41 oligonucleotide probes were designed to cover mutations in the regions of *katG* (35 probes), *furA* (2 probes), *fabG1-inhA promoter* (2 probes), and *fabG1* (2 probes). The details and performances of these tests have been reported in the references described above [12,15-17].

Restriction fragment length polymorphism (RFLP)

Experimental procedures for bacterial growth, DNA extraction, DNA digestion with *Pvu*II (Takara Bio Inc. Otsu, Japan), electrophoresis on a 1% agarose gel, and Southern blotting and membrane hybridization with a peroxidase-labeled 245-bp IS*6110* probe were performed using standardized methods [18] with slight modifications [14]. The hybridized probe was visualized with an ECL detection system (Amersham Biosciences). Fingerprinting images were analyzed with Fingerprinting™ II software (Bio-Rad Laboratories, Inc., Hercules, CA), and percent similarity among the isolates was determined according to the supplier's instructions. To classify strains into the same family on the basis of their genotyping profiles, a similarity index of 70%, slightly more stringent than 65% used in a previous report [19] was chosen in this study. Normalization was performed using molecular weight standards and the IS*6110*-fingerprinting patterns of two isolates run on each gel. Isolates with fewer than five IS*6110* copies were excluded from the cluster analysis.

DNA sequencing of INH resistance-related genes

The *furA-katG* operon and its upstream region were amplified by PCR using the specific primers and conditions described previously [16]. The primers used were −129furA (5′-GCTCATCGGAACATACGAAG-3′) and katG +50 (5′-GTGCTGCGGCGGGTTGTGGTTGATCGGCGG-3′). The *fabG1-inhA* operon and the upstream region of the *fabG1-inhA*operon were also amplified using previously reported primers [16]: −200fabG1 (5′-TTCGTA GGGCGTCAATACAC-3′) and inhA +40 (5′-CCGAA CGACAGCAGCAGGAC-3′). PCR products were used as templates for direct DNA sequencing. To detect mutations, DNA sequences were compared with those of H37Rv using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan).

Statistical analysis

Chi-square tests were used to compare proportions between two groups. Kappa statistics were used to determine the agreement between two tests. The following guidelines were used to interpret kappa coefficients: <0, poor agreement; 0–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; and 0.81–1.00, very good. *P* values <0.05 were considered statistically significant, unless otherwise noted. The Bonferroni correction was used when comparing the results for multiple drugs. JMP version 9.0.0 (SAS Institute, Inc., Cary, NC, USA) statistics software was used for analysis.

Results

Clinical isolates from AFB-positive sputum

Clinical isolates were collected from 346 consecutive previously-untreated patients with AFB-positive active TB in Hanoi, Vietnam. Of these patients, 270 (78.0%) were male, and their median age was 38 years (range: 17–84 years). Coinfection with HIV was found in 31 patients (9.0%).

Drug susceptibility profiles of MTB isolates

Sputum samples from 346 smear-positive patients were cultured, from which 339 MTB isolates (98.0%) were obtained. DST information for seven patients was not available. Among these, 127 (37.5%) were resistant to at least one of the four drugs tested; 93 (27.4%), 19 (5.6%), 96 (28.3%), and 11 (3.2%) isolates were resistant to INH, RMP, SM, and EMB, respectively; and 17 (5.0%) were multidrug-resistant (MDR) strains (Table 1). The PZase assay revealed that 8 (2.4%) of the 339 isolates were negative for PZase and were considered to be resistant to PZA (data not shown).

Eighty-eight INH-sensitive and 64 INH-resistant strains were randomly selected. LiPAs for RMP and INH were performed to confirm consistency with the results of culture-based DST and to identify profiles of genetic mutations associated with resistance to these drugs. Agreement between LiPAs and conventional DST was good or very good (kappa = 0.80 for RMP and kappa = 0.84 for INH 0.2 μg/mL; table not shown).

Mutations for RMP included *rpoB*:H526D, H526Y, S526D, and S531L. Only 11 isolates had one of these mutations, and 4 undefined RMP mutations were also observed in our study.

Mutations for INH were mostly *katG*:S315T (data not shown). This was more widely confirmed by subsequent DNA sequencing around the *katG* and *inh* genes (Additional file 1). LiPA for PZA was compared

Table 1 Drug susceptibility profiles of MTB isolates from previously-untreated patients

Patterns		n	% (95% CI)
Sensitive with all drugs		212	62.5 (57.3 - 67.5)
Any resistance	Subtotal	127	37.5 (32.5 - 42.7)
	INH	93	27.4 (23.0 - 32.4)
	RMP	19	5.6 (3.6 - 8.6)
	SM	96	28.3 (23.8 - 33.3)
	EMB	11	3.2 (1.8 - 5.7)
MDR		17	5.0 (3.2 - 7.9)

N = 339 isolates.
INH concentration (0.2 μg/mL).

with the results of the PZase assay; their consistency was moderately high (kappa = 0.55; table not shown). LiPA for FQ identified only 1 mutated strain carrying *gyrA*:A90V.

IS6110-probed RFLP and drug susceptibility

Sufficient DNA was extracted from 317 of the 339 isolates. Their IS*6110*-probed fingerprinting patterns are shown in Figure 1. Four clusters were identified when defined by >70% similarity in their RFLP patterns. These clusters accounted for 146 (46.1%) of the total isolates: 57 (18.0%) in cluster I; 70 (22.1%) in cluster II; 12 (3.8%) in cluster III; and 7 (2.2%) in cluster IV. Each cluster consisted of isolates collected from at least three districts in the city of Hanoi (data not shown).

