t-test was used to compare the CFU of TB of each group ($p < 0.05$). Human immune responses [human CTL activity and human T cell proliferation against alloantigen (CESS cells)] of IL-2 receptor γ-chain (-/-) NOD SCID PBL-hu mice were compared with those of CB17-NOD-SCID PBL-hu mice. (+), weak; (+++), very strong.

Table 3. In vivo anti-TB effect of 15kDa Granulysin Transgenic mouse

mouse	lung Number of M. tuberculosis 4 weeks after TB injection (log)
G1 15K Granulysin Tg	5.3 ± 0.35
G2 Wild Type C57BL/6	6.0 ± 0.5

Mean ± SD, n = 7; *p < 0.05 G1 vs. G2; Tukey-Kramer's HSD; In vivo inhibition of the growth of *M. Tuberculosis* in the 15K granulysin transgenic mice; In vivo anti-TB effect of 15K granulysin transgenic mouse; Five 15 K granulysin transgenic mice and five wild type C57BL/6 mice were injected with 5×10^5 H37Rv M. tuberculosis i.v. Four weeks after the challenge of *M. Tuberculosis*, mice were sacrificed. CFU of *M. Tuberculosis* in the lungs of these mice were assessed described in Material and Methods. Student's t-test was used ($p < 0.05$).

detailed immunological function has not been elucidated yet. We first revealed that the level of Ksp37 protein in the serum of patients with TB was lower than that of healthy volunteer. The result suggested the relation between the serum level of Ksp37 and TB disease. Next, we have established Ksp37 transgenic mice to elucidate the in vivo role of Ksp37 in the defense against the infection of M. tuberculosis. Ksp37 transgenic mice showed in vivo anti TB effect. Thus it was demonstrated that Ksp37 played an important role in anti-TB function in human as well as mice bodies. Finally, we examined the efficacy of Ksp37-vaccine in the mouse model of TB. Similar to granulysin-vaccine, Ksp37-vaccine augmented in vivo differentiation of CTL against TB (data not shown). In addition, simultaneous administration of Ksp37- and granulysin-vaccines induced CTL generation synergistically (data not shown). Therefore, these findings indicate that granulysin- and Ksp37-vaccine might provide very useful weapon as a novel TB vaccine, in the monotherapy or combination therapy.

The HSP65 vaccine showed a significant therapeutic effect against TB, as described previously: (1) Prolongation of survival of mice infected with XDR-TB; (2) Decrease in the CFU of TB in lung, liver and spleen of mice infected with MDR-TB as well as drug-sensitive TB (H37Rv); (3) Decrease in the CFU of TB in organs of mice challenged with TB in the in vivo humanized immune model of SCID-PBL/hu.

Here, we revealed the synergistic effects of the combination therapy of HSP65-vaccine and a first line chemotherapy drug Isoniazid (INH). It is very important to make a suitable regimen, which enables the treatment of the patient with TB to complete within a shorter period. In such circumstances, our data demonstrating the synergistic effect of the combinational therapy using a DNA vaccine and a chemotherapy drug will provide a new strategy for the treatment of TB.

We also revealed the importance of administration route of DNA vaccine. Generally, vaccines are administrated either intradermally (i.d.), intramuscullary (i.m.) or subcutaneously (s.c.). Our data suggested that the intradermal injection is suitable for the administration of our DNA vaccines. Therefore, in the monkey model we plan to conduct the efficacy study of intradermal injection of this vaccine. We will compare the efficacy of intradermal administration to conventional i.m administration.

In the recent study using cynomologus monkeys, it is suggested that i.d. vaccination of HSP65-vaccine showed stronger therapeutic effects against TB than i.m. vaccination on the basis of the prolongation of survival and ESR (Erythrocyte Sedimentation Rate).

