Annual Report

2016

NCGM-BMH
Medical Collaboration Center

July 2017
Tokyo, Japan-Hanoi, Viet Nam

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Preface

In this year, we are continuing the new collaboration scheme as well as other present collaboration activities such as researches and human resource development. In this year we have a big topics. That is, we invited Viet Namese's VIPs to NCGM, Japan. It was our great pleasure to receive them and this was a great opportunity for mutual understanding and deepening intimacy of the collaboration. In addition, Clinical Research was started as our new area of collaboration in this year.

In this regard, it is our pleasure that our activities are gradually expanding and becoming more active and meaningful for both sides.

I hope this Annual Report is useful to understand our collaboration.

July, 2017

Hidechika Akashi, MD, PhD, MPH, DTMH

Director,
Medical Collaboration Center (MCC)
National Center for Global Health and Medicine
(NCGM), Japan

Abbreviations

BMH	Bach Mai Hospital
NCGM	National Center for Global Health and Medicine
IMCJ	International Medical Center of Japan
MCC	NCGM - BMH Medical Collaboration Center
MOH	Ministry of Health, Viet Nam
MEXT	Ministry of Education, Culture, Sport, Science and Technology, Japan
J-GRID	Japan Initiative for Global Research Network on Infectious Diseases
MHLW	Ministry of Health, Labor and Welfare, Japan
JICA	Japan International Cooperation Agency
MOU	Memorandum of Understanding
HCMC	Ho Chi Minh City
NIHE	National Institute of Hygiene and Epidemiology
NHP	National Hospital of Pediatrics
NLH	National Lung Hospital
HLH	Hanoi Lung Hospital
NIHBT	National Institute of Hematology and Blood Transfusion
RIT-JATA	Research Institute of Tuberculosis-Japan Anti-Tuberculosis Association
WHO	World Health Organization
JFPIMRC	Japan Foundation for the Promotion of International Medical Research Cooperation
SARS	Severe Acute Respiratory Syndrome
DCC	Disease Control and Prevention Center of NCGM

Contents

I. General information on NCGM-BMH Medical Collaboration Center (MCC)

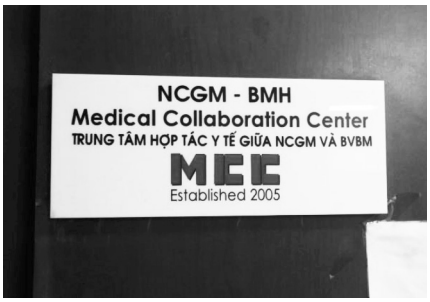
1. Background

Since the beginning of 1990's, National Center for Global Health and Medicine (NCGM) (former IMCJ) has been carrying out important roles in collaboration with health sector in Viet Nam for the purpose of the improvement of medical situation in the country. Particularly, collaboration with Bach Mai Hospital (BMH) has been implemented most actively and effectively. In the grant-aid and the technical cooperation projects in BMH, which was supported by Japan International Cooperation Agency (JICA), NCGM contributed to the successful implementation by dispatching experts and providing technical guidance.

Through the history of the past collaboration, NCGM has established close and reliable relationship with BMH and other leading medical institutions in Viet Nam. Using these bases, a new collaboration, which is conducted distinctly from ODA projects and focusing on research and human resource development, was designed.

In order to implement the new collaborative activities, the NCGM-BMH Medical Collaboration Center (MCC) was planed.

2. Establishment of MCC



In view of the successful outcome of BMH project (phase 1) and the efficient collaboration during the SARS outbreak in 2003, a plan to establish a medical collaboration center between NCGM and BMH, which functions separately from JICA projects, grew up in NCGM. The idea was put into practice when the research project on emerging and reemerging infectious diseases was proposed by the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT).

In recent years, emerging and reemerging infectious diseases have been threatening the world. In view of the rising fear of these diseases, the MEXT launched a new project in world-wide scale to cope with emerging and reemerging infectious diseases efficiently by setting up medical collaboration centers and conducting close collaboration there. The proposal of the MEXT project facilitated the realization of MCC in Viet Nam.

After several preliminary studies, NCGM and BMH decided to establish MCC within BMH based on the friendly and reliable relationship, which had been developed since the beginning of 1990's between the 2 medical institutions.

The Memorandum of Understanding (MOU) regarding the initiation of the project was signed by the Director of BMH and the President of NCGM in August 2005 followed by the official approval of the Ministry of Health, Viet Nam. In April 2010, NCGM changed its name (from International Medical Center of Japan; IMCJ to National Center for Global Health and Medicine; NCGM) due to its organizational reform (Independent Administrative Legal Entity). In view of this situation, both sides agreed to revise the MOU along with continuation of the current cooperative activities. After discussions between NCGM and BMH the revised MOU was drafted. In the new MOU, activities in MCC are clarified as research, training, medical case conference, technical cooperation, international conference/ meeting/seminars, personal exchange programs, and others, although in the current version description of activity is concentrated on researches. The new MOU, after getting approval of MOH, was signed by the representatives of BMH (Dr. Nguyen Quoc Anh) and NCGM (Dr. Takaaki Kirino) in June 2, 2010.

MCC office was established in the new building of BMH, which was constructed by Japan's grant-aid in 2000, as the managing center of various

Signing ceremony of the Memorandum of Understanding between NCGM and BMH in 2010

collaborative activities including the MEXT project and others. Based on the MCC, various activities were started in collaboration with BMH along with related medical institutions.

3. Objective of MCC

The objective of setting up MCC in Viet Nam is to implement various collaboration on medical science and medical care, such as researches, human resource development & technical exchange, information sharing, clinical case conference, etc. smoothly and effectively. The activities in MCC are conducted in close collaboration between BMH and NCGM, and related medical institutions and such collaborative activities are expected to benefit both Viet Nam and Japan. The contents of activities can include some advanced and sophisticated techniques, which had been difficult to conduct within the framework of JICA projects.

4. Related medical institutions

MCC is mainly collaborating with BMH, however based on the agreement described in MOU; some related medical institutions have been setting up under the approval of BMH.

Currently, five institutions in Hanoi and three institutions in Ho Chi Minh City are functioning as the main related medical institutions. In the future, more medical institutions might be added if necessary and efficient network building among them of are expected.

Table 1: Main medical institutions under collaboration (As of December 2015)

No.	Medical institution	Location	Collaborative study
1	National Institute of Tropical and Infectious Diseases	Hanoi	HIV/AIDS
2	National Hospital of Pediatrics	Hanoi	Clinical conference
3	National Lung Hospital (the former National Hospital of Tuberculosis and Lung Diseases)	Hanoi	Tuberculosis

No.	Medical institution	Location	Collaborative study
4	Hanoi Lung Hospital (the former Hanoi Hospital of Tuberculosis and Lung Diseases)	Hanoi	Tuberculosis
5	Ho Chi Minh Medical and Pharmaceutical University	HCMC	Tropical Medicine
6	Ho Chi Minh City Hospital of Tropical Medicine	HCMC	Tropical Medicine Medical education
7	National Institute of Nutrition	Hanoi	Diabetes and life style related disease
8	Cho Ray hospital	HCMC	Training program

5. MEXT's program

The Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) is implementing the MEXT "Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases" in Asian and African countries. The objective of these activities is to contribute to the emerging infectious diseases and other disease control from the world-wide viewpoint. As of December 2008, this program has been implemented in 11 research centers in 8 countries (Viet Nam, China, India, Thailand, Indonesia, Zambia, Ghana, Philippines). MCC is functioning as one of the important research centers for this program in Viet Nam. The period of this program is 5 years from April 2005 to March 2010. The next step started from April 2010 to March 2015, and now the third step has been started from April 2015.

Currently, the projects supported by MEXT account for the major part of the activities of MCC. Activities of the MEXT projects in Viet Nam include scientific researches (both basic and clinical researches), human resource development, etc. Equipment necessary for conducting these activities has also been provided to BMH and relevant medical institutions.

MEXT's program in Viet Nam consists of the following three research groups (Dr. Oka is the leader of these researches). These groups are implementing activities on emerging and reemerging infectious disease control based on the concept of MEXT project. The following three researches are three leading research subjects in MCC and under these research themes, sub-researches have been carried out.

1) Dr. Oka's group: HIV/AIDS
2) Dr. Ohmagari's group: Bacterial infections
3) Dr. Keicho's group: Tuberculosis

6. The program for international promotion of Japan's healthcare technologies and services

The program has been started from 2015, organized by the Bureau of International Health Cooperation and relevant departments of NCGM and funded by the Ministry of Health, Labour and Welfare, Japan. This program is in relation to the Memorandum of Cooperation in the field of healhcare between the Ministry of Health, Labour and Welfare of Japan and the Ministry of Health of Viet Nam signed on 18 March 2014 by both Ministers. This program aims at carrying out the major objectives of promotion, including sharing the experiences in Japan's public health insurance system and the transfer of cutting-edge medical technologies. Through this program, public health standards in the counterpart countries will be improved.

Under this program, 14 staff members of BMH, ten from Cho Ray hospital and two from Hue Center Hospital were invited to NCGM for training in different fields, such as clinical laboratory, neurosurgery, nursing management, hospital management etc. Specialists from NCGM also came to BMH for investigating the needs and discussing the content of training and collaborative activities. Healthcare staff working in quality management and patient safety in other related hospitals were also invited to NCGM for training. A forum on quality management and patient safety was held in Viet Nam, with the participation of several tens of healthcare staff working in these fields.

7. Other projects

In addition to the obove, the Ministry of Health, Labor and Welfare of Japan (MHLW) is also supporting research projects on various fields. As an oversea base of NCGM, MCC is functioning as a body to support NCGM research teams or individuals who want to implement a collaborative project in Viet Nam.

Within this scope, life-style related diseases such as diabetes and pediatric health issues are projects which have been implemented in collaboration with BMH and other medical institutions of Viet Nam so far. A community-based suvery on diabetes and obesity, followed by an intervention program has been implementing in Hanoi area and a lot of meaningful data have been obtained with the support from MCC.

In 2013, the Sister Renal Center Program was applied in cooperation between Nephro-Urology Department, BMH and Nephrology Department, NCGM to receive the support from the International Society of Nephrology. Under this program, in 2016, three Japanese groups including NCGM nephrologists and medical engineers came to BMH for training courses on kidney diseases and quality management of water for hemodialysis, with the trainees from BMH and other hospitals. Three staff of the Nephro-Urology Department, BMH were invited for one-week training in NCGM. Together with the Bureau of International Medical Cooperation of NCGM, MCC participated in supporting the implementation of this program.

8. Current MCC

In 2016, MCC has received many groups of researchers and specialists coming from NCGM and related institutions in Japan. In addition to logistic support for these groups, such as reservation of accommodation, arrangement of transportation vehicles, on-site coordination including making appointments with Viet Namese counterparts, MCC staff also participated in discussions on related activities held between the two sides. Together with Viet Namese counterparts, MCC staff also took part in conducting and monitoring on-site implementation of researches, as well as collecting and reporting data.