Within these four clusters, 55 and 49 (37.7% and 33.6%) of the isolates were resistant to INH at 0.2 μg/mL and 1.0 μg/mL, respectively; these proportions were significantly higher than the 19.9% and 17.0% among the non-clustered isolates ($P = 0.0004$ and $P = 0.0006$,

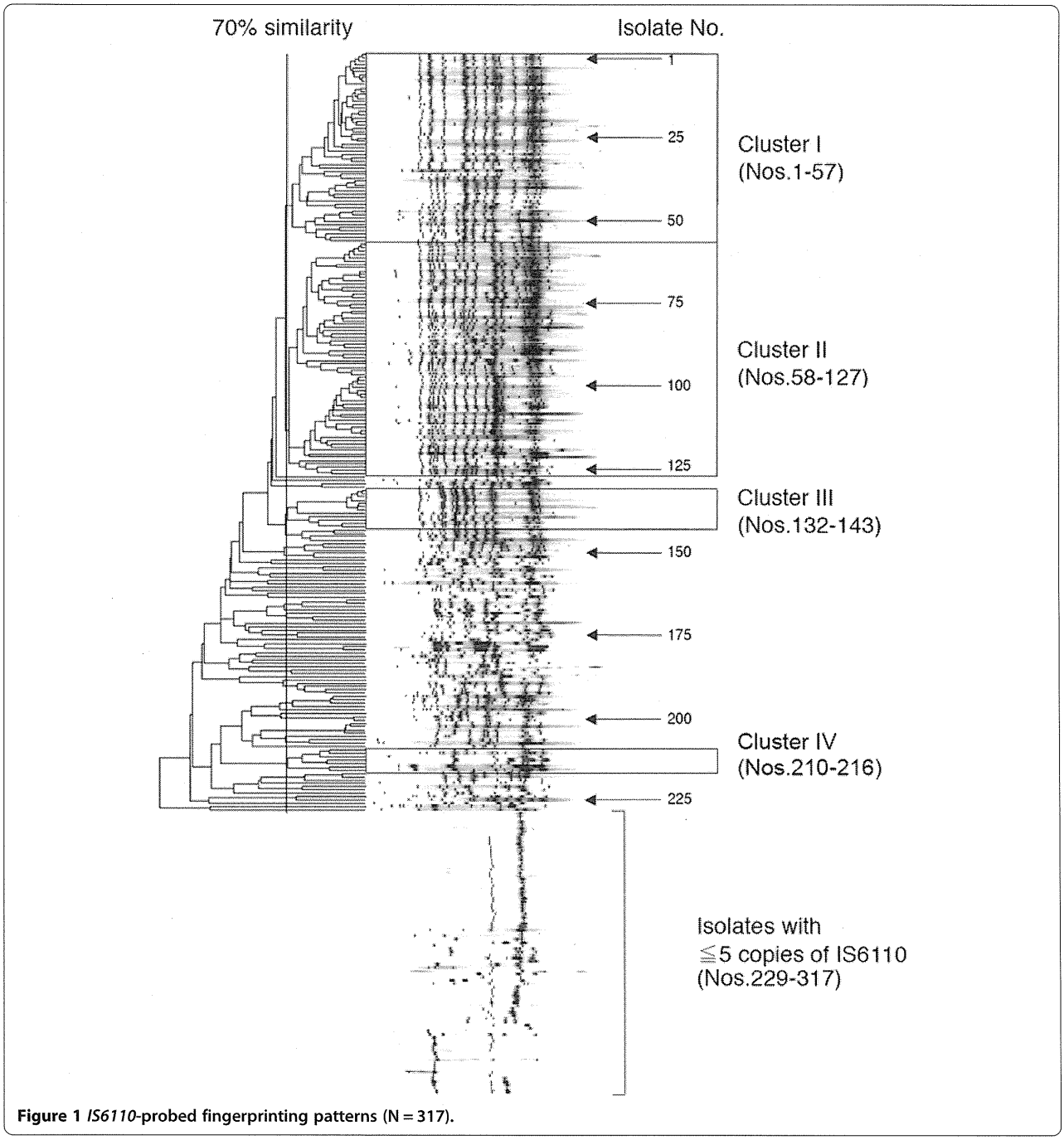

Figure 1 *IS6110*-probed fingerprinting patterns (N = 317).

respectively; Table 2). In fact, in the small clusters III and IV, the majority of the isolates were highly resistant to INH at 1.0 μg/mL (Additional file 1: Table S1). Although the proportions of isolates in these clusters that were resistant to RMP and other drugs also tended to be higher than those of non-clustered isolates, these differences were not significant, based on multiple comparisons statistical testing (Table 2). In addition, there were no significant associations between the clusters and specific *rpoB* mutations (data not shown).

Gene mutations responsible for INH resistance

Among all the INH-resistant isolates noted above, possible mutations within the *furA-katG* operon, the *fabG1-inhA* operon, and their upstream regions were investigated by PCR-based nucleotide sequencing (Additional file 1: Table S1).