DNA vaccine is a relatively new approach of immunization for infectious diseases.[3,4,18-21] We have developed a hemagglutinating virus of Japan envelope (HVJ-Envelope) using inactivated Sendai virus, as a nonviral vector for drug delivery.[22-24] It can efficiently deliver DNAs, siRNAs, proteins and anti-cancer drugs into cells both in vitro and in vivo.[25-27] Therefore, HVJ-Envelope was suitable as an efficient and safe vector for DNA vaccines.

The priority of development of vaccine(s) to prevent reactivation of TB will be increased, since large proportion of the world is latently infected with TB. The combination of HSP65-vaccine with conventional vaccine (BCG) showed synergistic effects in the mouse and monkey models of TB and prolonged the survival of animals. Therefore, it will be important to evaluate the current vaccines as post-exposure vaccines. Combination of several vaccines or combination of vaccines with drugs for chemotherapy might provide a new insight for the prevention of the reactivation of TB.

In conclusion, our data indicated the synergistic therapeutic effect of combination of HSP65-, granulysin- and Ksp37-vaccines or combination of these DNA vaccines and first line chemotherapy drug(s). Combinational therapy using vaccines and antibiotics might provide novel rationale against MDR-TB therapy. Furthermore, the efficacies of HSP65 vaccine and granulysin vaccine were confirmed in the murine therapeutic model for XDR-TB and cynomolgus monkey therapeutic model. These data will provide a rationale for moving this vaccine into clinical trial. HSP65-, granulysin- and Ksp37-vaccines might be useful

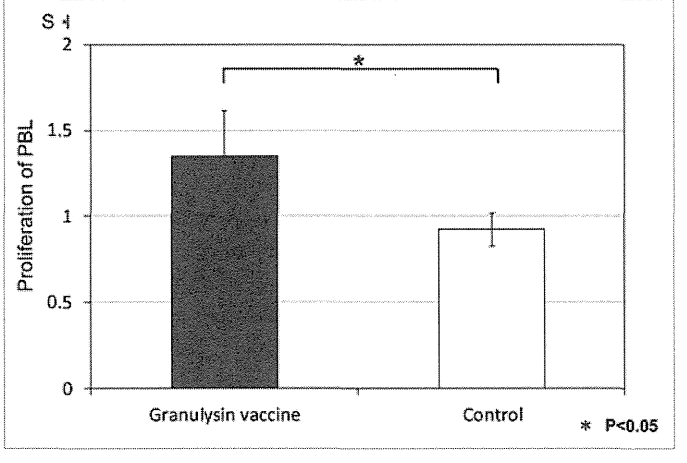

Figure 7. Proliferation of PBL from monkeys vaccinated with HVJ-Envelope/15K granulysin DNA by the stimulation with HSP65 antigen. Five × 10^2 M. tuberculosis (Erdman strain) were intratracheally into cynomolgus monkeys as described in Materials and Methods. Four weeks after challenge of TB, 400μg of HVJ-Envelope/15K granulysin were injected i.m. Six times every two weeks. The proliferation of PBL from monkeys vaccinated with HVJ-Envelope/15K granulysin on 13 weeks after TB challenge were assessed by the ^{3}H-TdR uptake of lymphocyte for 3 d culture.

vaccines against TB including XDR-TB and MDR-TB after the clinical trials.

Materials and Methods

Methods for the evaluation of the therapeutic efficacy of the vaccine on the *M. tuberculosis*-infected monkeys. Cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial Research Center. The animals were vaccinated nine times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400 ug i.m.), one week after the challenge with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies.[2,6,7]

The animals were vaccinated with HVJ-Envelope/15K granulysin DNA vaccine 6 times. Four weeks after challenge of TB, 400 μg of HVJ-Envelope/15K granulysin were injected i.m. six times every two weeks. Survival of monkeys treated with this vaccine were evaluated. All animal experiments were approved by the Leonard Wood Memorial Animal Care and Use Committee and the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee.

