別紙4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
永井英明	診断の進歩 QFT の新しい展開(解説)	永井厚志、巽浩一郎	Annual Review 呼吸器 2011	中外医学社	東京	2011	187-192

雑誌

発表者 氏　名	論文タイトル名	発表誌名	巻号	ページ	出版年
岡田全司	The study of novel DNA vaccines against tuberculosis: Induction of pathogen-specific CTL in the mouse and monkey models of tuberculosis.	Human Vaccines and Immunother-apeutics	9(3)	515-525	2013
岡田全司	Novel therapeutic vaccines [(HSP65+ IL-12)DNA-, granulysin- and Ksp37-vaccine] against tuberculosis and synergistic effects in the combination with chemotherapy.	Human Vaccines and Immunother-apeutics	9(3)	526-533	2013
岡田全司	Potential function of granulysin, other related effector molecules and lymphocyte subsets in patients with TB and HIV/TB coinfection.	International Journal of Medical Sciences	10(8)	1003-1014	2013
岡田全司	Siddiqi UR, Punpunich W, Chuchottaworn C, Jindaku S, Ashino Y, Saitoh H, Okada M, Chotpittayasunondh T, Hattori T.: Elevated anti-tubercular glycolipid antibody titers in healthy adults as well as in pulmonary TB patients in Thailand.	International Journal of Tuberculosis and Lung Diseases.	16(4)	532-538	2012
岡田全司	A Novel Therapeutic and Prophylactic Vaccine against Tuberculosis Using the Cynomolgus Monkey Model and Mouse Model.	Procedia in Vaccinology.	4	42-49	2011
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岡田全司	Novel therapeutic vaccine: Granulysin and new DNA vaccine against Tuberculosis.	Human Vaccines.	7	60-67	2011
岡田全司	Development of therapeutic and prophylactic vaccine against tuberculosis using monkey and granulysin transgenic mice models.	Human Vaccines	7	108-114	2011
永井英明	Multicolor flow cytometric analyses of CD4+ T cell responses to Mycobacterium tuberculosis-related latent antigens.	Jpn J Infect Dis	66	207-215	2013

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永井英明	Stratified Threshold Values of QuantiFERON Assay for Diagnosing Tuberculosis Infection in Immunocompromised Populations.	Tuberc Res Treat	2011	Article ID 940642	2011
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慶長直人	Primary drug-resistant tuberculosis.	present status and risk factors.	8(8)	e71867	2013
慶長直人	Clonal expansion of Mycobacterium tuberculosis isolates and coexisting drug resistance in patients newly diagnosed with pulmonary Tuberculosis.	BMC Res Notes	6	444	2013
慶長直人	Potential function of granulysin, other related effector molecules and lymphocyte subsets in patients with TB and HIV/TB coinfection.	Int J Med Sci	10(8)	1003-1014	2013
岡田全司	Ⅱ予防接種Q&A（B）BCG100.（結核予防ワクチン；DNAワクチン）結核予防ワクチン（DNAワクチン）の開発状況とその応用の可能性について教えてください。	小児内科	45(3)	281-283	2013
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岡田全司	ヒト結核感染に最も近いカニクイザルを用いた新規結核予防ワクチン開発及び臨床応用に向けて　「結核—古くて新しい感染症—」	最新医学	68(11)	2479-2487	2013
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岡田全司	新たな結核ワクチン	感染・炎症・免疫	41	46-51	2011

永井英明	新しい結核感染診断検査法 T-SPOT.TB の有用性	アニムス	19	37-42	2014
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Research Paper

Potential Function of Granulysin, Other Related Effector Molecules and Lymphocyte Subsets in Patients with TB and HIV/TB Coinfection

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Abstract

Background: Host effector mechanism against *Mycobacterium tuberculosis* (*Mtb*) infection is dependent on innate immune response by macrophages and neutrophils and the alterations in balanced adaptive immunity. Coordinated release of cytolytic effector molecules from NK cells and effector T cells and the subsequent granule-associated killing of infected cells have been documented; however, their role in clinical tuberculosis (TB) is still controversy.

Objective: To investigate whether circulating granulysin and other effector molecules are associated with the number of NK cells, *i*NKT cells, $V\gamma9^+V\delta2^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells, and such association influences the clinical outcome of the disease in patients with pulmonary TB and HIV/TB coinfection.

Methods: Circulating granulysin, perforin, granzyme-B and IFN-γ levels were determined by ELISA. The isoforms of granulysin were analyzed by Western blot analysis. The effector cells were analyzed by flow cytometry.

Results: Circulating granulysin and perforin levels in TB patients were lower than healthy controls, whereas the granulysin levels in HIV/TB coinfection were much higher than in any other groups, TB and HIV with or without receiving HAART, which corresponded to the number of $CD8^+$ T cells which kept high, but not with NK cells and other possible cellular sources of granulysin. In addition, the 17kDa, 15kDa and 9kDa isoforms of granulysin were recognized in plasma of HIV/TB coinfection. Increased granulysin and decreased IFN-γ levels in HIV/TB coinfection and TB after completion of anti-TB therapy were observed.

Conclusion: The results suggested that the alteration of circulating granulysin has potential function in host immune response against TB and HIV/TB coinfection. This is the first demonstration so far of granulysin in HIV/TB coinfection.

Key words: Granulysin, TB, HIV, HIV/TB Coinfection, Lymphocytes Subsets.

Introduction

Tuberculosis (TB) is the leading cause of death in human immunodeficiency virus (HIV)-infected individuals in countries with the highest TB burden [1]. Coinfection with HIV evidently affects the progression of *Mycobacterium tuberculosis* (*Mtb*) infection and induces *Mtb*-specific immune responses contributing to increased HIV replication through cellular activation [2, 3]. It is clear that the overall disruption of immune function in HIV infected patients is the sum of multiple factors including $CD4^+$ T cell depletion by direct infection of HIV-1 and chronic immune activation leading to dysfunction of the immune system [4]. Pathologically HIV/TB coinfection caused functional disruption of local immune responses leading to weakened granulomatous host response to *Mtb* [5]. However, immune activation induced by rapid reactivation of *Mtb* in chronic HIV infection has not been fully investigated to our knowledge.

Granulysin is a member of the saposin-like protein family and co-localizes in the granular compartments of human natural killer (NK) cells, double negative invariant NKT (*i*NKT), Vgamma (γ) 9^+ Vdelta (δ) 2^+ T cells and $CD8^+$ T cells along with granzymes and perforin [6]. It is a cationic small glycoprotein and synthesized as a secretary 15 kDa precursor which is then enzymatically processed into a granular 9 kDa protein. The 9 kDa isoform has characteristics of pro-inflammatory cytokine and cytolytic activity [7], which is able to induce cytolysis of various tumor cells, microbe-infected cells by release into the intercellular space between target and effector cells via granule exocytosis pathway upon stimulation [8] and mediates killing of extracellular and intracellular *Mtb* [9] via several effector molecules including perforin and granzymes. In contrast, the 15 kDa granulysin is constitutively secreted from NK and T cells via non-exocytotic pathway [10]. The $CD8^+$ T, NK and even $CD4^+$ T cells can express granulysin together with perforin and granzyme B co-localized in granules [11] and released into immunological synapse upon activation [12]. Granulysin-mediated lysis of *Mtb* infected cells has been performed mainly by $CD8^+$ and NKT cells expressing perforin and granulysin [9, 13, 14]. High frequency of $CD4^+$ T cells co-expressing granulysin was observed in children and adolescents [15]. Moreover, *i*NKT cells exhibiting antimycobacterial activity also expressed granulysin against *Mtb* inside monocytes/macrophages [14]. In addition, a reduced number of *i*NKT cells in peripheral blood were found in patients with pulmonary TB and HIV-1 infection [16]. In TB, granulysin and perforin could be detected in $V\gamma9^+V\delta2^+$ T cells, indicating their direct contribution to a protective host response against *Mtb* infection [17].