Of 89 INH (0.2 μg/mL)-resistant isolates, 76 (85.4%) carried a g944c mutation (AGC to ACC) that caused an S315T amino acid substitution in the *katG* gene and 70 (92.1%) of the isolates that carried the g944c mutation were highly resistant to INH at 1.0 μg/mL. Furthermore, g204c mutations in the *furA* operon were detected in 13.6% of all the isolates and were frequently accompanied by the g944c mutation in the *katG* gene, although this variant itself was not directly associated with culture-based INH resistance ($P = 0.072$; table not shown). In the upstream region of the *fabG1-inhA* operon, c-15t was observed in 7 isolates, and minor variations with <1% were observed within the *fabG1-inhA* operon. The combination of a g944c mutation in the *katG* gene with c-15t in the *inhA* promoter was observed only in 1 isolate.

Discussion

We investigated the drug susceptibility profiles of clinical isolates obtained from previously untreated patients with active pulmonary TB in Hanoi, the northern largest city in Vietnam, and found that a quarter of these isolates were highly resistant to INH, most of which had a single S315T mutation in the *katG* gene. These isolates with primary resistance to INH were enriched in the clusters identified by RFLP. They probably originated from a few genetically related clones and were recently transmitted into and spread within this area of Vietnam.

Table 2 The relationship between drug resistance and clustering of the clinical isolates in Hanoi

Resistant to	MTB isolates		P value*
	Clustered	Non-clustered	
	N = 146	N = 171	
INH (0.2 μg/mL)	55 (37.7%)	34 (19.9%)	0.0004
INH (1.0 μg/mL)	49 (33.6%)	29 (17.0%)	0.0006
RMP	13 (8.9%)	6 (3.5%)	0.0437
SM	49 (33.6%)	41 (24.0%)	0.0592
EMB	7 (4.8%)	4 (2.3%)	0.2338

*P values by chi-square test. P < 0.01 was regarded as statistically significant after considering multiple comparisons.

Among the isolates with resistance to the first-line drugs tested in this study, a high proportion of primary INH resistance (27.4%) was rather characteristic. This resistance level was higher than the average (19.1%) obtained during a 2006 nation-wide survey [5]. Such a high level of primary resistance is a serious concern because INH is a key drug by which newly diagnosed TB patients can be successfully treated. In Vietnam, culture-based DST has not yet been routinely performed for previously untreated patients, and the standard regimen for these patients remained 2S(E)HRZ/6HE for a long time [20]. In the years when RMP-based treatment was not easily accomplished during the maintenance phase in areas with inadequate resources, this regimen had certain significance and was thus endorsed by the WHO until recently [21].

However, when DST results are unknown and the above standard regimen is used for INH-resistant TB, treatment during the maintenance phase is no more than EMB monotherapy, which could increase the chances of failure, early relapse, and additional drug resistance [21,22]. Prescribing 2RHEZ/4RHE, a regimen that includes 6 months of RMP, has also been recently approved by the national TB program in Vietnam. Because treatment outcomes are largely affected by locale-specific factors, including patient adherence to the regimen and drug resistance profiles of the prevailing strains, further studies will be needed to confirm the optimal regimens in Vietnam [23].

To reduce the likelihood of failure, relapse, and additional acquired drug resistance in major cities, updating clinical laboratories for DST is an urgent need [21]. In addition to DST for first-line drugs, detecting resistance to second-line drugs, including FQ, has also become important recently [24], although the proportion appeared to be low (<1%) in our study. Even in a resource-poor setting, as per timely DST results, health care staff should treat and intensively follow up those patients with drug-resistant TB with the aim of complete cure in most of these cases and to prevent further spread of MDR-TB and generation of extensively drug-resistant TB.

Genetic analysis of our MTB isolates demonstrated that >85% of the INH resistance (92% with high-level resistance) was caused by a S315T mutation in the *katG* gene. The predominance of this mutation in INH resistance has been observed in most of the areas with high TB prevalence, although the proportion (85%) in our study was relatively high compared with what was reported in other studies [25-27]. Continuous use of INH may cause additional mutations and induce higher levels

of resistance [27,28]. Rapid detection of INH resistance at an early phase is important to break this chain of acquiring additional resistance. Predominance of the S315T mutation is potentially advantageous for providing molecular DST in a resource-limited setting because it might entice manufacturers to develop a simplified, maintenance-free genetic test specialized for detecting the relevant mutations at a reasonably low cost [29].

RFLP analysis demonstrated that primary resistance to INH was more often observed in clustered isolates than in non-clustered isolates. Resistance to other drugs also appeared to be associated with these clustered isolates, although the tendencies were not as clear as that for INH. This indicates that expansion of INH-resistant isolates presumably originated from a few genetically related clones and that they were transmitted into the city of Hanoi and they spread widely within a relatively short period. Rapid expansion of genetically related strains may also explain why a single INH-resistant mutation, S315T, was predominantly detected in this area.

In our study, other genotype data for these clinical isolates were not available. However, in Vietnam, particularly in the southern region, two families of strains, designated the Beijing genotype and a presumably indigenous East-African Indian (EAI) genotype, are known to be predominant [30]. According to the literature, strains with ≥15 IS*6110* copies may indicate typical Beijing strains [31], whereas most of the EAI strains in Vietnam have <5 copies [30]. The copy numbers and RFLP pattern profiles in large cluster II in our study were definitely consistent with those of Beijing strains, whereas approximately one-fourth of the isolates had a few copies that are observed in EAI. IS*6110* copy numbers in other clusters were those between these two families. An earlier study demonstrated that copy numbers of northern strains in Vietnam were relatively smaller than those of southern strains in which typical Beijing genotypes are frequently observed [6]. We will need to further characterize the strains originating from the Hanoi area in future genotypic studies.