MCC staff also supported administrative procedures for Viet Namese counterparts, who were invited to Japan for training. In 2016, more than 30 counterpart members have completed necessary procedures and successfully received training in Japan. Through this activity, MCC also acts as a bridge to link domestic health personnel and institutions, which is necessary for sustainable improvement.

In 2016, MCC also participated in arrangement of the training visits for NCGM trainees. Groups including doctors, nurses, technicians, and pharmacists came to BMH and other hospitals and healthcare centers in Hoa Binh province for the training on global health and medicine.

II. Activities

1. Research

List of collaborative researches in MCC, Viet Nam

Table 2 Collaborative researches in MCC, Viet Nam

No.	Main Researcher in NCGM	Affiliation in Viet Nam	Subject	Source of fund
1	Shinichi Oka	National Hospital of Tropical Diseases (NHTD), Bach Mai Hospital(BMH)	The cohort study of HIV-1-infected individuals in Northern Viet Nam	AMED
2	Kajio H Pham MT Nguyen KDV	Bach Mai Hospital (BMH)	Study on the contribution of obesity to diabetes and blood vascular diseases in Viet Nam	NCGM MHLW
3	Kajio H Anh NQ Lien DTK	Bach Mai Hospital (BMH)	Impact of a life style intervention in incident and prevalence of overweight and obesity among secondary school children in Hanoi	NCGM MHLW
4	Hiroyuki Shichino	National Hue Central Hospital (Hue), Ho Chi Minh Children Hospital 1	Support for Strengthening Medical Treatment Ability of the Childhood Cancer in Viet Nam	NCGM MHLW
5	Naoto Keicho Luu Thi Lien Pham Huu Thuong	Hanoi Lung Hospital (HLH), National Lung Hospital (NLH)	Research on tuberculosis in Viet Nam, Research on spreading Beijing-genotype strains of *Mycobacterium tuberculosis*, their drugresistance profiles and possible effects on treatment outcome	J-GRID MEXT
6	Naoto Keicho Luu Thi Lien Pham Huu Thuong	Hanoi Lung Hospital (HLH)	Research on latent tuberculosis infection among healthcare workers in Hanoi, Viet Nam	J-GRID MEXT
7	Shinsaku Sakurada Naoto Keicho Luu Thi Lien Pham Huu Thuong	Hanoi Lung Hospital (HLH)	Research on HIV/tuberculosis (TB) in Hanoi, Viet Nam	J-GRID MEXT
8	Fumihiko Hinoshita	Bach Mai Hospital (BMH)	Research on improvement of CKD and dialysis management in Hanoi, Viet Nam	NCGM ISN
9	Hiroshi Ohara	National Institute of Malariology, Parasitology and Entomology (NIMPE), Bach Mai Hospital (BMH)	Study on effective use of the surveillance results for drug resistant pathogens in infection control	NCGM

No.	Main Researcher in NCGM	Affiliation in Viet Nam	Subject	Source of fund
10	Norio Ohmagari Nguyen Quoc Anh	Bach Mai Hospital (BMH)	Research on Epidemiology, Diagnosis and Treatment for Healthcare Associated Infection and Antimicrobial Resistant Bacteria in Viet Nam	J-GRID
11	Teruo Kirikae	Bach Mai Hospital (BMH) Cho Ray Hospital	Molecular epidemiology of multidrug-resistant Gram-negative pathogens in medical settings in Viet Nam	J-GRID
12	Nguyen Gia Binh Vu Thi Tuong Van Noriko Nakajima Tsutomu Kageyama Jin Takasaki	Bach Mai Hospital (BMH)	International collaborative research on diagnosis and treatment based on clinicopathological study of highly pathogenic avian influenza virus infection	AMED

Research No.1

1.	**Title(in English)**	The cohort study of HIV-1-infected individuals in Northern Viet Nam
2.	**Title(in Japanese)**	ハノイにおける HIV 感染者のコホート研究
3.	**Main researcher**	Shinichi Oka (AIDS Clinical Center, National Center for Global Health and Medicine, Japan)
4.	**Co-Researcher(s)**	Junko Tanuma, Daisuke Mizushima, Ei Kinai, Hiroyuki Gatanaga, Shoko Matsumoto, Mika Sata, Masafumi Takiguchi, Nguyen Thi Huyen, Nguyen Hoai Dung, Tran Van Giang, Nguyen Vu Trung, Nguyen Van Kinh, Vu Thi Tuong Van, Doan Thu Tra, Do Duy Cuong
5.	**Resource of fund**	Japan Agency for Medical Research and Development (AMED)
6.	**Affiliation(s) in Viet Nam**	National Hospital of Tropical Diseases (NHTD) Bach Mai Hospital (BMH)
7.	**Period of the research**	October 2007- March 2020
8.	**Publications**	1. Tanuma J. et al. ***Kagaku Ryouhou no ryouiki vol4***, 2017. [ahead of print] 2. Tanuma J, et al. ***PLOS One*** 11 (3): e0150781, 2016. 3. Tanuma J, et al. Abstract 463, ***The annual Conference on Retroviruses and Opportunistic Infections***, Seattle, USA. Feb 13-16, 2017

9. Summary:

In 2007, we established a hospital-based cohort of HIV-infected individuals in Hanoi, Viet Nam under the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) network, which aimed to enhance the research collaboration on HIV between Japan and Viet Nam. We recruited participants in both the National Hospital for Tropical Diseases (NHTD) and Bach Mai Hospital (BMH) in urban Hanoi and 2,198 HIV-infected individuals have joined the cohort by the end of 2016. Data on demographics, clinical status and laboratory data has been prospectively collected every 6 months and it has now become the longest clinical dataset of HIV-infected patients in Northern Viet Nam.

In 2016, two major analysis were conducted in the cohort, one of which showed low mortality despite high prevalence of tuberculosis among patients on antiretroviral therapy (ART)2) and the other showed the durable virologic suppression during ART3). In the former study2), we found the mortality after ART initiation was comparable to that of Western countries, although AIDS-defining opportunistic infections were not rare while receiving ART. Almost half of deaths were attributable to AIDS and age over 50 years at ART initiation was statistically associated with shorter survival, while having injection drug use history was not associated with poor prognosis. This is the first in-depth data on survival of HIV patients receiving ART and provides the supportive evidence for the success of ART programs in urban Hanoi after the decade of rapid ART scale-up. In the latter study3)., we investigated the durability of ART success and explored the factors related to virologic failure (VF) and early immune recovery in the cohort. We found the viral suppression rate was 95.5% at 12 months and survival without VF was maintained over 90% for 42 months. A younger age and HCV-antibody positivity were associated with early VF, and the male sex, injection drug users, and HCV co-infection were related to impaired immune recovery at 24 months. EFV-containing regimens showed both a better virologic outcome and greater early CD4 recovery compared to d4T-containing regimens, which showed a higher rate of VF. These results strongly indicated that 90% viral suppression was achievable in Viet Nam, but suggested that support for adherence to ART among IDU and HCV co-infected patients should be increased. This study

	also supported the current global effort to scale up routine VL testing, enabling us to implement adherence support interventions more effectively. The results of both studies suggest the ART program in Viet Nam is highly successful and the UNAIDS goal of 90-90-90 by 2020 is achievable in the country. Other currently active researches include; 1. prevalence of drug resistance among those failing ART 2. incidence of adverse effects against ART 3. association between Social supports and depression 4. HIV/HBV co-infection 5. prevalence of rickets among children born from mothers receiving ART 6. quality of life measurement by the WHOQOL All of these studies will provide key information on the long-term prognosis of HIV-infected individuals in Viet Nam as well as offered a variety of opportunities for young investigators to work with colleagues from a different country in the field of HIV.

Research No.2

1	Title (in English)	Study on the contribution of obesity to diabetes and blood vascular diseases in Viet Nam
2	Title (in Japanese)	ベトナム人における肥満の糖尿病や心血管疾患への関与に関する研究
3	Main researcher	Kajio H (Department of Diabetes, Endocrinology and Metabolism, NCGM, Japan) Pham MT (BMH, Viet Nam) Nguyen KDV (Department of Diabetes and Endocrinology, BMH, Viet Nam)
4	Co-Researcher(s)	Japan: Matsushita Y (NCGM), Tsujimoto T(NCGM) Viet Nam: Do DL (BMH), Nguyen PA (BMH), Thuy PTP (MCC)
5	Resource of fund	1. The Grant of National Center for Global Health and Medicine, NCGM, Japan 2. The Grant of Ministry of Health, Labor and Welfare of Japan
6	Affiliation(s) in Viet Nam	Bach Mai Hospital
7	Period of the research	May 2011-
8	Publications	None
9	Summary:	

Obesity is supposed to contributing to the deterioration of metabolic abnormalities for diabetes and cardiovascular diseases (CVD). Recently, intra-abdominal adipose tissues, that are VATs, have been found to secret bioactive hormones, which partially regulate the functions of insulin-sensitive organs as well as the vascular functions. The amounts of these hormones are largely dependent on the degree of fat accumulation in VAT. The visceral fat area (VFA) determined as cross-sectional image at the umbilical level using CT or MRI was a superior predictor for the clustering of metabolic risk factors. However, the use of CT or MRI is limited because the methods are not simple or cost-effective. CT and MRI are often unsuitable for screening large number of participants. CT has a problem with X-ray exposure. Recently, several apparatus for the direct measurement have been developed to overcome these problems. Some of them are based on the bioelectrical impedance analysis (BIA). The advantages of BIA include its portability and ease of use, relatively low cost, minimal participant participation required, and safety (not for participants with a pacemaker), thus making it attractive for large-scale studies.

The aims of our study are to establish a system based on abdominal BIA by comparing with the result of CT scan, and to identify directly the association of obesity, especially visceral obesity, and diabetes and blood vascular diseases.

We started the recruitment of the participants in 2016. The participants are 300 subjects (150 males, 150 females), who are being recruited mainly in the outpatient and inpatient clinics of the department of endocrinology and metabolism at Bach Mai Hospital.