Methods for the evaluation of the efficacy of vaccines on the *M. tuberculosis*-infected mice. DNA vaccines encoding *M. tuberculosis* HSP65 and IL-12 were encapsulated into HVJ-Envelope.[3,6,8,28] HVJ-Envelope were prepared as described previously1.[4,29] The HVJ-Envelope complex was aliquoted and stored at -70°C until use. Groups of mice were vaccinated three times with 100 μl of HVJ-Envelope solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles after TB challenge.[28,29] At 30 d after intravenous challenge of *M. tuberculosis* H37Rv and MDR-TB, the number of CFU in the lungs, spleen, and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccines was evaluated.[28,29] DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine three times i.m. at 1, 8 and 15 d after the challenge of 5×10^5 CFU MDR-TB i.v. Therapeutic efficacy was also evaluated by chronic TB infection model of mice using aerosol challenge of TB (15CFU/mouse: Madison aerosol exposure chamber, University of Wisconsin). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8–10 weeks of age. All vaccinations and experiments on isolate tissue of animal were done under anesthetic state with sevoflurane. Infected animals were housed in individual micro-isolator cages in a Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee.

Methods for the establishment of SCID-PBL/hu model. IL-2 receptor γ-chain disrupted NOD-SCID-PBL/hu was constructed as described in our previous study.[4,5] CTL activity was assessed using the method as described previously.[30-32]

Methods for the establishment of granulysin transgenic mouse. Either 15K granulysin gene, 9K granulysin gene or secreted 9K granulysin DNA (15K Gra secretory signal DNA was fused into N-terminal of 9K granulysin DNA) were transferred to expressing plasmid DNA (pCAGGS) having CAG promoter. DNA fragment was injected to pronuclei embryo and grafted to 200 foster parents. Two types of 15K granulysin Tg mice, 3 types of 9K granulysin Tg mice and 6 types of secreted 9K granulysin Tg mice were made. Granulysin activity was assessed by monoclonal antibody targeting 15K granulysin and 9K granulysin. *Mycobacterium tuberculosis* H37Rv 5×10^5 CFU was intravenously injected to 15K granulysin Tg mice, 9K granulysin Tg mice, wild type (control) mice and normal C57BL/6 mice (8-12weeks).[3,7] From 2 to 12 weeks after injection, these mice were sacrificed. The lungs, the liver and the spleen of these mice were removed, homogenized and cultivated for 14 d on 7H11 agar medium. Then, the number of colony of *Mycobacterium tuberculosis* was measured.[28,29]

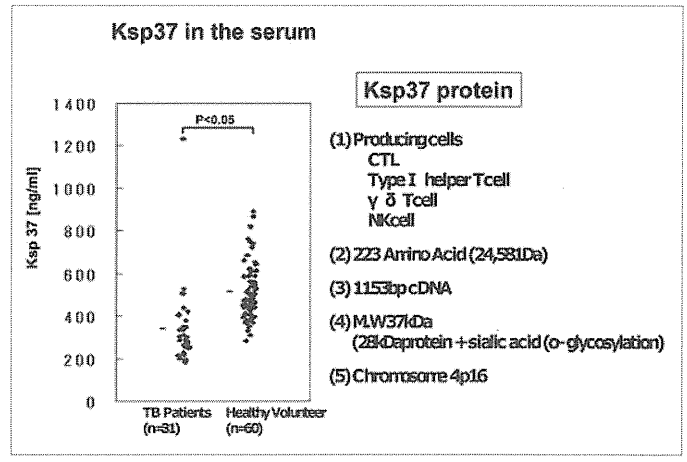

Figure 8. Killer specific secretory protein of 37kDa (Ksp37 protein) in the serum of patients with tuberculosis. Ksp37 protein in the serum of 31 patients with TB and 60 healthy volunteers were assessed by ELISA.