Our study had some limitations. The isolates analyzed in this study were collected from sputum smear-positive patients who visited TB clinics. Therefore, we may have only extracted features of MTB isolates from moderate to severe pulmonary TB cases. Nevertheless, understanding the current status of highly transmissible smear-positive TB is a priority for TB control because Vietnam is one of the high TB burden countries.

Conclusions

High levels of primary resistance to INH and emerging RMP resistance may be closely related to the problems of a rapidly developing city, such as the distribution of young workers with low incomes, undernutrition, poor hygiene, and crowding in a densely populated urban area with a floating population. Private acquisition and inappropriate use of anti-TB drugs through unofficial distribution routes are also difficult to manage in a large city such as Hanoi. It will be necessary to curb the transmission of drug-resistant MTB by considering effective counter measures. We will need to carefully monitor these trends further and search for the origins and transmission routes of these Southeast Asian MTB strains.

Additional file

Additional file 1: Table S1. Drug susceptibility testing and DNA sequencing of *M. tuberculosis* clinical isolates (N = 317).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NVH supervised on-site implementation of this study and drafted and revised the manuscript. HA performed the experiments and participated in technical transfer and supervision. TTBT carried out the drug susceptibility tests. TK performed the experiments. NTLH supervised on-site implementation of this study and drafted and revised the manuscript. SS and PHT monitored on-site data collection. LTL conceived and supervised this study. NK conceived the study, analyzed and interpreted data, and drafted and revised the manuscript. All authors read and approved this manuscript.

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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

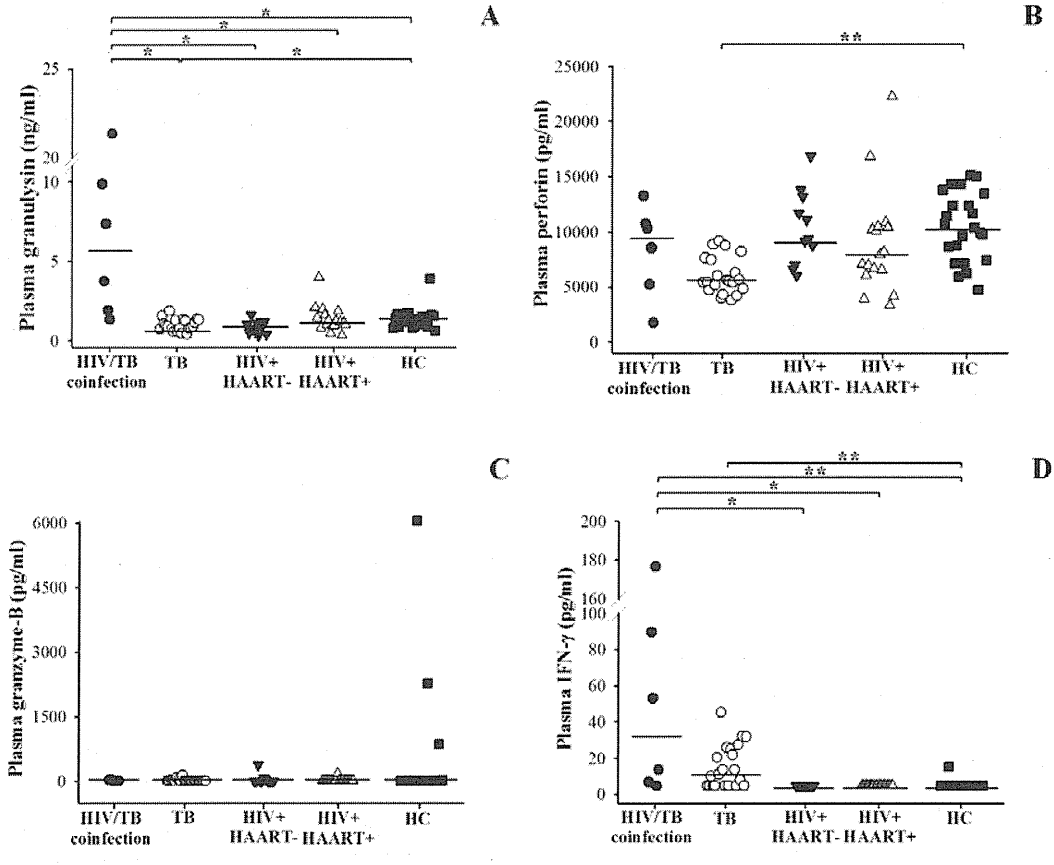

Fig 1. Circulating granulysin (ng/ml) (A), perforin (pg/ml) (B), granzyme-B (pg/ml) (C) and IFN-γ (pg/ml) (D) levels before anti-TB therapy in Thai patients with HIV/TB coinfection and TB in comparison with healthy controls (HC), HIV+HAART- and HIV+HAART+. Each dot represented one individual. A horizontal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