Research No.3

1	Title (in English)	Impact of a life style intervention in incident and prevalence of overweight and obesity among secondary school children in Hanoi
2	Title (in Japanese)	ハノイ市の中学生における肥満・過体重に対する生活習慣介入に関する研究
3	Main researcher	Kajio H (Department of Diabetes, Endocrinology and Metabolism, NCGM, Japan) Anh NQ (BMH, Viet Nam) Lien DTK (Department of Diabetes and Endocrinology, BMH, Viet Nam)
4	Co-Researcher(s)	Japan: Matsushita Y (NCGM), Tsujimoto T(NCGM), Hara M (Tokyo Metropolitan Hiroo General Hospital) Viet Nam: Thanh DVT (BMH), Thanh NTT (BMH), Thuy PTP (MCC)
5	Resource of fund	1. The Grant of National Center for Global Health and Medicine, NCGM, Japan 2. The Grant of Ministry of Health, Labor and Welfare of Japan
6	Affiliation(s) in Viet Nam	Bach Mai Hospital
7	Period of the research	Dec 2012-
8	Publications	In Preparation
9	Summary:	

In the developing countries, the increasing prevalence and incidence of overweight and obesity is a serious public health problem following the social and economic development of the country. The prevalence has increased at an alarming rate even in children. Overweight and obese children are likely to stay obese into adulthood and more likely to develop non-communicable diseases (NCD) like diabetes and cardiovascular diseases at a younger age. We studied the impact of a life style intervention against overweight and obesity among secondary school children in Hanoi from 2012 to 2016.

We recruited 821 children of 6th grade from 4 different schools (Cat Linh school, Nguyen Cong Tru School, Phan Chu Trinh School, Dong Da school), which were randomly selected from 2 urban districts in Hanoi. After allocation of 4 schools into two groups, two schools for the intervention group and the other two schools for the control group, we performed intervention activities for two years. We provided the participants with the pedometers, and the participants in the intervention group were also provided with the scales. As for the behavior intervention, we promoted the participants in the intervention group to continue the activities with self-monitoring, goal setting, and problem solving. The analysis of the results is now being performed.

We obtained the baseline data from 821 children of 4 schools. We found that the prevalences of children with obesity were 32.4% for males and 7.9% for females, respectively, following WHO standard cut-offs. The data demonstrated that family factors, such as education levels and level of overweight/obesity (OW/OB) of father/ mother, are significantly related with the OR of OW/OB for children. Birth weight and sleeping hour per day, more physical activities to lose weight, less food to lose weight and more vegetable to lose weight are also significantly related with the OR of OW/OB for children. We collected the data of the questionnaires from 739 students and 660 parents and the data of the health check from 731 students at the 2-year final surveillance. We are now under analysis.

We revealed the high prevalence of overweight or obesity in school children, and several factors influencing on the appearance of overweight or obesity. It is very important to identify the factors related to the behavioral changes of the students and their parents through the intervention introducing the reduction of the prevalence and incident of overweight and obesity in the children. The analysis would help us make the strategy for the intervention of NCD.

Research No.4

1	Title (in English)	Support for Strengthening Medical Treatment Ability of the Childhood Cancer in Viet Nam
2	Title (in Japanese)	ベトナムにおける小児がん医療の診療能力強化を目的とした支援
3	Main researcher	Hiroyuki Shichino (National Center For Global Health And Medicine, Japan)
4	Co-Researcher(s)	Noriko Sato, Junko Yamanaka, Hideko Uryu, Mizue Tanaka, Yuri Yoshimoto
5	Resource of fund	International Promotion of Japan's Healthcare Technologies and Services, NCGM Program International Health Research (A27-5) from Ministry of Health Labor and Welfare of Japan
6	Affiliation(s) in Viet Nam	National Hue Central Hospital (Hue), Ho Chi Minh Children Hospital 1
7	Period of the research	April 2016- March 2017
8	Publications	七野浩之 , 他：ベトナムの小児がん医療に対する国際医療支援悪経緯と概要．映像情報メディカル 49:56-61,2017 松井基浩、七野浩之：ベトナムの小児医療の現状．映像情報メディカル 49：66-70,2017
9	Summary:	

BACKGROUND:

Eighty percent of world childhood cancer patients are children in the developing countries. There are many problems such as misdiagnoses, delay of discoveries, lack of offer of treatment. Many childhood cancer patients were supposed to be untreated, and there were not the grasp of accurate number of the childhood cancer patients. And there were small numbers of specialists of pediatric cancer.

PURPOSE:

To support for strengthening diagnosis, medical treatment, supportive care abilities of the childhood cancer in the pediatrics,pediatric surgery,radiotherapy and radiological diagnosis of leading hospitals in Viet Nam.

METHODS:

Sending experts well-versed in childhood cancer, to provide training in the field of childhood cancer diagnosis, treatment, supportive care. Accepting healthcare providers from Viet Nam as trainees for studying childhood cancer. Making a new style of consulting system through the internet environment.

RESULTS:

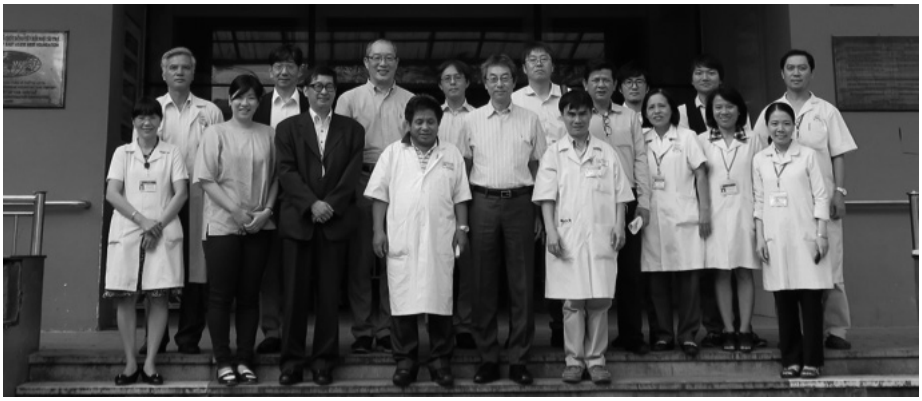
We sent total 14 Japanese experts to Hue central hospital, Ho Chi Minh Children Hospital 1, Ho Chi Minh Children Hospital 2 and National Children Hospital. And accepted 3 pediatric doctors from Hue and Ho Chi Minh into Japan. And also we started to use new consulting system in Hue and Ho Chi Minh.

CONCLUSION:

We could support to improve the medical treatment ability of the staff concerned with childhood cancer about such as a diagnosis, treatment, nursing care, supportive care. And also we thought we could increase the number of childhood cancer patients who had been diagnosed and treated step by step. Consulting system would be useful to keep in touch and continueing of study.

Ho Chi Minh Children Hospital 1

National Children Hospital in Ha Noi



Hue Central Hospital

Research No.5

1	Title (in English)	Research on tuberculosis in Viet Nam Research on spreading Beijing-genotype strains of *Mycobacterium tuberculosis*, their drug-resistance profiles and possible effects on treatment outcome
2	Title (in Japanese)	ベトナムにおける結核症に関する研究 結核菌北京型株の蔓延と多剤耐性に関わる研究
3	Main researcher	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA) Luu Thi Lien (Hanoi Department of Health) Pham Huu Thuong (Hanoi Lung Hospital)
4	Co-Researcher(s)	Vu Cao Cuong (Hanoi Department of Health) Nguyen Phuong Hoang (Hanoi Lung Hospital) Nguyen Van Hung (National Lung Hospital) Shinji Maeda (Hokkaido Pharmaceutical University School of Pharmacy) Minako Hijikata (NCGM/Research Institute of Tuberculosis, JATA) Nguyen Thi Le Hang (NCGM-BMH Medical Collaboration Center) Shinsaku Sakurada (Bureau of International Health Cooperation, NCGM)
5	Resource of fund	The Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT
6	Affiliation(s) in Viet Nam	Hanoi Lung Hospital (HLH), Viet Nam National Lung Hospital (NLH), Viet Nam
7	Period of the research	2015-2020
8	Publications	Hijikata M, Matsushita I, Le Hang NT, Thuong PH, Tam DB, Maeda S, Sakurada S, Cuong VC, Lien LT, Keicho N. **Influence of the polymorphism of the DUSP14 gene on the expression of immune-related genes and development of pulmonary tuberculosis.** Genes Immun. 2016 Jun;17(4):207-12.
9	Summary: **OVERALL PURPOSE:** • To strengthen collaborative research work on tuberculosis (TB) between Viet Nam and Japan. • To prevent generation and spread of drug-resistant TB and TB-HIV co-infection. **OUTPUT:** **A. NCGM-RIT-HLH collaboration** 1. Analysis of Hanoi-TB data containing clinical, genome-epidemiological, immunological and bacteriological information, and specimens. 2. Improvement of diagnosis, monitoring, treatment and prevention of TB and understanding process of TB infection and development. 3. Identification and reduction of risk factors to prevent spread of drug-resistant TB and TB-HIV co-infection. 4. Identification of possible risk factors to unfavorable anti-TB treatment outcomes. **B. NCGM-RIT-NLH collaboration** 1. Genome epidemiology of Mycobacterium tuberculosis (MTB) strains in Hanoi. 2. Analysis of drug-resistant MTB. 3. Analysis of reactivation and re-infection of MTB after anti-TB treatment.	

Research No.6

1	Title (in English)	Research on latent tuberculosis infection among healthcare workers in Hanoi, Viet Nam
2	Title (in Japanese)	ベトナムハノイ市の医療従事者における潜在性結核感染症の研究
3	Main researcher	Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA) Luu Thi Lien (Hanoi Department of Health) Pham Huu Thuong (Hanoi Lung Hospital)
4	Co-Researcher(s)	Vu Cao Cuong (Hanoi Department of Health) Do Bang Tam (Hanoi Lung Hospital) Minako Hijikata (NCGM/Research Institute of Tuberculosis, JATA) Nguyen Thi Le Hang (NCGM-BMH Medical Collaboration Center) Shinsaku Sakurada (Bureau of International Health Cooperation, NCGM)
5	Resource of fund	The Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT
6	Affiliation(s) in Viet Nam	Hanoi Lung Hospital (HLH), Viet Nam
7	Period of the research	2015-2018
8	Publications	Thuong PH, Tam DB, Sakurada S, Hang NT, Hijikata M, Hong LT, Ngoc PT, Anh PT, Cuong VC, Matsushita I, Lien LT, Keicho N. **Circulating granulysin levels in healthcare workers and latent tuberculosis infection estimated using interferon-gamma release assays.** BMC Infect Dis. 2016 Oct 18;16(1):580.
9	Summary: **OVERALL PURPOSE:** • To strengthen collaborative research work on tuberculosis (TB) between Viet Nam and Japan. • To study immunity of latent tuberculosis infection for a better prevention of tuberculosis. **OUTPUT:** 1. To understand human immunity of latent tuberculosis infection, and the process of TB infection and development. 2. Identification of risk factors of tuberculosis infection including occupational factors.	