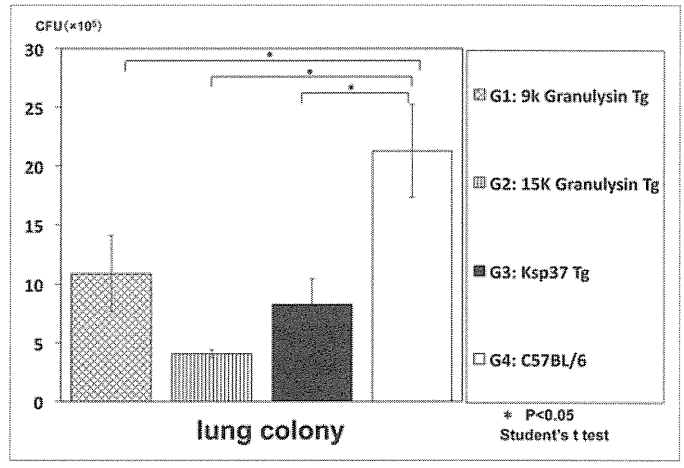

Figure 9. In vivo anti-TB effect of Ksp37 transgenic mice. Ksp37 Tg mice were established as described in Materials and Methods. Ksp37 Tg mice, 15K granulysin Tg mice, 9K graunlysin Tg mice and wild type C57BL/6 mice were infected with H37Rv TB by using intratracheal aerosol challenge using aerosol chamber.

Method for the establishment of Ksp37 transgenic mouse. Ksp37 gene were transferred to expressing plasmid DNA (pCAGGS) having CAG promoter. DNA fragment was injected to pronuclei embryo and grafted to 200 foster parents. Two types of Ksp Tg mice (#13, #14) were made. Ksp activity was assessed by monoclonal antibody targeting Ksp 37.

Reagents. Isoniazid (INH) was obtained from Sigma Co. Ltd (lot No. 117K0712). Rifampicin (RFP) was obtained from Sigma Co. Ltd (lot No. 087K18753). An amount of 0.03 mg/mouse of INH and 0.1 mg/mouse of RFP were administered to mice per os.

Statistical analysis. Student's t tests and Tukey-Krumer's test were used to compare log 10 value of CFU between groups following challenge of TB. Student's t tests were also performed to compare immune responses between groups in T cell proliferation assay. A P-value of < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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ORIGINAL INVESTIGATION

Association of *IFNGR2* gene polymorphisms with pulmonary tuberculosis among the Vietnamese

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Abstract Interferon-γ (IFN-γ) is a key molecule of T helper 1 (Th1)-immune response against tuberculosis (TB), and rare genetic defects of IFN-γ receptors cause disseminated mycobacterial infection. The aim of the present study was to investigate whether genetic polymorphisms found in the Th1-immune response genes play a role in TB. In our study, DNA samples were collected from two series of cases including 832 patients with new smear-positive TB and 506 unrelated individuals with no history of TB in the general population of Hanoi, Vietnam. Alleles of eight microsatellite markers located around Th1-immune response-related genes and single nucleotide polymorphisms near the promising microsatellites were genotyped. A set of polymorphisms within the interferon gamma receptor 2 gene (*IFNGR2*) showed a significant association with protection against TB ($P = 0.00054$). Resistant alleles tend to be less frequently found in younger age at diagnosis ($P = 0.011$). Luciferase assays revealed high transcriptional activity of the promoter segment in linkage disequilibrium with resistant alleles. We conclude that the polymorphisms of *IFNGR2* may confer resistance to the TB development of newly infected individuals. Contribution of the genetic factors to TB appeared to be different depending on age at diagnosis.

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Introduction

Tuberculosis (TB) remains one of the major health problems worldwide (Lopez et al. 2006): According to an estimate, approximately one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), and more than 9 million people develop active TB disease every year. Of these patients, 80% are from 22 high-burden countries including Vietnam (World Health Organization 2010).