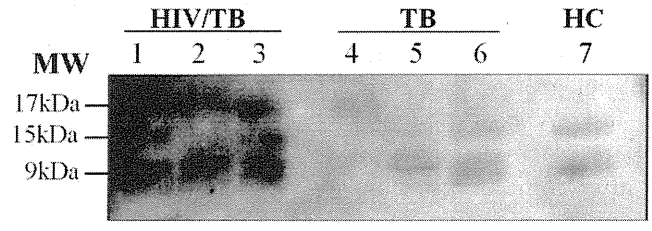

Fig 2. Isoforms of granulysin expression in plasma from Thai patients by Western blot analysis. Lane 1-3, HIV/TB coinfection plasma, 3 bands of ~17kDa, 15kDa and 9kDa isoforms; Lanes: 4-6, TB plasma, 1 band of ~17kDa isoform (Lane 4) and 2 bands of ~15kDa and 9kDa isoforms (Lane 5-6); Lane 7, 2 bands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

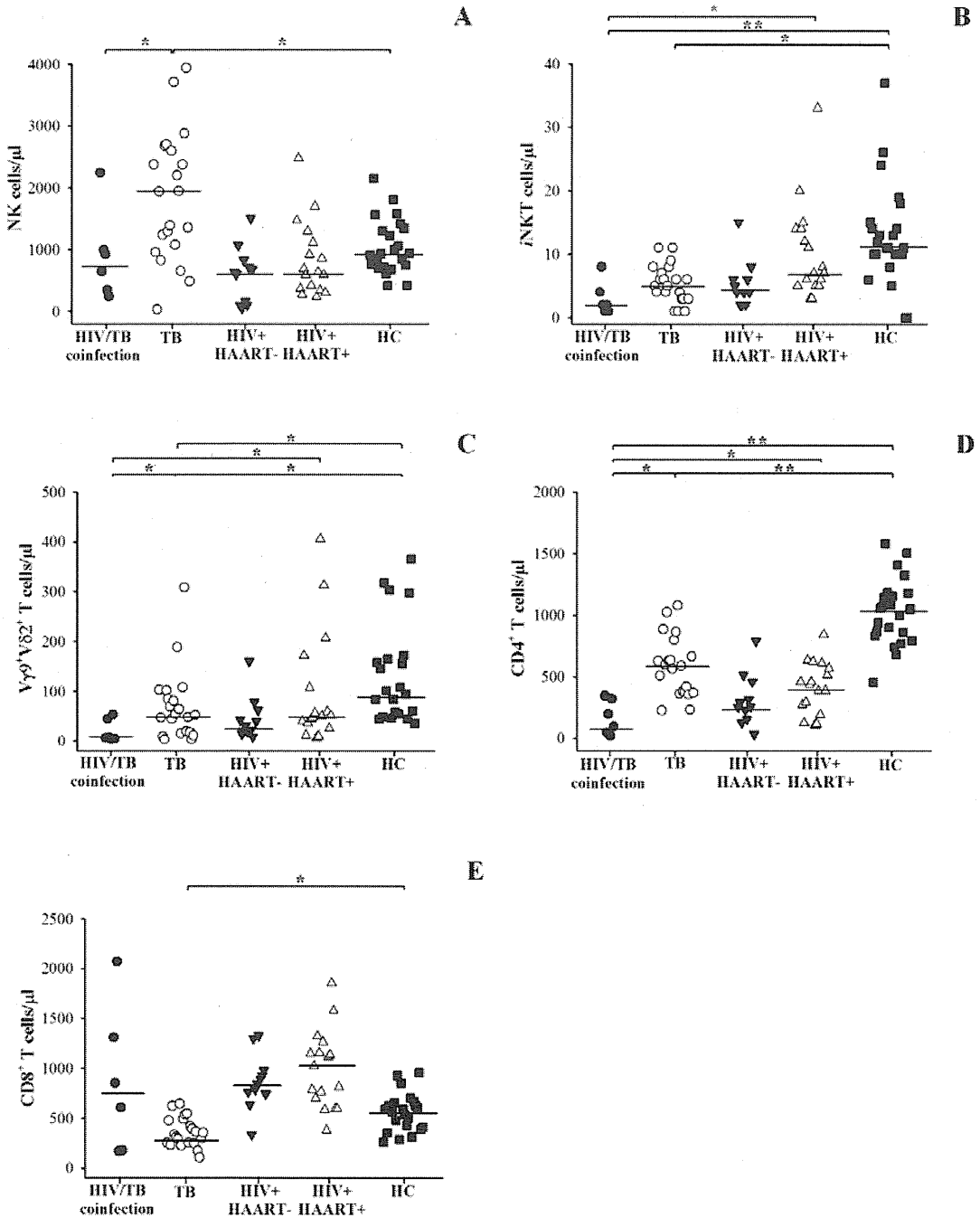

Fig 3. The number of NK cells (A), *i*NKT cells (B), $V\gamma9^+V\delta2^+$ T cells (C), $CD4^+$ T cells (D) and $CD8^+$ T cells (E) per microliter (μl) in Thai patients with HIV/TB coinfection and TB in comparison with healthy controls (HC), HIV+HAART- and HIV+HAART+ determined by flow cytometric analysis. 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). $V\gamma9^+V\delta2^+$ 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.