Research No.7

1	Title (in English)	Research on HIV/tuberculosis (TB) in Hanoi, Viet Nam
2	Title (in Japanese)	ベトナムハノイ市における HIV 合併結核の研究
3	Main researcher	Shinsaku Sakurada (Bureau of International Health Cooperation, NCGM) Naoto Keicho (NCGM/Research Institute of Tuberculosis, JATA) Luu Thi Lien (Hanoi Department of Health) Pham Huu Thuong (Hanoi Lung Hospital)
4	Co-Researcher(s)	Vu Cao Cuong (Hanoi Department of Health) Do Bang Tam (Hanoi Lung Hospital) Minako Hijikata (NCGM/Research Institute of Tuberculosis, JATA) Nguyen Thi Le Hang (NCGM-BMH Medical Collaboration Center)
5	Resource of fund	The Program of Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), MEXT
6	Affiliation(s) in Viet Nam	Hanoi Lung Hospital (HLH), Viet Nam
7	Period of the research	2015-2018
8	Publications	None
9	Summary: **OVERALL PURPOSE:** • To strengthen collaborative research work on tuberculosis (TB) between Viet Nam and Japan. • To study immunity and risk factors of HIV/TB for a better management of tuberculosis and HIV co-infection. **OUTPUT:** 1. To understand the immunity of HIV/TB co-infection. 2. To identify risk factors of HIV/TB co-infection.	

Research No.8

1	Title (in English)	Research on improvement of CKD and dialysis management in Hanoi, Viet Nam
2	Title (in Japanese)	ベトナム国ハノイにおける慢性腎臓病管理・透析の調査と質の向上に関する研究
3	Main researcher	Fumihiko Hinoshita (Department of Nephrology, NCGM)
4	Co-Researcher(s)	Manami Tada (Department of Nephrology, NCGM)
5	Resource of fund	NCGM (26-3), Sister Renal Center Program funded by International Society of Nephrology (ISN)
6	Affiliation(s) in Viet Nam	Bach Mai Hospital
7	Period of the research	January 2014- December, 2017
8	Publications	None
9	Summary:	

OVERALL PURPOSE:

- To establish further collaboration between Dept of Nephrology, NCGM and Dept of Nephro-Urology, BMH under the Sister Renal Center Program officially approved by International Society of Nephrology (ISN)
- To establish a sophisticated means of treating patients with CKD in the preservation period and retard the progression of CKD in the patients in BMH as well as local hospitals in Northern Viet Nam
- To improve management of hemodialysis at BMH and the dialysis facilities in Hanoi

ACTIVITIES:

- Nephrologists from NCGM had lectures on CKD management, DM nephropathy, diagnosis and treatment of glomerulonephritis at BMH
- Clinical engineers from NCGM had lectures on maintenance and management techniques of hemodialysis, focusing on hemodialysis water quality
- Two nephrologists and a nurse from BMH had a training at NCGM
- Upgrading from Level C to Level B under the Sister Renal Center Program was officially approved by ISN in January, 2016 according to the good evaluation of the activities between NCGM and BMH
- We conducted a survey on hemodialysis water quality at BMH and hemodialysis facilities in Hanoi

<u>Survey on hemodialysis water quality at BMH and hemodialysis facilities in Hanoi</u>

SUBJECTS AND METHODS:

Participating institutions included 11 hospitals in Hanoi and the surrounding province. First, site visits were made to understand the current situation at these hospitals, and workshops were held on the theme of maintenance and management techniques based on ISO 23500 standards and the guidelines of the Japanese Society for Dialysis Therapy. Thereafter, RO water hardness and residual chlorine were measured every day, and a water quality survey of RO water, endotoxins in dialysate, and viable bacteria tests was conducted.

RESULTS:

Four workshops were held by December 2016, with participants from 11 hospitals. Measurements were made at 8 hospitals. Most of the participants were nurses, followed by doctors and engineering technicians. There were no clinical engineers among the participants. The results of RO water measurements showed hardness of ≤ 25 ppm at 5 of the 8

	hospitals, and total residual chlorine of ≤ 0.1 mg/dl at all 8 institutions. Endotoxins in dialysate were measured at three hospitals with devices that used ETRF, which indicated levels of ≤0.001 EU/ml at two of the hospitals. Endotoxin levels in dialysate were more than 0.05 EU/ml at all 3 hospitals when ETRF devices were not used. No bacteria were detected in the dialysate of one hospital in the viable bacteria count study. Currently, in Viet Nam, there are no dialysis associations or clinical engineer systems, and each institution manages its equipment using its own standards. The measurements in this study revealed that RO water total residual chlorine was below the reference value, but there was inconsistency between hospitals in terms of water hardness. The results for endotoxins and viable bacteria were different depending on whether or not ETRF was used, and there seems to be a need for review of machine cleaning methods and replacement of consumables and other maintenance. From the above, we felt the need for future workshops to improve maintenance and management technique standards, and for sharing of information with machine administrators. Moreover, even though participants gained an understanding of the content and methods of maintenance and management in Japan, these practices may be economically unfeasible in Viet Nam under the current circumstances. Therefore, it may be useful to establish a dialysis association in Viet Nam and prepare evidence-based guidelines.

Research No.9

1	Title (in English)	Study on effective use of the surveillance results for drug resistant pathogens in infection control
2	Title (in Japanese)	院内感染対策における耐性菌サーベイランスの活用
3	Main researcher	Hiroshi Ohara
4	Co-Researcher(s)	KVu Huy Nam (Dept. of Planning, National Institute of Malariology, Parasitology and Entomology, Viet Nam) Jeevan B. Sherchand (Dept. of Public Health, Institute of Medicine, Tribhuvan University, Nepal) Pham Thi Thanh Thuy (Dept. of Infectious Diseases, Bach Mai Hospital) Chieko Matsubara (National Center for Global Health and Medicine)
5	Resource of fund	Grants of National Center for Global Health and Medicine (27-4)
6	Affiliation(s) in Viet Nam	National Institute of Malariology, Parasitology and Entomology (NIMPE), Bach Mai Hospital
7	Period of the research	October 2015- March 2018
8	Publications	None

9 Summary:

BACKGROUND AND PURPOSE:

Recently drug resistant pathogens have been spreading. This situation is not only causing issues in treatment, but also has been important factors of nosocomial infections. In developing countries these issues are enlarging but in many counties awareness among medical staff is still low and in actual fact the exact condition is not clear.

The researchers have investigated actual conditions of drug resistant pathogens and nosocomial infection control in Viet Nam and Nepal along with providing technical guidance. This study was designed aiming at contributing to making effective control system utilizing these basic information and latest information.

METHODS:

This study started with reviewing the results of the preceding researches and technical guidance (the researchers' researches and others, document reviews) on antimicrobial resistance of bacteria (AMR), drug resistance of malaria parasites and actual situation of nosocomial infection control. Thereafter, we will conduct comparative analysis between the 2 countries, discuss with health authorities on appropriate control measures and summarize as a proposal.

PROGRESS OF THE STUDY:

1. Viet Nam

Latest information on AMR was collected by interview with tertiary hospital staff and document reviews. High resistance rate for antibiotics have been reported (ex. 75% of Streptococcus pneumoniae is multi-drug resistant, 71% of Klebsiella pneumoniae is penicillin resistant and 92% is erythromycin resistant, high resistant rate in homophiles). As the cause of resistance, inappropriate use along with governance of antibiotics, abuse for livestock, and communication gap between medical and agricultural sectors etc. were suspected.

Latest information on drug resistance of malaria was also collected. During the past 20 years incidence of malaria has remarkably decreased in Viet Nam, however in recent years new issues such as emergence of new endemic areas in Cambodian border, artemisinin resistance malaria and so on, have been reported.

	2. Nepal We summarized the results of surveys on multi-drug resistant bacteria which we have conducted in Nepal up to 2016. These previous studies have revealed the spread of multi-drug resistant bacteria (New Delhi methalo-β-lactamase producers, etc.) in medical settings, however measures to address these situations have not been taken effectively. Furthermore, as the results of discussions with authorities in Nepal the following issues were pointed out: inappropriate use of antibiotic, ineffective feedback mechanism of the information on bacterial resistance, inappropriate nosocomoial infection control, poor antibiotic stewardship, weak intervention by the government, etc. As the results of the above researches in Viet Nam and Nepal (1, 2) along with discussion with authorities, the following challenges that we should address, were suspected: use of antibiotics and anti-malaria drugs along with their governance, feedback of resistance information to clinical settings, nosocomial infection control system, sales system of antibiotics, use of antibiotics in livestock farming, population movement, etc. Further investigations on these suspected issues are needed. As one of the investigations a survey on antibiotics stewardship was conducted in leading hospitals in Kathmandu City and reported at the annual assembly of the Japan Association for International Health. Regarding 1 and 2, the main researcher will describe in NCGM Technical Report.

Research No.10

1	Title (in English)	Research on Epidemiology, Diagnosis and Treatment for Healthcare Associated Infection and Antimicrobial Resistant Bacteria in Viet Nam
2	Title (in Japanese)	ベトナム拠点における医療関連感染症及び後期若耐性菌感染症に関する検討
3	Main researcher	Norio Ohmagari MD, MSc, PhD (Disease Control and Prevention Center, National Center for Global Health and Medicine) Assoc. Prof. Nguyen Quoc Anh MD., PhD. (Bach Mai Hospital)
4	Co-Researcher(s)	Teruo Kirikae, MD, PhD (NCGM) Tohru Miyoshi-Akiyama, PhD (NCGM) Tatsuya Tada, PhD (NCGM) Kayoko Hayakawa, MD., PhD (NCGM) Nozomi Takeshita, MD., PhD (NCGM) Satoshi Kutsuna, MD., PhD (NCGM) Maki Nagamatsu (NCGM) Mitsuhiro Tsuchiya, MSc (NCGM) Pham Thi Phuong Thuy BA. MPH (NCGM-BMH Medical Coloration Center) Prof. Nguyen Gia Binh, MD., PhD (Head of ICU) Doan Mai Phuong MD., PhD (Microbiology Dept., Bach Mai Hospital) Do Van Thanh (Infectious Dept. and International Dept. Bach Mai Hospital)
5	Resource of fund	Japan Initiative for Global Research Network on Infectious Diseases (Funded from Ministry of Education, Science and Technology, Culture and Sport of Japan)
6	Affiliation(s) in Viet Nam	Bach Mai Hospital
7	Period of the research	April 1, 2012 to March, 2017
8	Publications	None
9	Summary:	

1. Epidemiology and outcomes of ventilator-associated pneumonia in intensive care units in Viet Nam

BACKGROUND:

In recent years, there is a need for responses to nosocomial infections in developing countries. While it is necessary to introduce more rapid and appropriate treatment and preventive measures, the epidemiological data serving as the basis of such responses are dominated by those obtained in developed countries.

METHOD:

We conducted a prospective cohort study of patients diagnosed with VAP in the ICU of Bach Mai Hospital in Hanoi, Viet Nam, during the period from October 2015 to April 2016.