Reduction of perforin and granulysin levels related to granzyme A has been reported in lung tissue biopsy from patients with chronic TB, while higher expression in $CD8^+$ T cells was associated with bacteriological control, suggesting that perforin and granulysin could be used for evaluation of immune protection in human TB [18]. The primary effector function of $CD4^+$ T cells is believed to be the production of interferon-gamma (IFN)-γ and other cytokines to activate macrophages, which can then control or eliminate intracellular organisms [19]. It has been shown that $CD4^+$ T cells were the main sources of IFN-γ and the relative responses to early secreted antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 significantly increased in even chronically HIV-infected patients with decreased $CD4^+$ T cells, whereas acute HIV infection induced a rapid depletion of *Mtb*-specific $CD4^+$ T cells in asymptomatic TB [20, 21]. In active pulmonary TB, high circulating IFN-γ level was detected which decreased significantly after two months of therapy [22, 23]. Similar results were found in child TB patients [24]. These indicate the involvement of IFN-γ in curative immune response against *Mtb*.

Significantly lower plasma granulysin levels than controls have been demonstrated in adults with active pulmonary TB in highly TB endemic area in Indonesia which increased after two months of anti-TB therapy, reaching the values similar to those of controls and even further increased after completion of anti-TB therapy. Such granulysin levels were predominantly in patients expressed by IFN-γ negative T cells suggesting that their cellular source of IFN-γ and granulysin in TB are partly non-overlapping [12]. Patients with active pulmonary TB had low circulating granulysin but high IFN-γ levels, indicating their possible role in host defense against *Mtb* [25]. Earlier study demonstrated that higher plasma IFN-γ was

found in patients with HIV/TB coinfection than TB [26], suggesting a greater degree of immune activation in HIV/TB coinfection, particularly those with low $CD4^+$ T cells counts.

There is limited information so far regarding the role of granulysin and other cytolytic effector molecules related to NK cells, *i*NKT cells, T cells and their subpopulations against *Mtb* infection in TB and HIV/TB coinfection. This study aims to investigate whether circulating granulysin and other effector molecules are associated with the number of corresponding functional cells, NK cells, *i*NKT cells, $V\gamma9^+V\delta2^+$ T cells, $CD4^+$ T cells and $CD8^+$ T cells, and such association may influence the clinical outcome of the disease in patients with pulmonary TB and HIV/TB coinfection in northern Thailand where TB is endemic.

Materials and Methods

Study subjects

Six patients with HIV/TB coinfection and 21 TB patients were recruited from the outpatient and inpatient clinics of Chiang Rai Hospital and Mae Chan Hospital, north of Thailand. Pulmonary TB patients were categorized based on WHO criteria (WHO, 2009), defining whether or not the patients has previously received TB treatment. TB drug regimens were based on the recommendation of National Tuberculosis Program, Ministry of Public Health,Thailand. The patients with HIV/TB coinfection and TB were all newly diagnosed TB confirmed by microscopic examination of acid-fast bacilli (AFB) in sputum and positive cultures of *Mtb*, medical history and chest radiographic findings. All had never received any anti-TB therapy or taken anti-TB drugs for less than 7 days and never received any antiretroviral therapy, immune-suppressive drugs or other immunomodulators prior enrollment. None had diabetes mellitus or other acute infections. The patients with HIV/TB coinfection had not previously received highly active antiretroviral therapy (HAART), the standard drug treatment, and were positive for anti-HIV antibody by the particle agglutination assay (Serodia-HIV-1/2, Fujirebio Inc, Tokyo, Japan) and enzyme-linked immunosorbent assay (ELISA) (Enzygnost Anti-HIV 1/2 plus ELISA, or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Ill, USA) Dade Behring, Marburg, Germany). No patients were reported to be multidrug resistance (MDR) or extensively drug resistance (XDR) cases by drugs sensitivity tests at the time of enrollment. Eleven patients with HIV without receiving HAART (HIV+HAART-) and 17 with HIV receiving HAART (HIV+HAART+) were recruited from the HIV Care and Treatment Project (Daycare clinic). These patients had no previous TB episodes and had not received isoniazid preventive therapy (IPT) to sterilize latent TB infection (LTBI) and prevent progression to active TB at the time of enrollment. Their sputum smears were negative for AFB and *Mtb* cultures. They were negative (induration < 5 mm) by Tuberculin Skin Test (TST) and had no concomitant active AIDS-related opportunistic infections within 30 days prior enrollment. The clinical characteristics of individual HIV/TB coinfection are summarized in Table 1.

Table 1. Clinical characteristics of patients with HIV/TB coinfection.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex/Age	Male/42	Male/47	Male/44	Female/46	Male/30	Male/37
CXR findings at TB diagnosis	Non-cavitary	Non-cavitary, infiltrates, pleural effusion	Cavitary	Non-cavitary	Non-cavitary	Non-cavitary, pleural effusion
Presenting form of TB	Pulmonary	Pulmonary + extra-pulmonary (meningeal)	Pulmonary	Pulmonary + extra-pulmonary (colitis)	Pulmonary + extra-pulmonary (lymp node)	Pulmonary
Treatment regimen for TB	2HRZE/4HR	2HRZE/4HR	2HRZE/4HR	2HRZE/4HR	2HRZE/4HR	2HEOS/18HE
HAART initiation during study period* (regimen)	Yes (d4T,3TC,NVP)	No	No	No	No	Yes (d4T,3TC,EFV)
Outcomes after 6-9 mo of anti-TB therapy	Cured	Cured	Cured	N/A**	N/A**	Cured
Duration of TB treatment (month)	7	6	8	-	-	18

d4T = Stavudine; 3TC = Lamivudine; NVP = Nevirapine; EFV = Efavirenz; HAART = highly active antiretroviral therapy. *HAART initiated 2 months after starting anti-TB treatment. ** Unable to follow-up.

Twenty three healthy individuals recruited from Blood Bank of Mae Chan hospital was used as controls. They had no history of TB and no risk factors involving TB and their chest radiographs were normal. None of them had diabetes mellitus and all were negative for Hepatitis B surface antigen, Hepatitis C antigen and anti-HIV antibody.

This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand and the National Center for Global Health and Medicine, Japan, and the written informed consents were obtained from all patients and all healthy individuals.

Blood samples

Blood were collected in K_3EDTA vacutainers upon enrollment (n = 6 for HIV/TB coinfection and 21 for TB) and after completion of anti-TB therapy for 6-9 months when they were considered as cured (n = 3 for HIV/TB coinfection and 13 for TB). Plasma were separated by centrifugation and stored at -80°C.

Determination of granulysin concentration

The granulysin concentrations in plasma were determined by ELISA [25]. The test was done in duplicate. Briefly, a microtiter plate (Costar, USA) was coated with 100 µl/well containing 5 µg/ml mouse monoclonal anti-human granulysin (RB1) (MBL International Corporation, Nagoya, Japan) in 0.05 M carbonate-bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. The plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 and blocked with buffered protein solution with ProClin-150 at room temperature (RT) for 1 h. After being washed, the undiluted plasma was added and incubated for 2 h at RT and followed by washing. The bound antigens were detected with 0.1 µg/ml of mouse monoclonal anti-human granulysin biotin (RC8) (MBL International Corporation) and avidin-horseradish peroxidase (Av-HRP) conjugate (BD Biosciences Phamingen, San Diego, CA) diluted to 1:1000. After incubation for 1 h, the reactions were developed by coloring with tetramethylbenzidine (TMB) substrate (BD Biosciences Phamingen) for 20 min in the dark. Optical densities were measured at 450/570 nm wave-length by a microplate reader (Sunrise; Tecan, Männedorf, Switzerland). The granulysin concentrations were calculated from the standard curve using granulysin in culture supernatant from Cos7 cell transfected with gene encoding 15 kDa granulysin. The lower detection limit for granulysin is 0.047 ng/ml.

Determination of perforin, granzyme B and IFN-γ

ELISA was used to determine the concentration of plasma perforin and granzyme B (MABTECH AB, Sweden), and IFN-γ (BD Biosciences Phamingen, San Diego, USA) according to the manufacture protocols. The test was done in duplicate. The detection limits of perforin, granzyme B and IFN-γ assays were 78, 8.78 and 4.7 pg/ml, respectively.