Development of TB has been considered to be a two-stage process, infection with *M. tuberculosis* and progression to disease. In total, 5–10% of immunocompetent individuals initially infected with *M. tuberculosis* develop active TB during their lifetime (Frieden et al. 2003). When young vulnerable individuals fail to inhibit growth of the pathogen, they often develop the disease within 2 years of infection. In the remaining individuals, containment of *M. tuberculosis* is successful, though the agent is not

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)

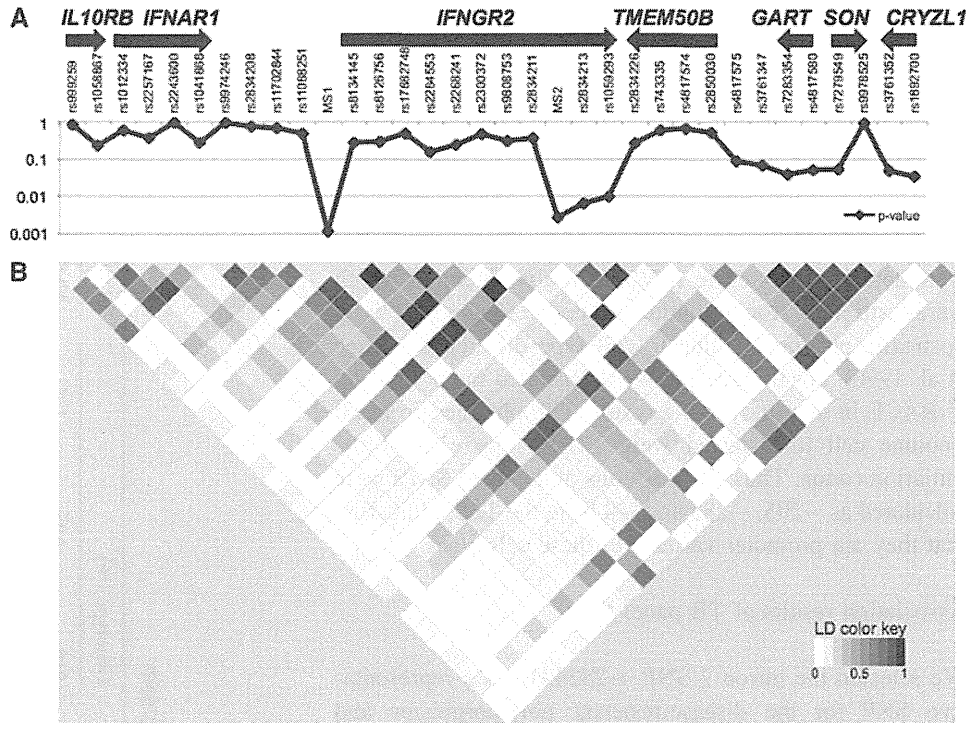

Fig. 1 Association *P* values and pairwise LD of genotyped polymorphisms around *IFNGR2* region. **a** Association results for 32 SNPs in 273 patients (panel A) and 506 controls are shown. *P* values of microsatellite markers in 98 patients and 200 controls are also included. Positions of genes are shown 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).

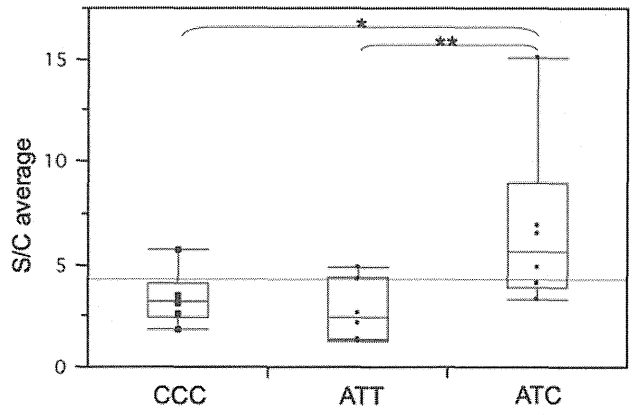

Fig. 2 Dual luciferase reporter assays. The ratios of Firefly luciferase activity (signal S) to Renilla luciferase activity (control C) are displayed using *box* and *whisker plots*. Three subcloned plasmids were prepared, and each subcloned plasmid was tested in triplicate and S/C values were averaged. 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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Circulating Levels of Adiponectin, Leptin, Fetuin-A and Retinol-Binding Protein in Patients with Tuberculosis: Markers of Metabolism and Inflammation

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Abstract

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

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

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

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

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Introduction

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

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

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

Table 1. Characteristics of study population.