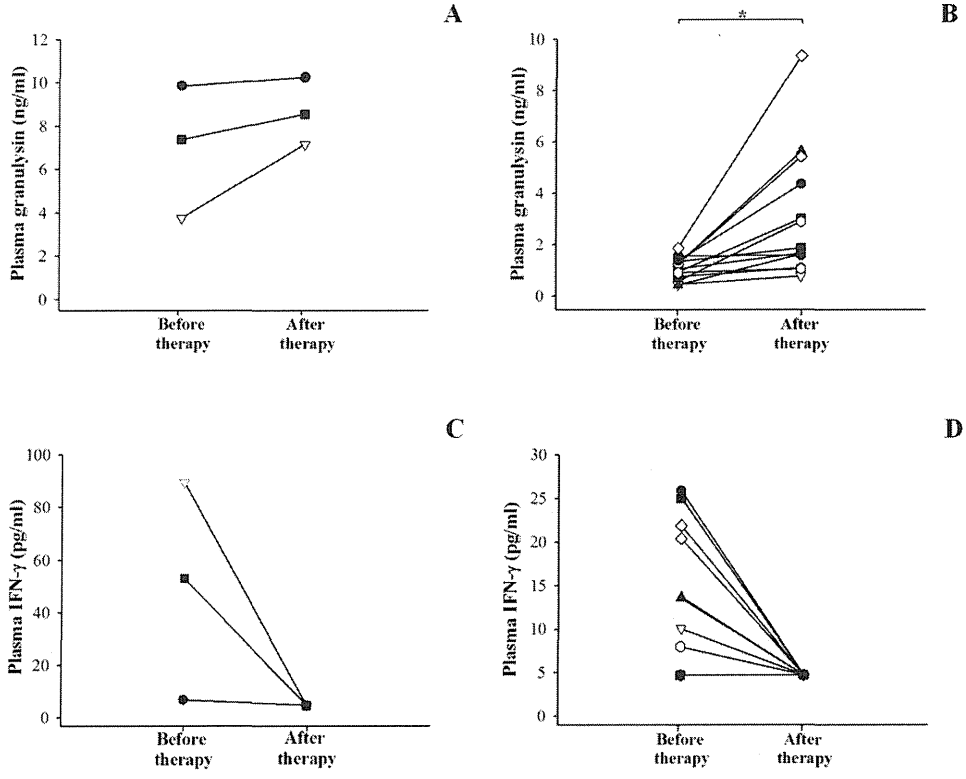

Fig 4. Circulating granulysin (ng/ml) and IFN-γ (pg/ml) in Thai patients with HIV/TB coinfection (A and C) and patients with TB (B and D) before and after completion of anti-TB therapy. Each dot represented one individual. *, $p < 0.05$.

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, Vγ9$^+$Vδ2$^+$ 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.

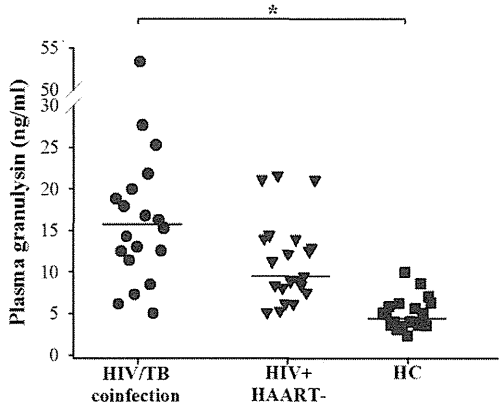

Fig 5. Circulating granulysin (ng/ml) in Japanese patients with HIV/TB coinfection before anti-TB therapy, HIV+HAART- and healthy controls (HC). Each dot represented one individual. A horizontal bar indicated the median of each group. *, p <0.05.

High levels of granulysin coexisted with relatively large number of CD8$^+$ T cells, but not proportional to *i*NKT, Vγ9$^+$Vδ2$^+$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, Vγ9$^+$Vδ2$^+$ 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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Primary Drug-Resistant Tuberculosis in Hanoi, Viet Nam: Present Status and Risk Factors

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Abstract

Introduction: Resistance of *Mycobacterium tuberculosis* (MTB) to anti-tuberculosis (TB) drugs presents a serious challenge to TB control worldwide. We investigated the status of drug resistance, including multidrug-resistant (MDR) TB, and possible risk factors among newly diagnosed TB patients in Hanoi, the capital of Viet Nam.

Methods: Clinical and epidemiological information was collected from 506 newly diagnosed patients with sputum smear- and culture-positive TB, and 489 (96.6%) MTB isolates were subjected to conventional drug susceptibility testing, spoligotyping, and 15-locus variable numbers of tandem repeats typing. Adjusted odds ratios (aORs) were calculated to analyze the risk factors for primary drug resistance.

Results: Of 489 isolates, 298 (60.9%) were sensitive to all drugs tested. Resistance to isoniazid, rifampicin, streptomycin, ethambutol, and MDR accounted for 28.2%, 4.9%, 28.2%, 2.9%, and 4.5%, respectively. Of 24 isolates with rifampicin resistance, 22 (91.7%) were MDR and also resistant to streptomycin, except one case. Factors associated with isoniazid resistance included living in old urban areas, presence of the Beijing genotype, and clustered strains [aOR = 2.23, 95% confidence interval (CI) 1.15–4.35; 1.91, 1.18–3.10; and 1.69, 1.06–2.69, respectively). The Beijing genotype was also associated with streptomycin resistance (aOR = 2.10, 95% CI 1.29–3.40). Human immunodeficiency virus (HIV) coinfection was associated with rifampicin resistance and MDR (aOR = 5.42, 95% CI 2.07–14.14; 6.23, 2.34–16.58, respectively).