Western blot analysis

The isoforms of granulysin with different molecular weight were analyzed by Western blot in 3 patients with HIV/TB coinfection and 3 with TB whose plasma were enough to be tested and one healthy controls (HC). The concentration of proteins with low molecular weight was performed by differential solublilization (DS)-method prior to SDS-PAGE and blotting [27]. Briefly, 36 µl of 7M urea/2M thiourea and 4 µl of 200 mM DTT were added to 20 µl of plasma and then mixed. The solution was dropped into 1.8 ml of purified acetone at 4°C with stirring and centrifuged at 19000 x g at 4°C for 15 min. Four hundred µl of 70% acetonitrile/12mM HCL were added to pellet and stirred at 4°C for 1 h. The solution was centrifuged at 19000 x g at 4°C for 15 min. The collected supernatant was subsequently dried by centrifugal concentrator (TAITEC, Koshigaya, Japan) and dissolved in 80 µl of 0.1% trifuluoroacetic acid. Equal volume of each sample was analyzed by SDS-PAGE, transferred onto 0.2 µm pore-size PVDF membrane (GE Healthcare, Buckinghamshire, UK) and then blotted with goat anti-granulysin polyclonal antibody (R&D, USA). Immunodetection was performed by incubation with HRP conjugated with rabbit anti-goat IgG (1:10000) (Cappel, MP Biomedicals, USA) and developed by ECL-prime detection reagents (GE Healthcare, USA).

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll-metrizonate density gradient centrifugation (Lymphoprep™ tube, AXIS-SHIELD PoC AS, Oslo, Norway) and suspended in cold 10% FBS in RPMI 1640 medium (Gibco, Invitrogen, USA). In this study, the monocytes depleted PBMCs were used instead of PBMCs due to the need of monocytes in the separation study. To remove monocytes, PBMCs were re-suspended in cold separation buffer, incubated with microbeads conjugated to mouse anti-human CD14 monoclonal antibody (Miltenyi Biotec, Germany) and passed through a magnetic cell separation system (MACS, Miltenyi Biotech) on LS

column. The viability of the cells determined by Trypan blue exclusion was ≥95%.

To determine the surface markers of NK cells ($CD56^+CD3^-$), *i*NKT cells ($V\alpha24^+CD3^+$), γδ T cells ($V\gamma9^+V\delta2^+CD3^+$), $CD4^+$ T cells ($CD4^+CD3^+$) and $CD8^+$ T cells ($CD8^+CD3^+$), the monocyte depleted PBMC were directly stained with a combination of fluorochrome-conjugated monoclonal antibodies (IMMUNOTECH, Beckman Coulter Company, France) for 30 min at 4°C in the dark and determined by flow cytometry using four-color immunofluorescent technique. Briefly, different cell populations were determined in $1x10^6$ monocyte depleted PBMCs per tube using the specific antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Texas Red-x (ECD) and phycoerythrin-cyanin5 (PC5) (IMMUNOTECH, Beckman Coulter Company, France) as follows: tube no. 1, FITC-labeled anti-Vγ9 (clone IMMU 360), PE-labeled anti-Vα24 (clone C15), ECD-labeled anti-CD3 (clone UCHT1) and PC5-labeled anti-CD8 (clone B9.11); tube no. 2, FITC-labeled anti-Vδ2 (clone IMMU389), PE-labeled anti-CD56 (clone N901), ECD-labeled anti-CD3 (clone UCHT1), and PC5-labeled anti-CD4 (clone 13B8.2). Mouse isotype IgG1-FITC (clone 679.1 Mc7), IgG1-PE (clone 679.1 Mc7), IgG1-ECD (clone 679.1 Mc7), and IgG1-PC5 (clone 679.1 Mc7) (IMMUNOTECH, Beckman Coulter Company, France) were used as isotype controls. After incubation, the erythrocytes were lysed with 500 µl of optilyse C lysis solution (Beckman Coulter, France) and incubated for 10 min at 4°C in the dark followed by adding 500 µl of PBS. The solutions were processed for flow cytometric analysis by four color detection EPICS® XL™ Flow cytometer (Beckman Coulter, Japan) and the data were analyzed using the XL SYSTEM II™ software. Data were displayed as four-color dot plots.

Statistical analyses

The data were analyzed using SPSS software version 18.0 (SPSS, Inc., Chicago, IL). The concentrations of granulysin, perforin, granzyme-B and IFN-γ in plasma and the surface markers expression on effector cells in each subject group were shown by median and interquartile range. Significant difference between two independent subject groups was compared by Mann-Whitney U test. Wilcoxon Signed Rank test was used to compare plasma granulysin and IFN-γ levels before and after completion of anti-TB therapy. The correlations among circulating granulysin, perforin, granzyme-B, IFN-γ and the number of NK cells, *i*NKT cells, $V\gamma9^+V\delta2^+$ T cells, $CD4^+$ T and $CD8^+$ T cells were analyzed using a Spearman's rank correlation test. P value < 0.05 was considered as statistical significance.

Results

High granulysin and IFN-γ in HIV/TB, but low granulysin and perforin levels in TB

The comparison of circulating granulysin, perforin and granzyme-B among patients with HIV/TB coinfection, TB, HIV+HAART- and HIV+HAART+ were shown in Figure 1 and Table 2. HIV/TB patients had significantly higher granulysin (median = 5.556 ng/ml, ranged 1.744-12.718) than TB patients (median = 0.905 ng/ml, ranged 0.735-1.272) (p = 0.001) and healthy controls (HC) (median=1.322 ng/ml, ranged 0.873-1.591) (p = 0.012) (Fig.1A), while TB patients had significantly lower than those of HC (p = 0.003).

No significance difference in perforin levels was found in HIV/TB coinfection (median = 9418 pg/ml, ranged 4328-11386) and HC (median = 10363 pg/ml, ranged 7388-13430), while the levels in TB (median = 5538 pg/ml, ranged 4749-7519) were significantly lower than HC (p <0.001) (Figure 1B). All study groups had granzyme-B levels as detection limit (Figure 1C). On average, IFN-γ levels were obviously higher in HIV/TB (median = 33.30 pg/ml, ranged 6.215-111.295) than TB patients (median = 11.08 pg/ml, ranged <4.7-25.43) (p < 0.001), and HC (median <4.7 pg/ml, ranged <4.7-15.09) (p <0.001), respectively (Figure 1D).

Three different isoforms of circulating granulysin in patients with HIV/TB coinfection

When the granulysin were analyzed by DS-method and Western blot analysis, three bands were detected corresponding to isoforms with molecular weight of 17 kDa, 15 kDa and 9 kDa in plasma of patients with HIV/TB coinfection (Figure 2).

Small number of *i*NKT cells, $V\gamma9^+V\delta2^+$ T cells and $CD4^+$ T cells but high number of $CD8^+$ T cells in HIV/TB coinfection

Compared to TB, the number of *i*NKT cells, $V\gamma9^+V\delta2^+$ T cells and $CD4^+$ T cells was small but the number of $CD8^+$ T cells was kept high in HIV/TB coinfection as shown in Figure 3 and individual data of HIV/TB patients in Table 2. Significantly higher number of NK cells in patients with TB (median = 1936 cells/µl, ranged 2016-2634) than HIV/TB patients (HIV/TB, median = 787 cells/µl, ranged 321-1303, p = 0.031) was observed.

Table 2. Levels of circulating granulysin, perforin, granzyme-B and IFN-γ and number of effector cells in patients with HIV/TB coinfection before anti-TB therapy.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Granulysin (ng/ml)	3.746	7.365	9.841	1.313	21.35	1.887
Perforin (pg/ml)	10763	10305	13255	8530	1722	5197
Granzyme-B (pg/ml)	41.33	<8.79	26	<8.79	<8.79	<8.79
IFN-γ (pg/ml)	89.54	53.04	6.72	<4.7	13.56	176.56
NK cells (cells/μl)	646	991	2239	346	244	928
*i*NKT cells (cells/μl)	8	2	4	2	1	1
$V\gamma9^+V\delta2^+$ T cells (cells/μl)	44	7	6	53	4	3
$CD4^+$ T cells (cells/μl)	46	198	344	321	94	19
$CD8^+$ T cells (cells/μl)	854	2068	1309	606	181	168