RESUTS:

During the observation period, 44 patients were diagnosed with VAP. The median age of the patients was 60 years (IQR: 48–68 years). Among these patients, 33 (75%) were male, and 24 (55%) had such underlying diseases as diabetes, chronic heart disease, chronic renal disease, or COPD. Among the identifiable causative agents, the most frequent ones were A. baumanii (11 patients), K. pneumoniae (7 patients), and P. aeruginosa (4 patients). The number of patients who required treatment with colistin based on the results of drug sensitivity tests was 20 (45%). The number of deaths within 7 days after

onset was 6 (14%) and that within 30 days was 16 (36%). The median number of days spent in the ICU after onset was 9 days (IQR: 3–17 days).

CONCLUSION:

The study results suggest that both the frequency of VAP due to multiple-drug-resistant bacteria and the mortality rate are high in ICUs in Viet Nam. Further research, including a study of the proper empiric therapy, is considered necessary for improving the prognosis.

2. Prospective cohort study of epidemiological findings with nosocomial bloodstream infections in the ICU of a critical care medical center in Viet Nam (Bach Mai Hospital in Hanoi)

BACKGROUND:

Nosocomial infections are a challenging issue for medical facilities around the world. The mortality of bloodstream infection (BSI), the leading nosocomial infection, is high. In Viet Nam, however, no sufficient surveillance of nosocomial infections is performed at medical facilities and the epidemiological information on nosocomial BSI is limited.

METHOD:

We conducted a prospective cohort study of patients diagnosed with BSI in the ICU of a critical care medical center in Viet Nam (Bach Mai Hospital in Hanoi) during the period from December 2013 to August 2015.

RESULTS:

During the observation period, 100 patients were diagnosed with BSI. We defined nosocomial infection in case of patients whose blood for culture was collected 48 hours or longer after admission, or those whose blood for culture was collected before 48 hours after admission, but who had other medical exposure, such as to patients with a nosocomial infection. We analyzed 90 cases of patients with BSI associated with a nosocomial infection, but not with contamination. Among these patients, 59 patients were male (66%) with a median age at the time of the diagnosis of 57 years (IQR: 41–72 years); and 53 patients (59%) had such underlying diseases as diabetes, chronic cardiac disease, chronic renal disease, COPD, blood tumor, or solid tumor. The sources of infection were: CRABSI in 27 patients (30%); PLABSI in 2 (2.2%); others in 32 (35.6%); and unknown in 29 (32.2%). The major causative agents were: Candida spp. in 26 patients (29%); Enterococcus spp. in 19 (19%); E. coli in 13 (13%); A. baumanii in 10 (10%); K. pneumoniae in 10 (10%); MRSA in 4 (4%); and MSSA in 3 (3%). The total hospitalization period was 17 (9-23) days. Of 65 patients who were followed up on day 30 after blood culture collection, 31 patients (48%) were alive and 34 (52%) had died. Univariate logistic regression analysis indicated the following risk factors for death: having any underlying disease (odds ratio: 4.3; 95% CI: 1.5-12.8; $p < 0.01$); and having chronic heart disease (odds ratio: 3.4; 95% CI: 1.1-10.5; $p = 0.03$). Multivariate logistic regression analysis with age and gender as covariates indicated: having chronic heart disease (odds ratio: 3.5; 95% CI: 1.0–11.9; $p = 0.05$).

CONCLUSION:

The epidemiology of nosocomial BSI in an ICU in Viet Nam was different from that in developed countries; we hope that it will be further researched in the future.

Educational activities:

Training Course on Case Management of Tropical Infectious Diseases was held at Ho Chi Minh City, Viet Nam (December 2016).

Research No.11

1	Title (in English)	Molecular epidemiology of multidrug-resistant Gram-negative pathogens in medical settings in Viet Nam
2	Title (in Japanese)	ベトナムの医療施設における多剤耐性グラム陰性菌の分子疫学解析
3	Main researcher	Teruo Kirikae
4	Co-Researcher(s)	Norio Ohmagari, Miyoshi-Akiyama Tohru, Tatsuya Tada
5	Resource of fund	J-GRID
6	Affiliation(s) in Viet Nam	Bach Mai Hospital, Cho Ray Hospital
7	Period of the research	2015-2016
8	Publications	• Tada T, Tsuchiya M, Shimada K, Nga TTT, Thu LTA, Phu TT, Ohmagari N, Kirikae T. Dissemination of Carbapenem-resistant Klebsiella pneumoniae clinical isolates with various combinations of Carbapenemases (KPC-2, NDM-1, NDM-4, and OXA-48) and 16S rRNA Methylases (RmtB and RmtC) in Viet Nam. ***BMC Infect Dis.*** 2017 Jul 4;17(1):467 • Tada T, Nhung PH, Shimada K, Tsuchiya M, Phuong DM, Anh NQ, Ohmagari N, Kirikae T. Emergence of colistin-resistant Escherichia coli clinical isolates harboring mcr-1 in Viet Nam. ***Int J Infect Dis.*** In press
9	Summary: Twenty-seven clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* with MICs ≥4 mg/L for imipenem or meropenem were obtained from inpatients in a hospital in Viet Nam. All the isolates harbored one gene encoding a carbapenemase, including KPC-2, NDM-1, NDM-4 or OXA-48. Of the isolates, 13 were resistant to arbekacin with MICs ≥256 mg/L and to amikacin with MICs ≥512 mg/L. These isolates harbored a gene encoding a 16S rRNA methylase, either RmtB or RmtC. Eighteen and four isolates belonged to the international clones ST15 and ST16, respectively. This is the first report of KPC-2, NDM-4 and OXA-48 producers in a medical setting in Viet Nam. These results suggest that carbapenem-resistant *K. pneumoniae* isolates belonging to international clones have been spreading in medical settings in Viet Nam, and that these isolates harbored genes encoding various combinations of carbapenemases and 16S rRNA methylases. Moreover, mcr-1 harboring *Escherichia coli* isolates were obtained in a medical setting in Hanoi, Viet Nam. The mcr-1 was first detected on a plasmid in colistin-resistant *Escherichia coli* from livestock and patients in China. We reported the emergence of colistin-resistant *E. coli* clinical isolates harboring mcr-1 on the chromosomes in Viet Nam. To our knowledge, this is the first report of hospital-acquired *E. coli* isolates harboring mcr-1 in a medical setting in Viet Nam.	

Research No.12

1	Title (in English)	International collaborative research on diagnosis and treatment based on clinicopathological study of highly pathogenic avian influenza virus infection
2	Title (in Japanese)	高病原性鳥インフルエンザ感染症の臨床病理学的解析に基づく診断・治療に関する国際連携研究
3	Main researcher	Nguyen Gia Binh (Bach Mai Hospital) Vu Thi Tuong Van (Bach Mai Hospital) Noriko Nakajima (NIID, Japan) Tsutomu Kageyama (NIID, Japan) Jin Takasaki (DCC, Respiratory Medicine, NCGM, Japan)
4	Co-Researcher(s)	**Viet Nam:** Dao Xuan Co, Nguyen Dang Tuan, The Pham Thach (ICU), Truong Thai Phuong, Le Thi Ngan (Microbiology), Do Van Thanh (International and ID Dept.), Do Duy Cuong (Infectious disease), Phan Thu Phuong (Respiratory Center) **Japan**: Shoji Kawachi (teikyo Univ.), Ikuyo Takayama(Center for Influenza Virus Research, NIID), Tadaki Suzuki, Akira Ainai (Dept. of Pathology, NIID), Pham Phuong Thuy (MCC).
5	Resource of fund	Japan Agency for Medical Research and Development, AMED
6	Affiliation(s) in Viet Nam	Bach Mai Hospital
7	Period of the research	September 2015 to March 2019
8	Publications	None
9	Summary:	

Study 1. RT-LAMP study in intensive care unit (ICU), Infectious disease department (ID), and Respiratory Center in BMH (2015-2017)

Study 2. The Study for Severe or Fatal Influenza in ICU, BMH (2016-2019)

Seasonal and avian influenza viruses differ in their sites of infection in human respiratory organs. While seasonal influenza viruses mainly affect epithelial cells in the upper respiratory tract, avian influenza viruses primarily infect alveolar epithelial cells, causing acute severe respiratory failure. However, depending on specific viral, host, and co-infection factors, seasonal influenza viruses can also cause severe pneumonia, ARDS, encephalopathy, or myocardial diseases. The aim of this collaborative research is to elucidate the mechanism and role of these factors.

In the study 1, we isolated different influenza virus clones from the upper airway and the lower airway derived from the same patient. We aim to determine specific viral factors by examining the association between specific genomic sequences and the severity of the diseases in the study 2.

One of the primary host factors contributing to severe or fatal influenza is reported to be hypercytokinemia, called "*Cytokine Storm*", which leads to dysregulated systematic inflammation. In addition, the receptor for advanced glycation end-products (RAGE) and angiopoietin II (Ang-2) were featured as a biomarker of ARDS related to the epithelium damage and endothelial damage, respectively. By integrating inflammatory mediators/biomarkers with the clinical informatics in the influenza patients, we aim to identify the key mediators associated with severity. Additionally, the results from this study

	may be helpful in establishing a novel anti-inflammatory therapy for the severe or fatal influenza. Bacterial factors contributing to influenza pathogenesis have been discussed since the late 18th century. We revealed that multiple viral infection and bacterial co-infections were observed in hospitalized patients with respiratory infectious diseases in ICU, respiratory department and infectious disease department in Bach Mai Hospital in the study 1. Co-infection with other pathogens should be examined as a possible severity factor. In addition, in fatal cases, the pathological and molecular biological examination with post-mortem biopsied lung tissues would be necessary to elucidate the pathogenesis of progression of severe influenza.

2. The International Promotion of Japan's Healthcare Technologies and Services in 2016

This program has been commissioned by the Ministry of Health, Labour and Welfare Japan since fiscal 2016. The purpose is to extend Japanese healthcare and services as well as experiences on health systems to the world. Areas of the program include (1) Japanese health technologies, medical devices, and medicines, (2) management of health facilities, (3) health regulation, medical insurance, medical environment management, (4) health information systems, and (5) global health issues such as emerging and re-emerging infectious diseases, an aging society, maternal and child health, nutrition, non-infectious diseases, and disaster response. The program consists of two methods; dispatch of Japanese specialists and acceptance of foreign trainees in Japan.

The five programs were carried out in Viet Nam by NCGM as follows:

- The project for strengthening Radiological technology and Pharmacy department at the Hospitals
- Strengthening Management Capability for Quality and Safety in Healthcare
- Project to Support Improvement of the Quality of Stroke Care in the Socialist Republic of Viet Nam
- Support for Strengthening Medical Treatment Ability of the Childhood Cancer
- Strengthening clinical capacities through Medical Collaboration Center in Viet Nam

3. Other activities, topics

International Nursing Practicum for Nursing Students at the National College of Nursing, Japan

We conducted a one-week nursing practicum in Viet Nam as part of the compulsory subject of International Nursing Practicum for fourth-year undergraduate students in collaboration with Hai Duong Medical Technical University (HMTU), Viet Nam.