Conclusion: Isoniazid and streptomycin resistance was observed in more than a quarter of TB patients without treatment history in Hanoi. Transmission of isoniazid-resistant TB among younger people should be carefully monitored in urban areas, where Beijing strains and HIV coinfection are prevalent. Choosing an optimal treatment regimen on the basis of the results of drug susceptibility tests and monitoring of treatment adherence would minimize further development of drug resistance strains.

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Introduction

Resistance of *Mycobacterium tuberculosis* (MTB) to anti-tuberculosis (TB) drugs, particularly to isoniazid (INH) and rifampicin (RMP), which results in multidrug-resistant (MDR)-TB, presents a serious challenge in the control of TB worldwide [1,2]. The World Health Organization (WHO) estimates that the prevalence of MDR-TB varies from 0% to 65.1% across the world [1]. Despite progress in disease surveillance, more than 80% of MDR-TB patients are unaware of their disease status, indicating that the transmission status of MDR-TB is mostly unknown in high-TB burden countries [1].

Drug-resistant TB, including MDR-TB, develops as a result of inadequate treatment of an individual who was initially infected

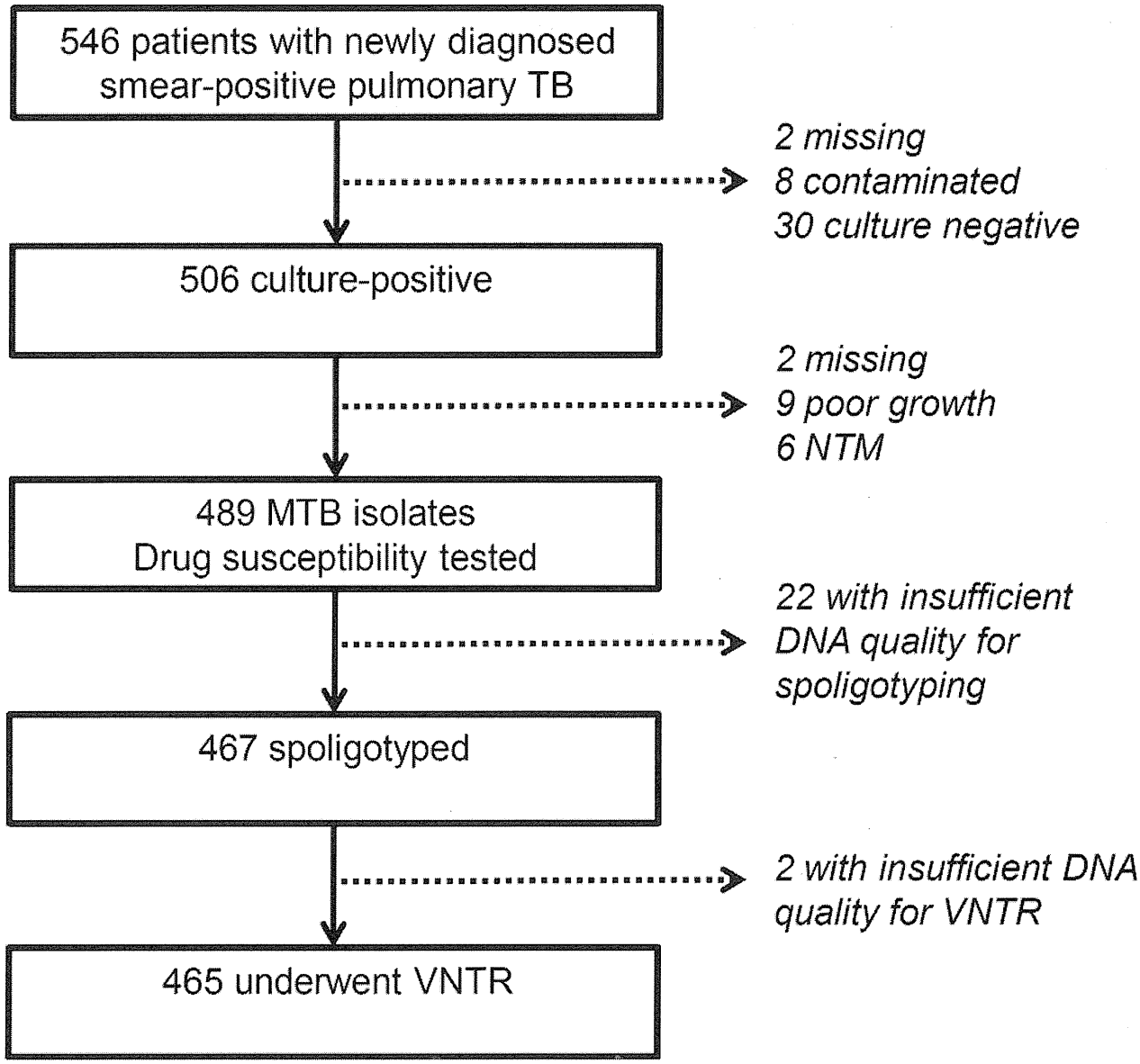

Figure 1. Study flow. TB: tuberculosis; MTB: *Mycobacterium tuberculosis*; NTM: nontuberculous mycobacterium; VNTR: variable numbers of tandem repeats; DNA: deoxyribonucleic acid.
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with a fully or partly sensitive strain or by direct transmission of a drug-resistant strain from one individual to another [3]. Although previous treatment is the strongest risk factor of MDR-TB, other risk factors such as younger age, male gender, and human immunodeficiency virus (HIV) coinfection have also been reported [4-6]. Further analysis may provide information on the dynamics of its transmission and better countermeasures against increasingly drug-resistant TB.