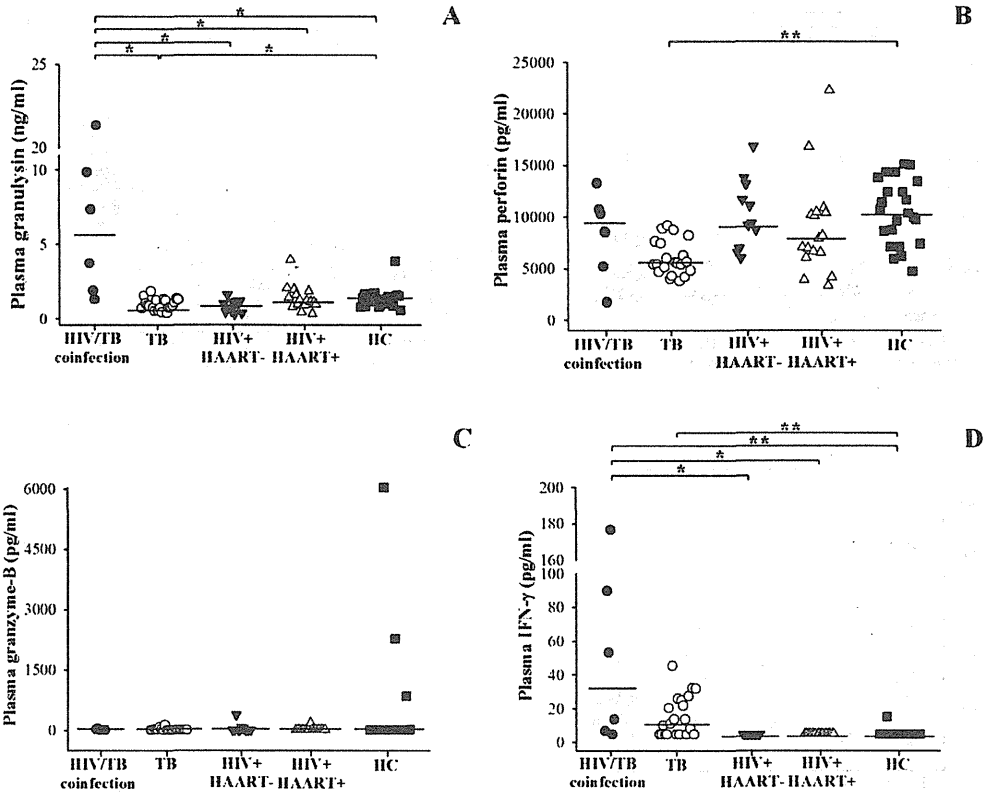

Fig 1. Circulating granulysin (ng/ml) (A), perforin (pg/ml) (B), granzyme-B (pg/ml) (C) and IFN-γ (pg/ml) (D) levels before anti-TB therapy in Thai patients with HIV/TB coinfection and TB in comparison with healthy controls (HC), HIV+HAART- and HIV+HAART+. Each dot represented one individual. A horizontal bar indicated the median of each group. *, p <0.05; **, p <0.01.

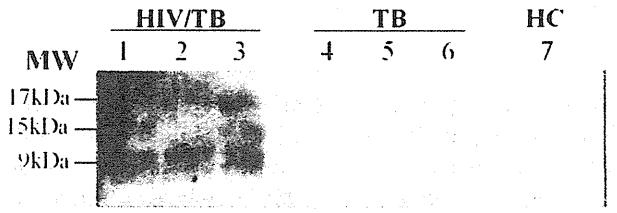

Fig 2. Isoforms of granulysin expression in plasma from Thai patients by Western blot analysis. Lane 1-3, HIV/TB coinfection plasma, 3 bands of ~17kDa, 15kDa and 9kDa isoforms; Lanes: 4-6, TB plasma, 1 band of ~17kDa isoform (Lane 4) and 2 bands of ~15kDa and 9kDa isoforms (Lane 5-6); Lane 7, 2 bands of HC ~15kDa and 9kDa isoforms.

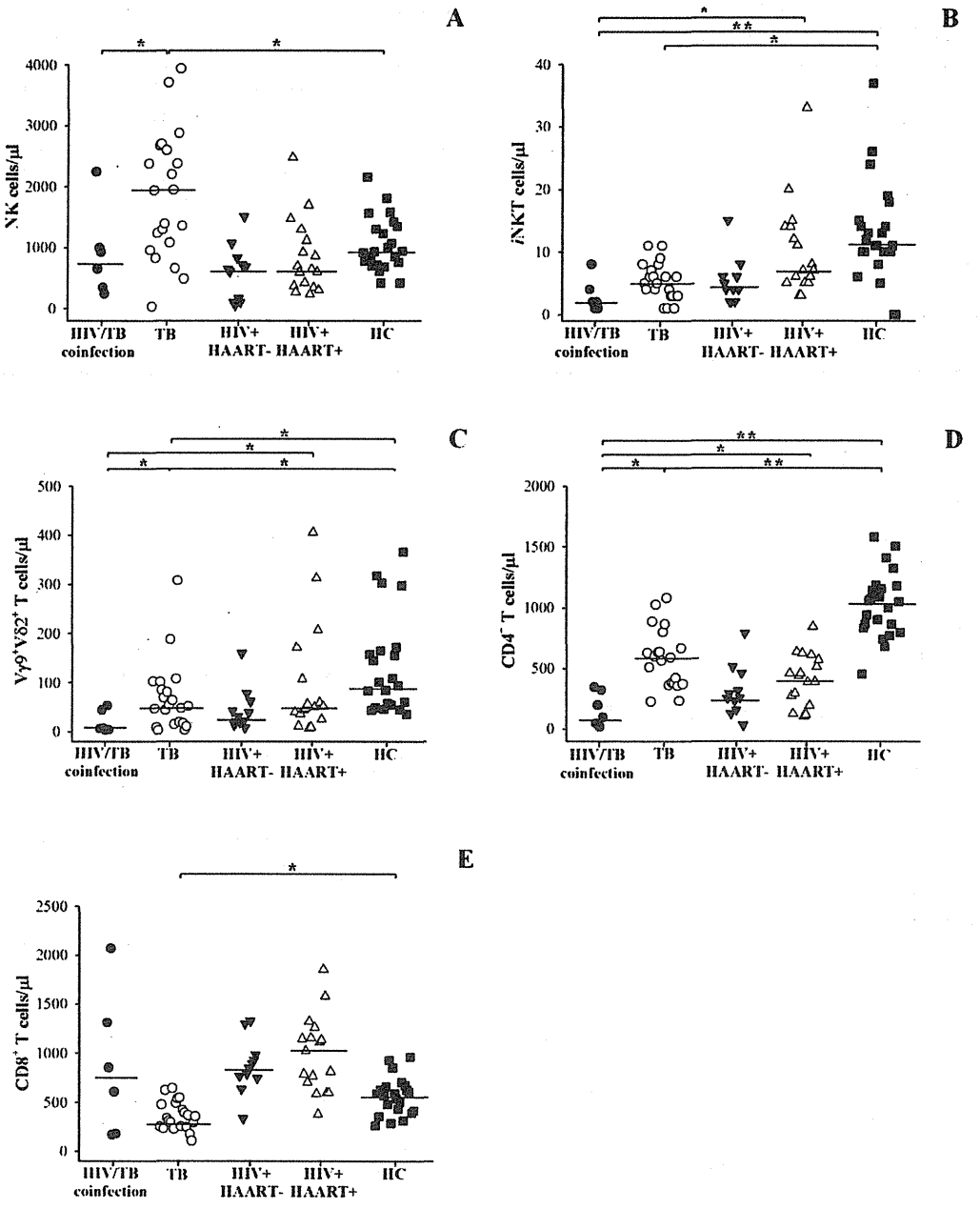

Fig 3. The number of NK cells (A), *i*NKT cells (B), Vγ9$^+$Vδ2$^+$ T cells (C), CD4$^+$ T cells (D) and CD8$^+$ T cells (E) per microliter (μl) in Thai patients with HIV/TB coinfection and TB in comparison with healthy controls (HC), HIV+HAART- and HIV+HAART+ determined by flow cytometric analysis. Each dot represented one individual. A horizontal bar indicated the median of each group. *, p <0.05; **, p <0.01.

Relatively smaller number of *i*NKT cells (median = 2 cells/μl, ranged 1-5) and CD4$^+$ T cells (median = 146 cells/μl, ranged 39-327) were found in HIV/TB than TB patients (median of *i*NKT cells = 5 cells/μl, ranged 3-7, p = 0.029; median of CD4$^+$ T cells = 589 cells/μl, ranged 375-732, p = 0.001). Vγ9$^+$Vδ2$^+$ T cells in HIV/TB coinfection (median = 7 cells/μl, ranged 6-46) also tended to be lower than those in TB (median = 52 cells/μl, ranged 16-94), although it was not significantly different. In addition, small number of CD8$^+$ T cells was remarkable in TB (median = 339 cells/μl, ranged 249-485), whereas the CD8$^+$ T cells in HIV/TB coinfection were kept high in circulation (median = 730 cells/μl, ranged 178-1499).