The International Nursing Practicum is designed to enhance students' abilities to understand the current situation of nursing and health care practice in developing countries, whereby promoting the development of nursing theory with international perspectives to facilitate international health cooperation in nursing. As a prerequisite, students are required to complete the international nursing theory course.

One hundred students were divided into 14 groups, and each group was assigned several presentation topics to work toward the goals of the practicum. Before departing for Hai Duong, Viet Nam, where the practicum took place, students rehearsed their presentation in English in order to improve the quality of presentation and share their knowledge among groups in preparation for the practicum.

On the first day of the practicum, students gave their presentation in front of the faculty members and undergraduate students at HMTU and NCNJ. They then visited several institutions in Hai Duong province, such as provincial hospital, district hospital, specialty hospital, leprosy village, social welfare institution, and community health center.

On the last day of the practicum, each group presented the summary of students' experiences at HMTU. Back in Japan at NCNJ, a poster presentation was held in the entrance hall, which gave students an opportunity to summarize what they had learned through the practicum in both Japan and Viet Nam, as well as to inform other junior students and faculty members of their valuable experiences.

Student evaluation revealed that most students wished to contribute what they had learned to nursing activities in Japan and promotion of international health cooperation.

PLOS | ONE

RESEARCH ARTICLE

Incidence of AIDS-Defining Opportunistic Infections and Mortality during Antiretroviral Therapy in a Cohort of Adult HIV-Infected Individuals in Hanoi, 2007-2014

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Data Availability Statement: The data used for our analyses cannot be made publicly available because we did not receive a permission for such disclosure from the local ethics committee. Data are available from the database of Hanoi Cohort study via the corresponding author after getting the authorization of the Institutional Ethics Committee.

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Abstract

Background

Although the prognosis for HIV-infected individuals has improved after antiretroviral therapy (ART) scale-up, limited data exist on the incidence of AIDS-defining opportunistic infections (ADIs) and mortality during ART in resource-limited settings.

Methods

HIV-infected adults in two large hospitals in urban Hanoi were enrolled to the prospective cohort, from October 2007 through December 2013. Those who started ART less than one year before enrollment were assigned to the survival analysis. Data on ART history and ADIs were collected retrospectively at enrollment and followed-up prospectively until April 2014.

Results

Of 2,070 cohort participants, 1,197 were eligible for analysis and provided 3,446 person-years (PYs) of being on ART. Overall, 161 ADIs episodes were noted at a median of 3.20 months after ART initiation (range 0.03–75.8) with an incidence 46.7/1,000 PYs (95% confidence interval [CI] 39.8–54.5). The most common ADI was tuberculosis with an incidence of 29.9/1,000 PYs. Mortality after ART initiation was 8.68/1,000 PYs and 45% (19/45) died of AIDS-related illnesses. Age over 50 years at ART initiation was significantly associated with shorter survival after controlling for baseline CD4 count, but neither having injection drug use (IDU) history nor previous ADIs were associated with poor survival. Semi-competing

(http://www.amed.go.jp/en/). SO was the receiver as the primary investigator. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interest exist.

risks analysis in 951 patients without ADIs history prior to ART showed those who developed ADIs after starting ART were at higher risk of death in the first six months than after six months.

Conclusion

ADIs were not rare in spite of being on effective ART. Age over 50 years, but not IDU history, was associated with shorter survival in the cohort. This study provides in-depth data on the prognosis of patients on ART in Vietnam during the first decade of ART scale-up.

Introduction

Antiretroviral therapy (ART) has resulted in a remarkable decline in acquired immunodeficiency syndrome (AIDS)-related death among HIV-infected individuals worldwide [1–6]. As prognosis has improved, reports from resource-rich countries have shown that the causes of death in HIV-infected individuals have changed, with cancers or cardiovascular diseases or liver-related diseases becoming the leading causes of mortality. [7–10].

Although a detailed understanding of causes of death and associated risk factors is crucial to the appropriate management of HIV-related diseases and co-morbidities, the specific causes of death have not been well described in resource-limited settings. Additionally, all-cause mortality of HIV-infected individuals is still higher in resource-limited than resource-rich countries [2]. Despite the high efficacy of ART, opportunistic infections (OIs) can develop while the patient is on ART, either due to the unmasking of subclinical infection that occurs with immune recovery, or due to prolonged immunosuppression. Treatment failure also facilitates the development of OIs at any time during ART. As a result, AIDS-defining illnesses (ADIs) have remained major morbidities in HIV-infected individuals in resource-limited settings, even in the era of ART [11–13]. Furthermore, previous reports have shown high mortality rates among injection drug users (IDUs) from drug overdose, suicide, accidents, violence, or liver-related diseases [14, 15]. In Vietnam, where a large part of the HIV epidemic has been driven by IDUs, the mortality rate among IDUs with or without HIV infection was reported to be as much as 13-fold higher than that in the general population [16]. Thus, the overall prognosis of HIV-infected individuals in Vietnam may partly reflect the social and epidemiological characteristics of IDUs. However, few studies have addressed the incidence of AIDS, mortality, or specific causes of death in HIV-infected individuals receiving ART in Vietnam [17].

In this prospective cohort study of HIV-infected adults on ART in two large hospitals in urban Hanoi, Vietnam, we aimed to describe the incidence of ADIs, specific causes of death, mortality rates, and risk factors associated with the development of ADIs and shorter survival time, from 2007 through 2014.

Methods

Study Population and Data Collection

A prospective cohort study of HIV-infected adults was conducted in two large hospitals in urban Hanoi, Vietnam: Bach Mai Hospital (BMH) and the National Hospital of Tropical Diseases (NHTD). Patients attending the two HIV clinics were recruited from April 2011 through October 2012 in BMH and from 2007 to 2013 in NHTD by contacting all who were on ART. Participants were enrolled after providing written informed consent as set out in the study

protocol approved by the ethics committee and the institutional ethical review boards. Participants in the cohort had different histories with respect to ART prior to enrollment. We excluded from the present analysis those who had received ART for more than one year prior to enrollment. Information was obtained on ADIs that occurred before and after ART, non-ADI clinical events, medication and laboratory data using standardized forms at enrollment and at each follow-up visit scheduled six-monthly until the end of April 2014. The causes of death were classified according to the Coding of Causes of Death in HIV (CoDe) [18]. The Center for Disease Control's (CDC) list for AIDS-defining illnesses [19] was used for coding all ADIs except wasting syndrome, for which it was difficult to determine the date of onset. However, wasting syndrome was used in the classification of causes of death as an AIDS-related death [18]. A second or third episode of tuberculosis (TB) in a same person was counted as a new event if it occurred after completing treatment and if more than a year had passed since the diagnosis of a previous TB episode. Diagnosis, prophylaxis, treatment of OIs, and ART were based on the Vietnamese national guidelines [20–22], which were updated twice during the study period according to updates in the World Health Organization (WHO) ART guidelines [23, 24]. The CD4 count for ART indication changed from 200/mm^3 to 250/mm^3 in 2009 [21] and to 350/mm^3 in 2011. Zidovudine (AZT) or stavudine (d4T) was replaced with tenofovir (TDF) in the preferred first-line regimen in 2011 [22]. Follow-up was censored when patients were transferred to other hospitals or lost from clinical care in the study sites for more than 12 months.

The study protocol was approved by the ethics committee in the Vietnamese Ministry of Health (No:1666/QD-BYT) and the institutional ethical review boards in BMH, NHTD and the National Center for Global Health and Medicine (NCGM) in Tokyo, Japan (NCGM-G-001074-01).

Statistical Analysis

Incidence rate of ADIs were calculated by dividing the number of patients who developed an event by the number of person-years (PYs) on ART. In order to identify the factors for incidence of ADIs, we fitted a Poisson regression model. We estimated the effects of potential risk factors on all-cause mortality by fitting a Cox proportional hazards model. These analyses were conducted by including one covariate at a time (univariate analysis) or all covariates at the same time (multivariable analysis) into the regression models. In addition, we estimate survival functions for ADIs and death using joint semi-competing risks analysis [25, 26] to investigate the impact of developing ADIs on occurrence of subsequent deaths among those without a history of ADI before ART. Such analyses make explicit use of information on the timing of death following ADI, which would be ignored in a traditional competing risks analysis [27]. This, in-turn, permitted the investigation of how the risk of death changed over time, depending on whether a new ADI event occurred. We computed the explanatory hazard ratio, defined by the ratio of the risk of death with and without a new ADI at any given point in time [26]. For comparison, we also presented results from univariate Weibull regression analyses of ADIs and death. For all statistical analyses, differences were considered significant if the p value was less than 0.05. Analyses were performed using STATA version 12 (StataCorp LP, TX, U.S.A.) and R Statistical Software version 3.2.0 (Foundation for Statistical Computing, Vienna, Austria) [28].

Results

Characteristics of the Study Population

In total, 2,070 individuals were enrolled to the cohort from October 2007 until the end of 2013. Of those, 190 who had never received ART and 683 who had been on ART for more than a

year at enrollment were excluded from the analysis. The remaining 1,197 were assigned to the present analysis, and contributed to 3,446 PYs. Of these, 951 had not developed ADIs at the time of starting ART; they contributed 2,763 PYs. The characteristics of the participants are shown in Table 1. Overall, 63% of the study participants were men and the median age was 32 years. Sexual contact was reported as a possible route of infection in 74% participants; 28% declared previous injection drug use; while the proportion of hepatitis C (HCV) coinfection, which strongly indicates possible multiple-needle-sharing exposure, was 42%. Twenty participants had other possible risk factors, including tattooing, accidents that happened while medical care was being administered, or receiving blood products. Of these participants, three also reported sexual contact as a possible route of infection. Sixty-six (5.5%) refused to answer or did not answer questions on HIV risk factors. The majority of patients with injection drug use experience were men and the percentage of IDUs was considerably greater in men than in women (44.5% in men vs. 1.3% in women; $p<0.001$). ART was started a median of 2 months after HIV diagnosis at a median CD4 count of 110/mm3. The median CD4 count at baseline increased significantly after the Vietnamese guideline on ART indication were changed in 2011 (median 75/mm^3 before 2011 vs. 269/mm^3 after 2011; $p<0.001$). According to these guidelines, the majority of the participants started ART regimens that included a nucleos(t)ide reverse transcriptase inhibitors (NRTI) backbones of either AZT plus lamivudine (3TC), d4T plus

Table 1. Characteristics of Study Participants.