Viet Nam is one of the 22 countries with a high TB burden and is one of the 27 countries with a high MDR-TB burden [1]; the prevalence of any drug resistance and MDR-TB among newly diagnosed cases in a 2006 countrywide survey was 30.7% and 2.7%, respectively [7]. Although drug resistance, including MDR, and potential risk factors have been investigated in some areas [8-10], host-, pathogen-, and environment-related factors, such as patients' HIV status; residential area; and genotypes of the MTB isolates, have not been comprehensively assessed in Viet Nam. We conducted this study to estimate the status of primary anti-TB drug resistance, including MDR, among newly diagnosed TB patients in Hanoi, the capital and second largest city of Viet Nam, and to investigate the role of the above risk factors in resistance to each of the first-line drugs.

Table 1. Characteristics of the study population (n = 489).

		Number	%
Age (median, range)		(38.6,	16.6–85.4)
Gender	Male	386	78.9
	Female	103	21.1
Body mass index	<16	70	14.3
	16–18.4	201	41.1
	18.5–24.9	213	43.6
	≥25	4	0.8
	Not available	1	0.2
Residential area	Suburban	100	20.4
	New urban	228	46.6
	Old urban	161	32.9
Smoking habit	Smoker	189	38.7
	Ex-smoker	134	27.4
	Nonsmoker	165	33.7
	No answer	1	0.2
HIV status	Positive	44	9.0
	Negative	443	90.6
	Not available	2	0.4

HIV: human immunodeficiency virus

Table 2. Patterns of INH, SM, RMP, and EMB resistance (n = 489).

Pattern		Number	%
Sensitive with all drugs		**298**	**60.9**
Any resistance	**Total**	**191**	**39.1**
	INH	138	28.2
	RMP	24	4.9
	SM	138	28.2
	EMB	14	2.9
Monoresistance	**Total**	**101**	**20.7**
	INH	49	10.0
	RMP	2	0.4
	SM	50	10.2
	EMB	0	0.0
Polyresistance, non-MDR	**Total**	**68**	**13.9**
	INH + SM	65	13.3
	INH + EMB	1	0.2
	INH + SM + EMB	1	0.2
	RMP + SM	0	0.0
	RMP + EMB	0	0.0
	RMP + SM + EMB	0	0.0
	SM + EMB	1	0.2
MDR	**Total**	**22**	**4.5**
	INH + RMP	1	0.2
	INH + RMP + EMB	0	0.0
	INH + RMP + SM	10	2.1
	INH + RMP + EMB + SM	11	2.2

INH: isoniazid; RMP: rifampicin; SM: streptomycin; EMB: ethambutol; MDR: multidrug resistance

Materials and Methods

Ethics statement

Written informed consent was obtained from each participant. In the case of minors, the parents provided written informed consent. This study was approved by the ethical committees of the Ministry of Health, Viet Nam, and National Center for Global Health and Medicine, Japan, respectively.

Study sites, recruitment of patients, and sample collection

As part of our prospective study project, we included 7 of the 14 districts in Hanoi as the catchment area, where more than half of new smear-positive TB patients in the city were diagnosed and treated in the area during the study period. Among the districts, two were located in the old city area established before 1954 and had a population density that ranged from 25,000 to 26,000 individuals /km^2 in 2009. As such, they were categorized as "old urban" areas. The remaining five districts were originally regarded as suburban areas. Of these, three were recently upgraded to urban areas on the basis of rapid economic development and had a population density that ranged from 2,800 to 5,300 individuals/km^2, although the migrating population was not counted. We categorized these three areas as "new urban." The two other areas remained "suburban," and their population densities ranged from 1,500 to 2,500 individuals/km^2.

Patients were considered eligible if they were 16 years or older, resided in the abovementioned catchment areas, suffered from smear-positive pulmonary TB without a history of TB treatment, and agreed to participate in this study. Eligible patients who visited the local TB care units were recruited consecutively from July 2007 to March 2009. Information about no previous TB treatment was based on interviews conducted by pre-trained health care staff and medical records kept for registration with the National TB Program in district TB centers.

Before initiating anti-TB treatment, sputum specimens were cultured and subjected to identification of MTB, drug susceptibility tests, and DNA extraction for molecular typing. Blood samples were obtained for HIV testing and complete blood count. Bacterial load estimated in sputum smear was used to assess the severity of the disease.

Identification of MTB and drug susceptibility testing

After undergoing solid cultures on Löwenstein–Jensen media, MTB isolates from sputum specimens were subjected to a niacin test. For drug susceptibility testing, the WHO standard proportional method was used to identify resistance to INH, RMP, streptomycin (SM), and ethambutol (EMB) [11]. The test media contained INH (0.2 μg/mL), RMP (40 μg/mL), SM (4 μg/mL), and EMB (2 μg/mL). Resistance to pyrazinamide (PZA) was tested using a pyrazinamidase assay, in which pyrazinamidase activity was determined using Wayne's method with minor modifications [12]. The H37Rv strain of MTB, which is susceptible to PZA and positive for pyrazinamidase, was used as the positive control. The BCG strain of *M. bovis*, which is resistant to PZA and negative for pyrazinamidase, served as the negative control.