NK cells, CD8+ T cells, granulysin, perforin and IFN-γ in patients with HIV/TB coinfection and TB

The number of NK, *i*NKT, Vγ9+Vδ2+ T, CD4+ T and CD8+ T cells were correlated with granulysin, perforin, granzyme-B and IFN-γ levels at the time of enrollment. In HIV/TB patients, NK cells and CD8+ T cells were not significantly correlated with granulysin, but both cell types positively correlated with perforin ($p = 0.045$, $r = 0.714$ and $p = 0.036$, $r = 0.771$, respectively). In TB patients, NK cells showed negative correlation, whereas CD8+ T cells was positively correlated with granulysin ($p = 0.011$, $r = -0.499$), $p = 0.049$, $r = 0.398$, respectively). For IFN-γ, a trend to increase in relation to the numbers of NK cells in patients with both HIV/TB coinfection and TB were seen. For the rest, no significant correlations were found among effector molecules and cell populations.

Increased circulating granulysin and decreased IFN-γ levels in HIV/TB coinfection and TB after completion of anti-TB therapy

After 6-9 months of anti-TB therapy, only 3 HIV/TB patients and 13 TB patients could be followed-up when they were considered as treatment success (Figure 4). In patients with HIV/TB coinfection, the granulysin levels after completion of anti-TB therapy (median = 8.535 ng/ml) showed a trend to increase compared to those before therapy (median = 7.365 ng/ml) ($p = 0.208$), although significant difference was not found (Figure 4A). Whereas in TB patients, the granulysin levels after completion of anti-TB therapy (median = 1.861 ng/ml) were significantly higher than those before therapy (median = 1.048 ng/ml) ($p = 0.002$) (Figure 4B).

In contrast, the IFN-γ levels after completion of anti-TB therapy were significantly lower in HIV/TB (median <4.7 pg/ml) (Figure 4C) and TB patients (median <4.7 pg/ml) (Figure 4D) than those before therapy (median = 53.04 pg/ml for HIV/TB, $p = 0.037$ and 10.04 pg/ml for TB, $p = 0.012$).

Clinical profiles in relation to effector molecules and cells in patients with HIV/TB coinfection

Among 6 HIV/TB patients, 4 were considered as cured, whereas 2 could not be followed-up (Table 1). Among these, 3 patients with cured after 6-8 months of TB treatment had high granulysin and perforin levels and high number of NK cells (Table 2). Obviously, they had very high CD8+ T cells. While one patient with cured after 18 months of TB treatment had quite low CD8+ T cells, but high NK cells and even very high IFN-γ levels, but the granulysin and perforin levels were lower than other 3 patients.

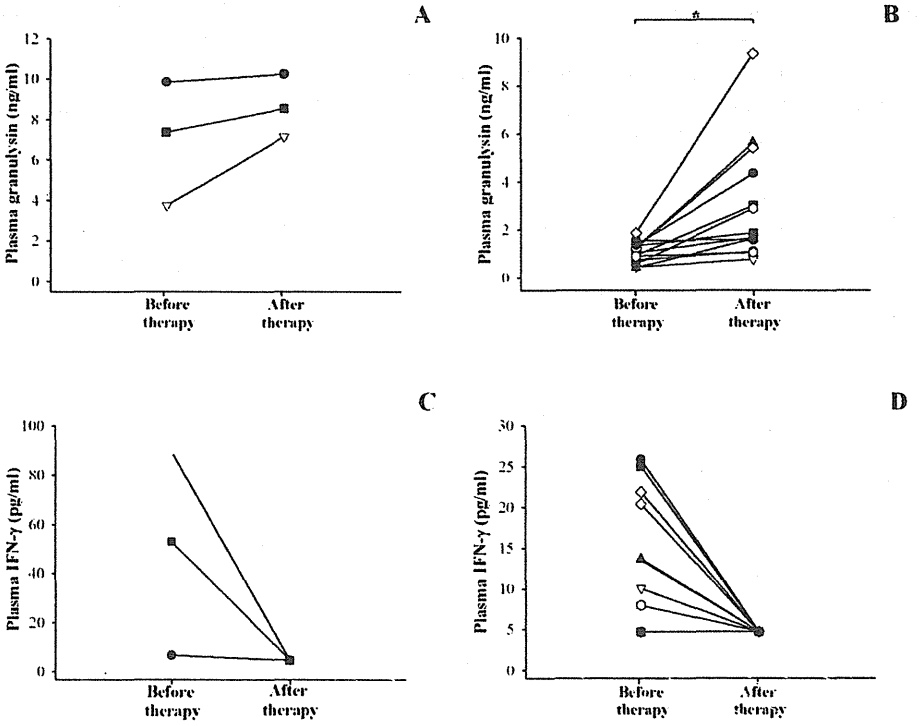

Fig 4. Circulating granulysin (ng/ml) and IFN-γ (pg/ml) in Thai patients with HIV/TB coinfection (A and C) and patients with TB (B and D) before and after completion of anti-TB therapy. Each dot represented one individual. *, $p < 0.05$.

Discussion

In this study, circulating granulysin and other immune molecules, perforin, granzyme-B and IFN-γ in relation to the numbers of NK cells, *i*NKT cells, $V\gamma9^+V\delta2^+$ T cells, $CD4^+$ T and $CD8^+$ T cells in HIV/TB coinfection, TB and other control groups before and after completion of anti-TB therapy were investigated. Before anti-TB therapy, the extremely higher granulysin in HIV/TB coinfection and slightly lower granulysin in active pulmonary TB than HC were noted, and both increased after completion of anti-TB therapy, presumably indicating its protective role of host defense against *Mtb* infection. Low granulysin levels in active TB, may be explained by rapid consumption due to ongoing effector immune response, or reduced during active disease due to the reduction of T cell subsets dedicated to its production [12, 25]. Interestingly, the results of higher granulysin and perforin levels, higher number of NK cells and obviously higher number of $CD8^+$ T cells in HIV/TB coinfection with cured after 6-8 months of TB treatment than the one with cured after 18 months upon therapy, indicated the effective role of NK cells in innate and $CD8^+$ T cells in adaptive immunity. However, the patient with 18 months cured had high NK cells and obviously high IFN-γ levels suggesting the effective role in innate immunity. These results in Thai patients with HIV/TB coinfection were in accordance with the findings in Japanese patients with HIV/TB coinfection which also showed the higher plasma granulysin levels (median = 15.222 ng/ml, ranged 11.372-19.946, n = 19) than healthy individuals (median = 4.869 ng/ml, ranged 2.262-9.983, n = 19) (Figure 5) (Data provided by Dr. Shinichi Oka and his colleagues, AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan on November 1st, 2012). The explanation of the high granulysin in HIV/TB coinfection might be possibly due to (i) exposure to many antigens or with complication occurrences once those patients were concurrently infected with both pathogens as chronic infection which may influence quite high granulysin levels or (ii) the results of immune activation in HIV/TB coinfection or (iii) high number of $CD8^+$ T cells may play a major cell for granulysin production.

In this study, three bands with ~17 kDa, 15 kDa and 9 kDa were identified in plasma from HIV/TB coinfected patients. Though 9 kDa form of granulysin is cleaved from 15 kDa precursor by protease, it is known that 9 kDa form cannot be detected in normal plasma [10]. It is assumed that ~17 kDa band may correspond to full length granulysin with signal sequence. Release of granulysin with signal peptide is questionable, except that from disrupted cells due to necrosis. So far, this is the first demonstration of granulysin in patients with HIV/TB coinfection using DS method to concentrate peptides and low molecular weight proteins in plasma.

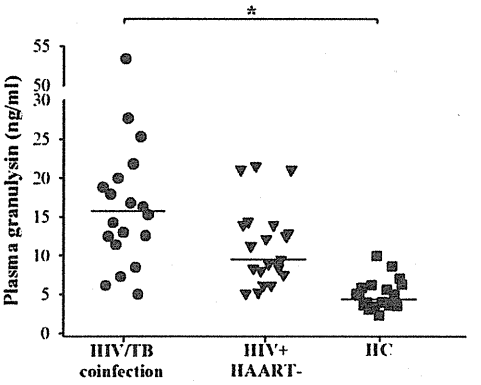

Fig 5. Circulating granulysin (ng/ml) in Japanese patients with HIV/TB coinfection before anti-TB therapy, HIV+HAART- and healthy controls (HC). Each dot represented one individual. A horizontal bar indicated the median of each group. *, $p < 0.05$.