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

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

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

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

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

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

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

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

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

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

Methods

Study design

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

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

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

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

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

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

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

[a]Estimated means of plasma concentrations were compared after logarithmic transformation, being adjusted for gender, age and BMI as covariates. The data shown are transformed back to the original unit.
*Statistically significant when the significance level is set as $P<0.008$ based on the Bonferrroni correction.
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Measurements of markers of metabolism and inflammation

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

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

Statistical analysis

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

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

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

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

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

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

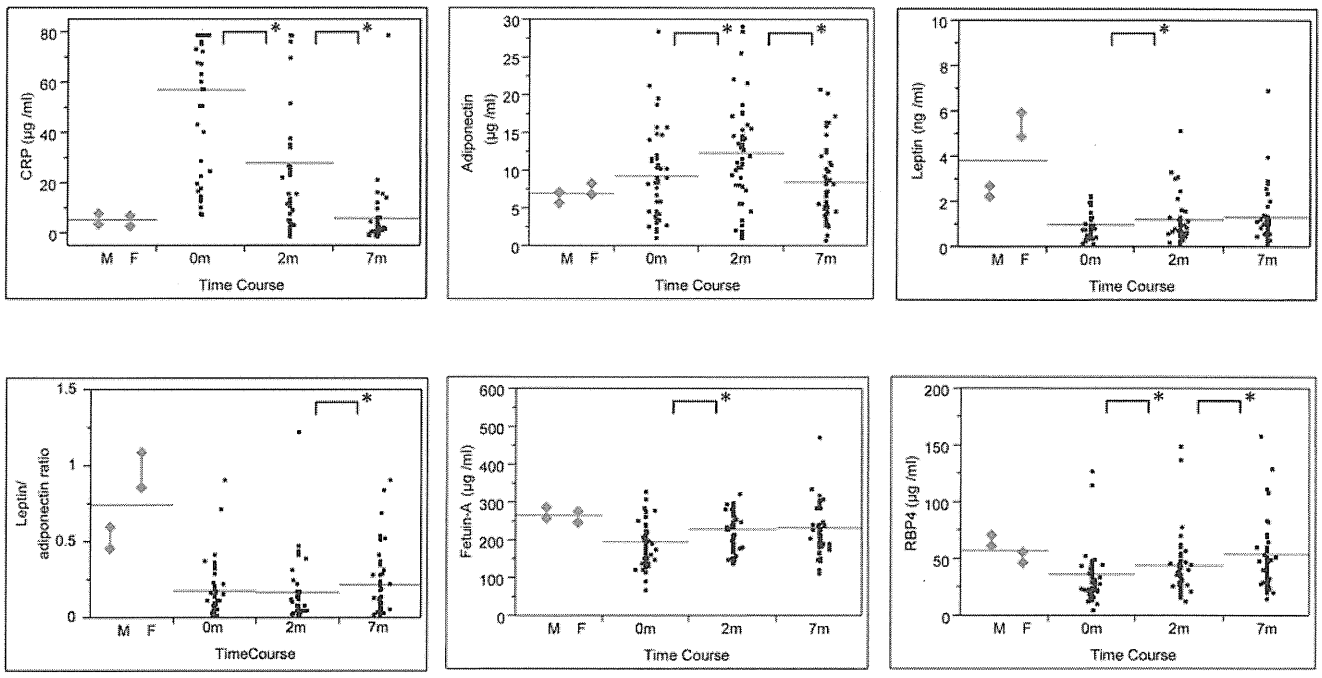

Figure 1. CRP and tested marker levels in patients with active TB before (0 month), during (2 months) and at the end (7 months) of anti-TB treatment (N = 46). Vertical bars with diamonds on the left side (M and F) indicate reference values, means ± SEM of the values in men (N = 41) and women (N = 43)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

Characteristics of study population

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

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

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

Pairwise correlations between four tested markers

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

Supporting Information

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

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Author Contributions

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

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