			ADIs at baseline				
	Total (n = 1,197)		Yes (n = 246)		No (n = 951)		*p*
Age at starting ART, median (range)	32	(18–73)	33	(19–73)	32	(18–70)	<0.001
Gender male, n (%)	750	(62.7)	195	(79.3)	555	(58.4)	<0.001
HIV risk factor							
Sexual contact, n (%)	890	(74.4)	174	(70.7)	716	(75.3)	0.20
Injection drug use, n (%)	340	(28.4)	101	(41)	239	(25.1)	<0.001
Other/unknown, n (%)	91	(7.6)	14	(5.7)	77	(8.1)	0.36
HBs antigen positive, n (%)[a]	161	(13.5)	31	(12.6)	132	(14.3)	0.40
Anti-HCV antibody positive, n (%)[a]	441	(42.0)	124	(50.4)	317	(39.5)	<0.001
CD4 count at baseline, median (range)[a]	110	(1–693)	42	(1–550)	144	(1–693)	<0.001
Initial ART regimen, NRTI							
AZT+3TC/FTC	576	(48.1)	93	(37.8)	483	(50.8)	<0.001
d4T+3TC/FTC	318	(26.6)	72	(29.3)	246	(25.9)	0.32
TDF+3TC/FTC	294	(24.6)	81	(32.9)	213	(22.4)	0.001
Others	9	(0.8)	0		9	(0.9)	
Initial ART regimen, third drug							
NVP	548	(45.8)	62	(25.2)	486	(51.1)	<0.001
EFV	635	(53.0)	183	(74.4)	452	(47.5)	<0.001
Others	14	(1.2)	1	(0.4)	13	(1.4)	
Time from ART start to enrollment, median months (range)	5.2	(0–12)	4.6	(0–12)	5.4	(0–7.9)	0.20
Time from HIV diagnosis to ART, median months (range)	2.0	(0–211)	1.2	(0–135)	2.3	(0–211)	<0.001
Time on ART, median months (range)	32	(0.6–91)	30	(0.6–91)	33	(1–91)	0.10

Note:

[a]–Data were unavailable in 20 subjects for HBs antigen, in 149 for Anti-HCV antibody and in 167 for CD4 count at baseline.

ADI: AIDS-defining illness; NRTI: nucleoside reverse transcriptase inhibitor; AZT zidovufine; 3TC: lamivudine; FTC: emtricitabine; d4T: stavudine; TDF: tenofovir; NVP: nevirapine; EFV: efavirenz

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3TC or TDF plus 3TC (48.1%, 26.6%, and 24.6%, respectively) plus the non-nucleoside reverse-transcriptase inhibitor (NNRTI) nevirapine (NVP) or efavirenz (EFV)– 45.8% and 53%, respectively. Those who experienced ADIs before ART were less likely to have AZT plus 3TC and more likely to have TDF plus 3TC as an NRTI backbone and EFV as the third drug in their initial ART regimens. Those eligible for analysis were enrolled to the cohort at a median of 5.2 months after ART initiation: 678 (56.6%) joined the cohort within the first six months of ART. Of 1,195 participants who had HIV viral load results available for at least two time points, 1,141 (95.5%) had achieved viral suppression, at less than 200 copies/ml, prior to censoring. Compared to those without any ADI episodes before ART, participants who had experienced ADIs before starting ART were older, had lower CD4 counts at baseline, and had started ART in an earlier calendar year. In addition, a significantly greater proportions were men, IDUs or had tested positive for anti-HCV antibodies (Table 1).

Incidence of AIDS-Defining Opportunistic Infections and Risk Factors

Estimated probabilities of participants not developing new ADIs, and estimated overall survival probabilities after starting ART are illustrated in Fig 1a and 1b for all 1,197 participants and in Fig 2a and 2b for the 951 without previous ADIs before ART, respectively. In total, 161 episodes of ADIs were observed after starting ART in 137 patients (46.7/1000PYs, 95% confidence interval [CI] 39.8–54.5) at a median of 3.2 months on ART (range 0.03–75.8). The number of episodes and the incidence rate of each ADI are shown in Table 2. The most common ADI was TB (28.4/1,000 PYs, 95% CI 23.1–34.7), followed by toxoplasmosis and pneumocystis pneumonia.

From the univariate analyses, male gender, injection drug use and anti-HCV-antibody positivity were significantly associated with higher risk of developing new ADIs, while the multivariable analyses showed only age older than 50 years old and CD4 counts less than 200 /mm^3 at baseline to be significant among the 951 patients without ADI history at baseline (Table 3). ADIs prior to ART was not statistically associated with the risk of acquiring new ADIs.

Mortality and Causes of Deaths

In total, 42 (3.5%) participants died after median 1.13 years on ART (range 0.05–7.27 years) with median CD4 count 126/mm^3 (range 3–479/mm^3) at death, giving a mortality rate of 11.9/1,000 PYs (95% CI 8.78–16.5). Nineteen participants who died (45.2%) had viral loads of less than 200 copies/ml. Of the 33 patients for whom the causes of death was successfully identified, 19 died due to ADIs and 14 died due to other causes (Table 4). There was no significant differences in survival time on ART between the 19 AIDS-related deaths and 14 non-AIDS deaths (median of 1.05 years in AIDS-related deaths vs. 1.61 years in non-AIDS deaths; $p = 0.29$).

The Cox proportional hazards model showed that male gender, older age, injection drug use, baseline CD4 less than 200/mm^3, and ADIs prior to ART were associated with shorter survival in univariate models, while only older age (over 50 years old in all 1,197 patients and over 40 years old in 951 patients without ADIs prior to ART) was significant in multivariate models (Table 5). In the semi-competing risks analysis of the 951 patients without ADIs prior to ART, deaths following new ADI events appeared to be more likely to occur within a short period after starting ART (Fig 2e), while the risk of deaths without acquiring ADIs showed a gradual increase overtime (Fig 2d). In Fig 3, we see that the explanatory hazard ratio is substantially larger than 1 immediately after ART initiation, implying that the development of the ADIs considerably increases the risk of death at the beginning of ART. Subsequently, however, the explanatory hazard ratio rapidly declined and the risk of death without acquiring ADIs outweighed that following ADIs after 4.1 months of ART.

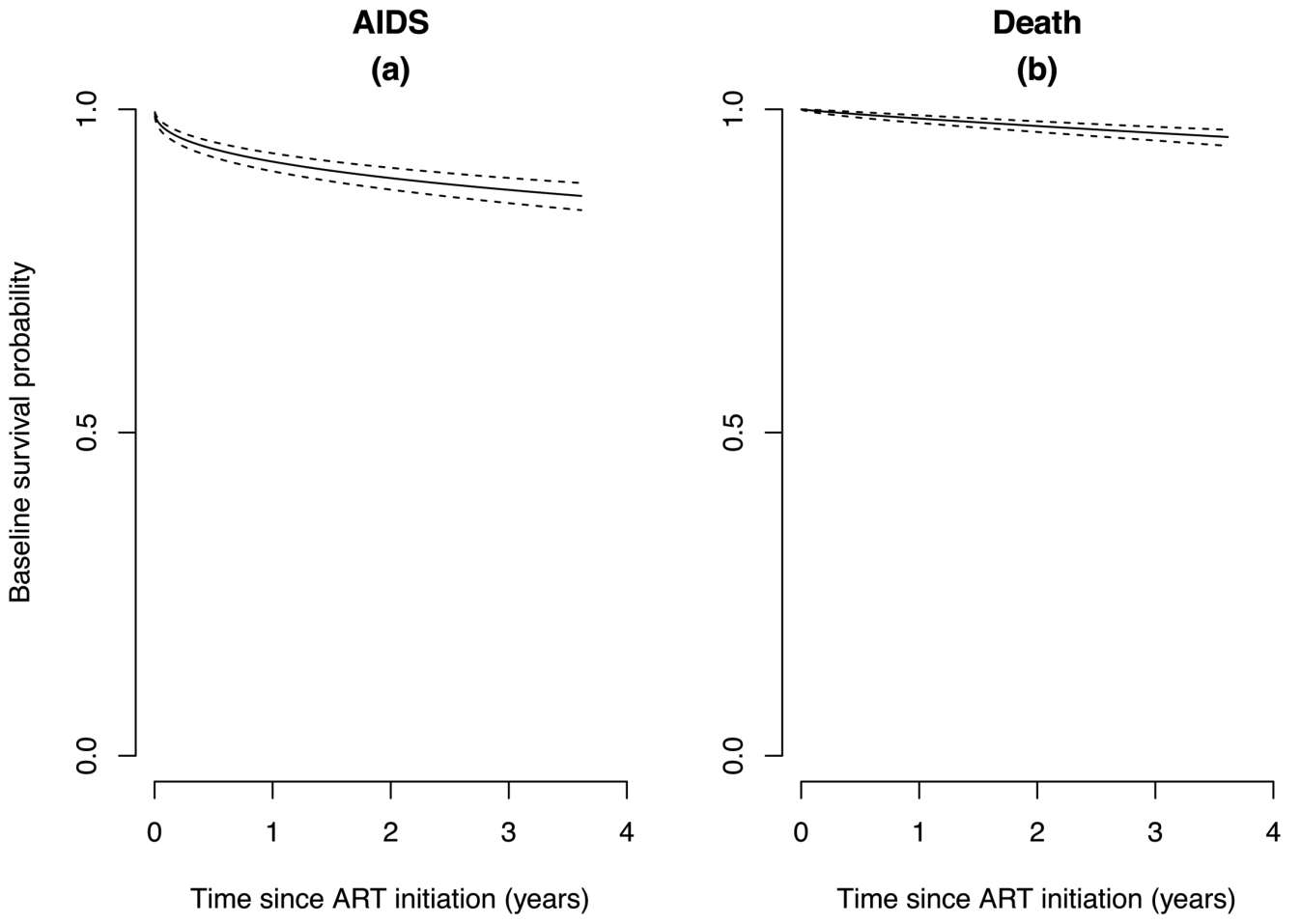

Fig 1. Survival Probabilities on ART in All Study Participants. (a) Survival without new AIDS events on ART. (b) Overall survival on ART. Dotted lines indicate ranges of 95% CIs.

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Discussion

In this study, we described the incidence of ADIs and mortality after ART initiation in a cohort of HIV-infected adults in two large hospitals in urban Hanoi, Vietnam. Although 11.4% of study participants developed ADIs after starting ART, the overall mortality rate was low, irrespective of ADI history prior to ART. Almost half the deaths were attributable to ADIs and older age at baseline was statistically associated with shorter survival. IDU history was not a significant factor for developing ADIs or for increased mortality. This study provides in-depth data on the prognosis of HIV-infected individuals who started ART in Vietnam, where there has been the extensive ART scale-up over the last decade.