High levels of granulysin coexisted with relatively large number of $CD8^+$ T cells, but not proportional to *i*NKT, $V\gamma9^+V\delta2^+$T, $CD4^+$ T cells and NK cells in patients with HIV/TB coinfection in this study. In fact, the decreased $CD4^+$ T cells in patients with HIV/TB coinfection were previously shown to be associated with failure of granuloma formation to contain *Mtb* infection, thereby causing mycobacterial dissemination [28]. Furthermore, activation of HIV-1 by *Mtb* components may be particularly important in viral expansion at the times when *Mtb* growth becomes exponential, and TB overwhelms host defenses [29]. Compared to HIV/TB coinfection, the significantly decreased *i*NKT, $V\gamma9^+V\delta2^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells and increased NK cells in patients with TB before anti-TB therapy indicated the possibility that granulysin may be compromised by T cell subset with significantly correlated with $CD8^+$ T cells. Obviously, increased NK cells were correlated with decreased granulysin, suggesting that it may not be released from NK cells, or granulysin inside the cells does not degranulated later upon activation. However, it could not be assured that (i) how much granulysin contained intracellularly and released to extracellular space since only free granulysin in circulation was measured in this study, (ii) how frequent expression of granulysin was induced in accumulated immune cells including $CD8^+$ T cells at the TB-affected

sites, particularly in patients with HIV/TB coinfection, (iii) the exact duration of being infected with *Mtb* until development to active disease with sputum AFB positive, (iv) how much granulysin was released in latent and early stage of active TB, and (v) varied granulysin levels may depend upon the nutrition and health status in individuals. Similarly, low perforin in TB were seen. The persistence of clinical disease was evidenced to be associated with deficient expression of perforin and granulysin at the local site of TB infection [18]. Although significant infiltration of $CD4^+$ and $CD8^+$ T cells was evidenced in TB lesions in patients with persistent inflammation [18], however, the levels of either perforin or granulysin remained low in TB lesions including severely impaired expression of these cytolytic effector molecules inside the distinct granules [18]. In the present study, either NK cells or $CD8^+$ T cells were significantly correlated with the elevation of perforin in patients with HIV/TB coinfection but not in TB, indicating their potential functions in HIV/TB coinfection, as previously shown in individuals chronically infected with HIV-1 who have increased extracellular perforin levels compared with uninfected individuals, while the impaired functional activity of CTLs and NK cells during HIV-1 infection has been attributed to the decreased intracellular levels of perforin and granzyme B [30]. It is suggested that the induction of perforin has a distinct pathway from that of granulysin in HIV/TB coinfection in this study. However, in TB, no differences in granzyme B levels were found which is supported by similar results in slow or fast TB responders upon TB treatment at any time points and healthy individuals with PPD positive [31].

In contrast to granulysin, perforin and granzyme-B, the elevated circulating IFN-γ seen in patients with both HIV/TB coinfection and TB before anti-TB therapy which decreased after completion of therapy inferred a role of IFN-γ in effective immunity against *Mtb* infection. The results were similar to the previous report on significantly higher plasma IFN-γ levels in patients with active pulmonary TB than healthy individuals which decreased after treatment, suggesting that the levels may result from local production and spill-over of IFN-γ from activated lymphocytes sequestered at the site of *Mtb* infection [22, 24, 32]. In human and mouse models, IFN-γ is evidenced to be normally synthesized from $CD4^+$ T cells activated upon recognition of *Mtb* antigen on antigen presenting cells [22] as well as by *Mtb* antigens specific $CD8^+$ T cells [33]. Although IFN-γ producing $CD4^+$ T cells of Th1 type is of major importance, however, other T cells notably $CD8^+$ T cells and perhaps γδ T cells or CD1-restricted T cells participate in immune function as well [34]. However, increased $CD4^+$ and decreased $CD8^+$ T cells in TB in this study conversed to HIV/TB coinfection. Elevated IFN-γ levels in HIV/TB coinfection might be possibly due to the persistence of immune activation and chronically HIV associated TB. In addition, *Mtb* infection may support the HIV-1 replication and dissemination through dysregulation of host cytokines, chemokines and their receptors [29]. HIV/TB coinfection could inhibit cell mediated responses to *Mtb* through interruption of IL-2 signaling as well [35]. The deleterious effects of HIV infection in $CD4^+$ T cells impair immune function as resulting in a failure to contain mycobacterial infection and restrict the replication of the microbe [36].

In this study, IFN-γ had a trend to increase along with NK cells in patients with both HIV/TB coinfection and TB, suggesting the possible production of IFN-γ from NK cells during initiation against *Mtb* infection which high NK cells were shown. It is also possible as recently shown that NK cells secrete IFN-γ which stimulates monocytes to produce IL-15 and IL-18, which in turn facilitates expansion of $CD8^+$ T cells producing IFN-γ in response to *Mtb*-infected monocytes [37]. Since NK cells produce IFN-γ in early *Mtb* infection [38], therefore, this pathway is likely to be important in facilitating expansion of $CD8^+$ T cells during immune response to *Mtb in vivo* [37].

In conclusion, the alteration of circulating granulysin, perforin and IFN-γ has potential function in host immune response in TB and HIV/TB coinfection. Circulating granulysin and perforin levels in TB were lower than healthy controls, whereas the granulysin levels in HIV/TB coinfection were much higher than in any other disease groups. Increased granulysin and decreased IFN-γ levels in HIV/TB coinfection and TB after completion of anti-TB therapy were noted. Slightly high perforin levels in HIV/TB coinfection indicated the immune activation in TB associated with HIV infection. Three distinct isoforms with ~17kDa, 15kDa and 9kDa of granulysin were recognized in plasma of HIV/TB coinfection. The number of $CD8^+$ T cells kept high but NK cells and other possible cellular sources of granulysin were decreased in HIV/TB coinfection, which in contrast to what seen in TB in which low $CD8^+$ T cells but high NK cells were found, suggesting their different sources of granulysin, which in turn, play a crucial role in host defense against tuberculosis and in association with HIV infection. This is the first demonstration so far of granulysin in HIV/TB coinfection.

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Abbreviations

CFP: culture filtrate protein; CTL: cytotoxic T lymphocyte; ELISA: enzyme-linked immunosorbent assay; ESAT: early secreted antigenic target; HAART: highly active antiretroviral therapy; HIV: human immunodeficiency virus; IFN-γ: interferon gamma; IL: interleukin; *i*NKT: invariant NKT; MDR: multi-drugs resistance; *Mtb*: *Mycobacterium tuberculosis*; NK: natural killer; PBMCs: peripheral blood mononuclear cells; TB: tuberculosis; Th1: T-helper type 1; TLR: toll-like receptor; TNF: tumor necrosis factor; XDR: extensively drugs resistance

Competing Interests

The authors have declared that no competing interest exists.

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Simple Multiplex PCR Assay for Identification of Beijing Family *Mycobacterium tuberculosis* Isolates with a Lineage-Specific Mutation in *Rv0679c*

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The Beijing genotype of *Mycobacterium tuberculosis* is known to be a worldwide epidemic clade. It is suggested to be a possibly resistant clone against BCG vaccination and is also suggested to be highly pathogenic and prone to becoming drug resistant. Thus, monitoring the prevalence of this lineage seems to be important for the proper control of tuberculosis. The Rv0679c protein of *M. tuberculosis* has been predicted to be one of the outer membrane proteins and is suggested to contribute to host cell invasion. Here, we conducted a sequence analysis of the *Rv0679c* gene using clinical isolates and found that a single nucleotide polymorphism, C to G at position 426, can be observed only in the isolates that are identified as members of the Beijing genotype family. Here, we developed a simple multiplex PCR assay to detect this point mutation and applied it to 619 clinical isolates. The method successfully distinguished Beijing lineage clones from non-Beijing strains with 100% accuracy. This simple, quick, and cost-effective multiplex PCR assay can be used for a survey or for monitoring the prevalence of Beijing genotype *M. tuberculosis* strains.

The *Mycobacterium tuberculosis* Beijing genotype, first identified by van Soolingen et al. (1), is known to be a worldwide epidemic clade (2–4). Its possible resistance to BCG vaccination, in addition to its tendency to have a multidrug-resistant (MDR) phenotype, might give a selective advantage to the wide geographic distribution of the Beijing genotype strains (3, 5–7). Although some of the Beijing genotype strains show hypervirulence in animal infection models (7–9), neither the virulence factor nor the phenotypically specific factor of this lineage has been elucidated. The origin of the Beijing lineage is thought to be east Asia, where the prevalence of this clade is from around 40% to >90% (1, 3, 4, 10–13). However, in some other global areas, i.e., countries in the former Soviet Union and South Africa, the prevalence of the Beijing lineage has increased markedly in a short period, and some increases were suggested to be related to MDR (4, 11, 14). In those areas, higher clonality of the circulating strains was suggested, and most were categorized as being in the modern or typical Beijing clade, which is defined as a strain having one or two IS*6110* insertions in the noise transfer function (NTF) chromosomal region (11, 15). On the other hand, a higher variety of strains can be observed in east Asian countries. Especially in Japan and Korea, the majority of the strains belong to another cluster called the ancient or atypical Beijing clade (12, 16). Details regarding the higher pathogenicity of the Beijing lineage are controversial. Some studies have suggested that the modern Beijing clade is more prone to be pathogenic, tends to be drug resistant, and is likely able to escape from BCG vaccination (4, 8, 11, 14); however, some of the ancient Beijing clones were also shown to have higher pathogenicity (17) or a tendency toward acquiring drug resistance (16).

Since Beijing lineage prevalence has a great impact on the tuberculosis (TB) control program, several methods to distinguish this clade have been developed. First, van Soolingen et al. (1) identified this clade by its specific IS*6110* restriction fragment length polymorphism (RFLP) signatures. Soon after, these strains were shown to have a specific spoligotype pattern lacking spacer numbers 1 to 34, and this has been proposed as the definition of the clade (18, 19), since IS*6110* RFLP genotyping is time-consuming, and comparing results between laboratories is difficult. The deletion of spacers observed in the Beijing spoligotype is caused by the insertion of IS*6110* in the direct repeat (DR) region (18). Since this typical spoligotype pattern has become a specific marker of the Beijing genotype, some PCR methods to detect this specific deletion, named region of difference 207 (RD207), have been developed (20–22). In addition to RD207, another deleted region named RD105 was also shown to be a good marker for discrimination of the Beijing genotype, although this deletion is common for all the east Asian lineages, including the non-Beijing strains (10, 23); however, most of these published detection methods require expensive real-time PCR equipment and high-cost reagents (24). The conventional PCR assay targeting RD207 still seems to be at a disadvantage, since it relies on an unstable inser-

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tion sequence that is likely to be a target of homologous recombination.

Instead of unstable repetitive structures, single nucleotide polymorphisms (SNPs) were recently considered to be a robust target for defining the accurate position of a strain on the phylogenetic tree, since horizontal gene transfer or gene recombination between different strains is rare in the *M. tuberculosis* complex (MTC) (12, 24, 25). Filliol et al. (26) drew phylogenetic trees of the MTC using several typing methods and showed that the dendrogram drawn with SNPs most accurately reflected the true evolution of the MTC. Some of those SNPs are suggested to be specific to the Beijing or east Asian lineages. In a search for membrane proteins that are suitable for vaccine antigens and/or are targets for the specific detection of the MTC, we found a candidate protein encoded by the *Rv0679c* gene. This protein was expressed on the cell surface as a lipoarabinomannan-associated protein (27, 28), and the coding sequence has an SNP that seems to be specific to the Beijing clade. In this study, we confirmed the lineage specificity of this SNP and developed a simple and low-cost multiplex PCR assay to distinguish the Beijing lineage strains.

MATERIALS AND METHODS

Preparation of genomic DNA from *M. tuberculosis* isolates. *M. tuberculosis* was isolated from the sputa or other clinical specimens of patients by conventional procedures using *N*-acetyl-L-cysteine (NALC)–NaOH. A total of 619 isolates obtained in Japan (n = 145), Bangladesh (n = 122), Nepal (n = 110), Myanmar (n = 198), and China (Heilongjiang Province, n = 44) were used in this study. Some of these isolates were the same as those in previous studies, and the details are described elsewhere (13, 29–31). Colonies grown on egg-based medium (either Ogawa or Löwenstein-Jensen medium) were resuspended in distilled water and boiled for 20 min, and the supernatant was used in the Bangladeshi and Myanmar samples. In the Japanese and Nepalese samples, colonies were suspended in 0.5 ml of 10 mM Tris-HCl, 1 mM EDTA (Tris-EDTA [TE] buffer [pH 8]), and 0.5 ml chloroform; 0.5 g glass beads of 0.17-mm diameter was added; and they were disrupted with a bead beater (MicroSmash; Tomy Seiko Co. Ltd., Tokyo, Japan). After centrifugation at 10,000 × *g* for 5 min, DNA in the supernatant was precipitated by ethanol, and the precipitated genomic DNA was resuspended in TE buffer for further use. In China, bacteria grown in a BACTEC *Mycobacterium* growth indicator tube (MGIT) (Becton, Dickinson and Company, Franklin Lakes, NJ) were used, and DNA was extracted by lysozymes and the phenol-chloroform method (13). All the DNA samples extracted in each country were brought to Japan, and the following steps were carried out in the Hokkaido University Research Center for Zoonosis Control. To determine the specificity of the method, DNAs extracted from five reference MTC strains (i.e., *M. tuberculosis* H37Rv, *Mycobacterium africanum* ATCC 25420, *Mycobacterium orygis* Z0001, *Mycobacterium microti* TC 89, and *Mycobacterium bovis* BCG Tokyo 172) and 30 nontuberculous mycobacterial (NTM) species, including *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii*, were used.

Gene sequencing and comparison. A subset of 197 *M. tuberculosis* samples, 68 from Japan, 92 from Bangladesh, and 37 from Nepal, were chosen from the total 619 clinical isolates, and the *Rv0679c* gene fragment was amplified by PCR. The PCR mixture contained GoTaq PCR buffer (Promega Co., Madison, WI), 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.3 μM each primers og0001 and og0002 (Table 1), 0.5 M betaine, 1 ng genomic DNA from *M. tuberculosis*, and 0.5 units of GoTaq polymerase. Amplification was carried out by applying 35 cycles of denaturation at 95°C for 10 s, annealing at 57°C for 10 s, polymerase reaction mixture at 72°C for 40 s, and a final extension at 72°C for 5 min. The amplified DNA fragment was subjected to sequence analysis with BigDye Terminator v3.1 (Life Technologies Co., Carlsbad, CA) reagents by a sequencer, the 3130 genetic analyzer (Life Technologies Co.), according to the manufacturer's protocol. The *Rv0679c* sequence was also compared with those of 80 whole-genome sequenced MTC strains registered in the GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) or TB (http://genome.tbdb.org/annotation/genome/tbdb/MultiHome.html) (32) databases by the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Genotyping. The spoligotype of *M. tuberculosis* clinical isolates was determined as described previously (33). Briefly, the DR region was amplified with a primer pair, and the PCR products were hybridized to a set of 43 spacer-specific oligonucleotide probes, which were covalently bound to the membrane. The spoligo-international type (SIT) was determined by comparing spoligotypes against the international spoligotyping database (SpolDB4) (3).

The detection of an RD105 deletion was performed by multiplex PCR in Beijing clones and by conventional PCR in east Asian strains other than those of the Beijing type, since the deletion pattern is different between those two groups (10). The reaction mixture consisted of GoTaq PCR buffer (Promega), 0.2 mM each dNTP, 0.3 μM (each) two or three primers (Table 1), 0.5 M betaine, 1 μl extracted DNA sample, and 0.5 units of GoTaq polymerase. The target was amplified by 35 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 40 s, with a final extension at 72°C for 5 min. RD207 deletion was detected by two PCR assays described by Warren et al. (22), and TbD1 was detected by PCR using the Huard et al. (25) protocol (Table 1). The amplified DNA fragment was subjected to agarose gel electrophoresis with ethidium bromide (EtBr) to see the size of the band under a UV transilluminator.

The multilocus sequence type (MLST) was determined with 9 SNPs, which were described by Filliol et al. (26) and were selected for Beijing subtyping by Iwamoto et al. (16). Each locus was amplified with a primer pair (Table 1), and the product was subjected to sequencing. SNPs were detected by comparing the sequences with those of H37Rv (34). The sequence type (ST) was identified according to Filliol et al. (26).

Beijing lineage identification by multiplex PCR. Multiplex PCR for the identification of the Beijing lineage was performed under the following conditions. The PCR mixture, in a final volume of 15 μl, contained 1× PCR buffer (1.5 mM Mg; TaKaRa Bio, Inc., Shiga, Japan), 0.5 μl dNTP solution mix (10 mM each dNTP; New England BioLabs, Inc., Ipswich, MA), 0.5 μl each of Fw and R1 primers, 0.2 μl R2 primer (primer solutions in 10 μM; Table 1), 1.5 μl of 5 M betaine, 0.45 μl of 25 mM $MgCl_2$ (to make a final Mg concentration of 2.25 mM), 1 ng of sample DNA, and 0.5 units of TaKaRa Hot Start *Taq* polymerase (TaKaRa). Amplification was carried out with the first denaturation at 95°C for 1 min followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 66°C for 10 s, extension at 72°C for 15 s, and the final extension at 72°C for 3 min. The amplicon was subjected to electrophoresis in a 2% agarose gel that included EtBr. DNA samples extracted from the isolate BCG Tokyo 172 and a well-characterized clinical isolate (Beijing OM-9) were used as controls for the non-Beijing and Beijing banding patterns, respectively. Sensitivity was determined with serially diluted genomic DNA obtained from these BCG and Beijing control strains. A specificity study was performed with genomic DNA samples (2 ng/μl each) from the MTC and NTM strains described above.

RESULTS

Spoligotyping and MLST. A total of 619 clinical isolates were subjected to spoligotyping, and 393 were identified as being in the Beijing lineage and 226 as a non-Beijing group (Table 2). The non-Beijing group consisted of a variety of strains belonging to the following lineages: east African-Indian (EAI), central Asian (CAS), Latin American Mediterranean (LAM), Haarlem, S, T, X, and non-Beijing east Asian (3). Ninety-four of the Beijing isolates were subjected to MLST analysis and were subtyped into 8 sequence-type classes, namely, ST26, ST3, STK, ST25, ST19, ST10, ST22, and ST8, which are listed in evolutional order from ancient to modern Beijing types (16, 26).

TABLE 1 Primers used in the study

Target	Primer name	Nucleotide sequence	Purpose	Reference
Rv0679c	og0001	CCGGGAACTAGGAATGGTAA	Sequencing	This study
	og0002	AGCAACCTCGCAATCTGAC	Sequencing	This study
	ON-1002 (Fw)	GTCACTGAACGTGGCCGGCTC	Multiplex PCR for Beijing type identification	This study
	ON-1258 (R1)[a]	TCGGTCACCGTTTTTGTAGGTGACCGTC	Multiplex PCR for Beijing type identification	This study
	ON-1127 (R2)	AGCAACCTCGCAATCTGACC	Multiplex PCR for Beijing type identification	This study
RD105	RD105-F (−239~−218)	GGAAAGCAACATACACACCACG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-R	AGGCCGCATAGTCACGGTCG	Multiplex PCR for east Asian type determination[b]	This study
	RD105-M (+304~323)	TCCTGGGTGCCGAACAAGTG	Multiplex PCR for east Asian type determination[b]	This study
	RD105EA-F (−80~−60)	TCGGACCCGATGGCTTCGGTG	PCR for east Asian type determination[c]	This study
	RD105EA-R (61~42)	TGATCACGGTTCGCCCGCAG	PCR for east Asian type determination[c]	This study
RD207	RD207-1F (Warren)	TTCAACCATCGCCGCCTCTAC	PCR for Beijing type identification (set 1)	22
	RD207-1R (Warren)	CACCCTCTACTCTGCGCTTTG	PCR for Beijing type identification (set 1)	22
	RD207-2F (Warren)	ACCGAGCTGATCAAACCCG	PCR for Beijing type identification (set 2)	22
	RD207-2R (Warren)	ATGGCACGGCCGACCTGAATGAACC	PCR for Beijing type identification (set 2)	22
TbD1	TbD1F	CGTTCAACCCCAAACAGGTA	PCR for ancestral *M. tuberculosis* determination	25
	TbD1R	AATCGAACTCGTGGAACACC	PCR for ancestral *M. tuberculosis* determination	25
797736[d]	Beijing ST-1F	GACGGCCGAATCTGACACTG	MLST for Beijing lineage	This study
	Beijing ST-1R	CCATTCCGGGTGGTCACTG	MLST for Beijing lineage	This study
909164[d]	Beijing ST-2F	CGTCGAGCTCCCACTTCTTG	MLST for Beijing lineage	This study
	Beijing ST-2R	TCGTCGAAGTGGACGAGGAC	MLST for Beijing lineage	This study
1477596[d]	Beijing ST-3F	GTCGACAGCGCCAGAAAATG	MLST for Beijing lineage	This study
	Beijing ST-3R	GCTCCTATGCCACCCAGCAC	MLST for Beijing lineage	This study
1692067[d]	Beijing ST-5F	GATTGGCAACTGGCAACAGG	MLST for Beijing lineage	This study
	Beijing ST-5R	TGGCCGTTTCAGATAGCACAC	MLST for Beijing lineage	This study
1892015[d]	Beijing ST-6F	GCTGCACATCATGGGTTGG	MLST for Beijing lineage	This study
	Beijing ST-6R	GTATCGAGGCCGACGAAAGG	MLST for Beijing lineage	This study
2376133[d]	Beijing ST-7F	TCTTGCGACCCGATGTGAAC	MLST for Beijing lineage	This study
	Beijing ST-7R	GAGCGCAACATGGGTGAGTC	MLST for Beijing lineage	This study
2532614[d]	Beijing ST-8F	CCCTTTTCTGCTCGGACACG	MLST for Beijing lineage	This study
	Beijing ST-8R	GATCGACCTTCGTGCACTGG	MLST for Beijing lineage	This study
2825579[d]	Beijing ST-9F	CCTTGGAGCGCAACAAGATG	MLST for Beijing lineage	This study
	Beijing ST-9R	CTGGCCGGACGATTTTGAAG	MLST for Beijing lineage	This study
4137829[d]	Beijing ST-10F	CGTCGCTGCAATTGTCTGG	MLST for Beijing lineage	This study
	Beijing ST-10R	GGACGCAGTCGCAACAGTTC	MLST for Beijing lineage	This study

[a] Beijing-type specific mutation-detection primer. Underlined 2-base sequences at the 5′ end are not complementary sequences.
[b] This assay was used for Beijing genotype strains.
[c] This assay was used for non-Beijing genotype strains.
[d] This SNP nucleotide position on the *H37Rv* genome is according to references 26 and 34.

Sequence analysis of the *Rv0679c* gene of *M. tuberculosis* isolates. Nucleotide sequences of the full-length *Rv0679c* gene obtained from 197 clinical *M. tuberculosis* isolates collected in Japan, Bangladesh, and Nepal were compared with the *Rv0679c* sequence in *M. tuberculosis* H37Rv (34). Only a single nucleotide difference of cytosine to guanine at position 426, which leads to an amino acid change at codon 142 from Asn (AAC) to Lys (AAG), was detected in 87 isolates, all of which were identified as being in the Beijing lineage by spoligotyping and, supportively, by RD207 PCR (22) (data not shown). One Bangladeshi isolate showed a mixed peak of C and G at position 426 and was revealed as a mixed culture of Beijing and another strain by RD105 and RD207 detection PCR (Table 2). None of the non-Beijing isolates had the mutation, and vice versa. In public databases, 14 strains reported from several countries were revealed to have this mutation, and all were confirmed as being in the Beijing lineage by checking for the RD207 deletion *in silico* (18). None of the other 66 MTC strains, which were determined to be non-Beijing, had this mutation. The 498-bp *Rv0679c* sequence was well conserved among the MTC strains, and the following three strains in the database showed alterations: *M. tuberculosis* strains C and T17 and *Mycobacterium canettii* CIPT 140010059.