The overall incidence of ADIs during ART was as high as 46.1/1,000PYs in this cohort. There have been a few reports on the incidence of OIs during ART in HIV-infected individuals thus far [11–13], one of which was from the Swiss HIV Cohort Study, reporting 36/1,000PYs [29]. The ADI incidence is largely influenced by regional factors, such as endemic levels of specific opportunistic infections, the ART indication in the regional guidelines, and accessibility of healthcare. A possible explanation for the higher ADI incidence in our cohort (vs. the Swiss

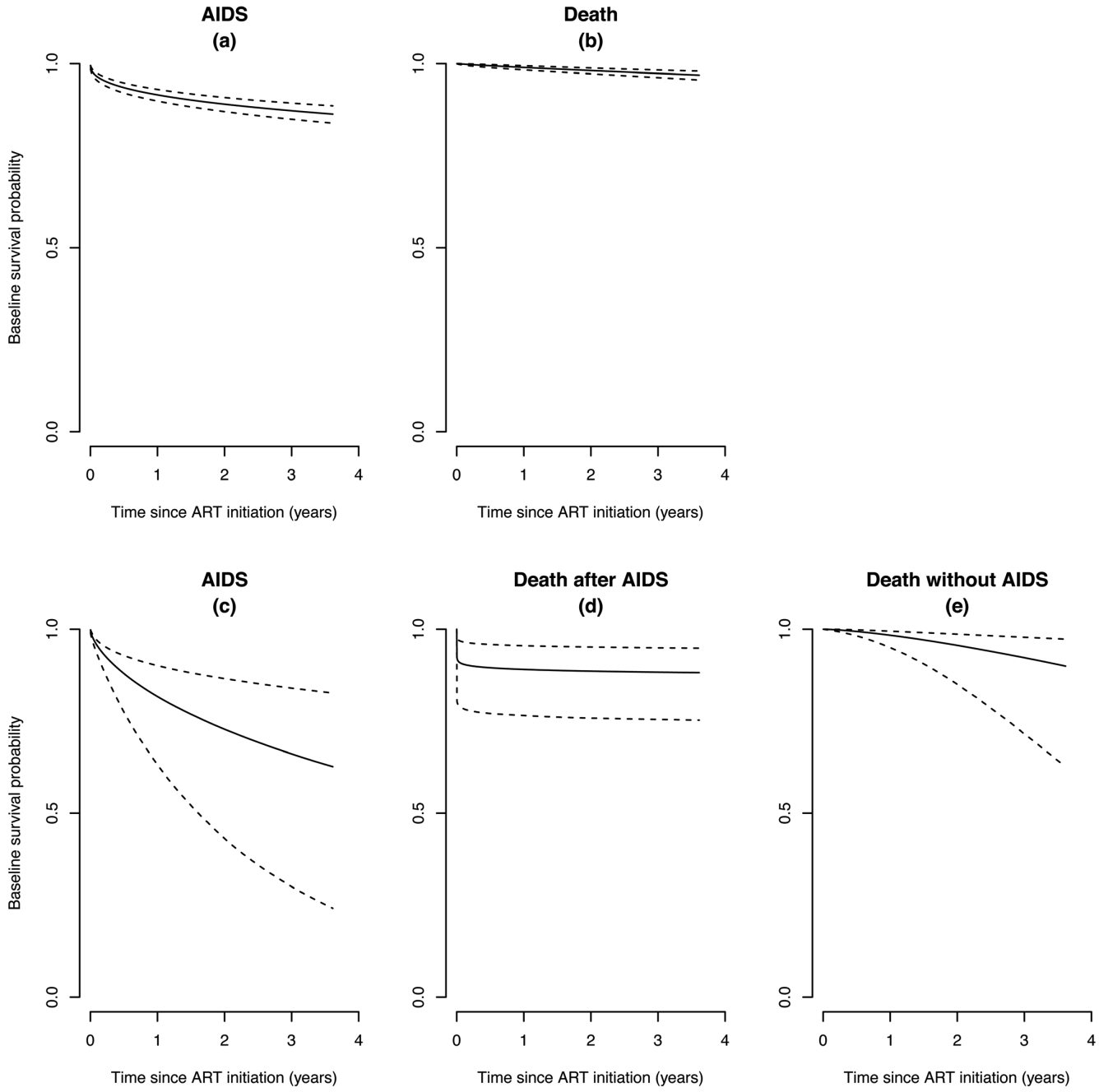

Fig 2. Survival Probabilities on ART in Subjects without AIDS History before ART. (a) Survival without AIDS on ART in Weibull regression analysis. (b) Overall survival on ART in Weibull regression analysis. (c) Survival without AIDS on ART in semi-competing risks analysis. (d) Time to death without acquiring AIDS during ART in semi-competing risks analysis. (e) Time to death following new AIDS events during ART in semi-competing risks analysis. Dotted lines indicate ranges of 95% CIs.

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Table 2. Incidence Rates of AIDS-Defining Opportunistic Infections during ART.

AIDS-defining illnesses (ADIs)	Number of episodes, Incidence Rate (/1000 PYS), (95%CI)					
	All (n = 1,197; 3,446 person-years)			No prior ADIs at baseline (n = 951; 2,763 person-years)		
Any ADIs	161	46.7	(39.8–54.5)	130	47.1	(39.3–55.9)
Tuberculosis	98	28.4	(23.1–34.7)	79	28.6	(22.6–35.6)
Toxoplasmosis of brain	16	4.64	(2.65–7.54)	14	5.01	(2.77–8.50)
Pneumocystis pneumonia	10	2.90	(1.39–5.34)	8	2.89	(1.25–5.70)
Esophageal candidiasis	7	2.03	(0.82–4.19)	7	2.53	(1.02–5.22)
Cytomegalovirus retinitis	6	1.74	(0.64–3.79)	6	2.17	(0.80–4.72)
Mycobacterium avium complex	6	1.74	(0.64–3.79)	4	1.45	(0.39–3.71)
Recurrent bacterial pneumonia	5	1.45	(0.47–3.39)	4	1.45	(0.39–3.71)
Cryptococcus	4	1.16	(0.32–2.97)	4	1.45	(0.39–3.71)
Cervical cancer	2	0.58	(0.070–2.10)	2	0.72	(0.088–2.61)
Progressive multifocal leukoencephalopathy	2	0.58	(0.070–2.10)	0	-	-
Disseminated HSV infection	2	0.58	(0.070–2.10)	0	-	-
Primary CNS lymphoma	1	0.29	(0.007–1.62)	0	-	-
Non-Hodgkin's lymphoma	1	0.29	(0.007–1.62)	1	0.36	(0.009–2.01)
Salmonella septicemia	1	0.29	(0.007–1.62)	1	0.36	(0.009–2.01)

CNS: central nervous system

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cohort) is the more severe immunodeficiency at baseline and the different OI distribution in our cohort, particularly the higher incidences of TB, toxoplasmosis and cryptococcal meningitis, none of which are common in resource-rich countries. Conversely, a study from Thailand had reported a substantially higher incidence of ADIs on ART (82/1,000PYs) than our results [13]. Although the pattern of OIs was similar to the Thai study, our patients might have been

Table 3. Factors Associated with Acquiring AIDS-Defining Opportunistic Infections during ART.

Variables	Univariate models, IRR (95%CI), *p*						Multivariate models, IRR (95%CI), *p*					
	All (n = 1,197)			No prior ADIs at baseline (n = 951)			All (n = 1,197)			No prior ADIs at baseline (n = 951)		
Gender male	2.23	(1.52–3.25)	<0.001*	2.80	(1.83–4.28)	<0.001*	1.47	(0.85–2.56)	0.19	1.86	(0.97–3.57)	0.06
Age 20–29	1						1			1		
Age 30–39	1.32	(0.91–1.90)	0.14	0.89	(0.37–2.17)	0.804	1.34	(0.82–2.20)	0.31	1.40	(0.80–2.44)	0.24
Age 40–49	1.29	(0.75–2.20)	0.36	1.32	(0.42–4.16)	0.636	1.60	(0.84–3.04)	0.15	1.59	(0.75–3.37)	0.23
Age over 50	1.46	(0.80–2.67)	0.22	0.91	(0.24–3.52)	0.892	1.83	(0.81–4.14)	0.15	2.46	(1.01–5.95)	0.046*
Injection drug use	1.71	(1.25–2.34)	0.001*	1.93	(1.36–2.74)	<0.001*	1.31	(0.75–2.30)	0.34	1.15	(0.61–2.16)	0.68
HBs antigen positive	1.31	(0.87–1.98)	0.19	1.43	(0.92–2.23)	0.114	1.28	(0.75–2.16)	0.36	1.28	(0.70–2.35)	0.43
Anti-HCV antibody positive	1.83	(1.31–2.55)	<0.001*	2.25	(1.55–3.27)	<0.001*	1.19	(0.68–2.10)	0.55	1.54	(0.81–2.92)	0.19
CD4<200 at baseline	9.73	(3.98–23.9)	<0.001*	5.70	(2.32–14.0)	<0.001*	4.42	(3.10–19.1)	<0.001*	6.94	(2.74–17.0)	<0.001*
Prior ADIs at baseline	0.92	(0.62–1.36)	0.68	-			0.69	(0.43–1.10)	0.12	-		

IRR: incident rate ratio; CI: confidential interval; ADI: AIDS-defining illness; HBs antigen: hepatitis B surface antigen; HCV: hepatitis C virus

Note:

* Statistically significant (P<0.05)

doi:10.1371/journal.pone.0150781.t003

Table 4. Causes of Deaths.

Causes of Deaths, n (%)	All		Prior ADIs at baseline			
			Yes		No	
Total	42		18		24	
AIDS-defining illnesses (ADIs)	19	(45.2)	9	(50)	10	(41.7)
TB	7		4		3	
Wasting Syndrome	6		2		4	
PML	2		2		0	
Pneumonia	2		1		1	
Cervical cancer	1		0		1	
Toxoplasma	1		0		1	
Others	14	(33.3)	4	(22.2)	10	(41.7)
Renal failure	4		1		3	
Cancer (not cervical cancer)	2		1		1	
Stroke	2		0		2	
Influenza H1N1	2		1		1	
Penicillium marneffei infection	1		0		1	
Liver failure	1		0		1	
Accident	1		0		1	
Drug overdose	1		1		0	
Not identified	9	(21.5)	5	(27.8)	4	(16.6)

ADI: AIDS-defining illness; TB: tuberculosis; PML: progressive multifocal leukoencephalopathy

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recruited to the study at an earlier clinical stage. Furthermore, more patients were initiated on ART after the WHO guideline increased the recommended CD4 count for ART indication from 200 to 350/mm^2 in 2010 [24] and thus had remarkably higher baseline CD4 counts than patients in the Thai study. Importantly, this change in CD4 counts for ART indication significantly increased the median baseline CD4 count, leading to a decline in ADI incidence in our cohort. Since immune status at ART initiation is one of the most crucial factors determining the incidence of ADIs during ART, earlier treatment should continue to be promoted to reduce morbidity of patients on ART.

Comparing mortality rates between different studies is challenging because of differences in selection criteria and socio-demographic backgrounds of participants. A cohort study of 894 IDUs in Thai Nguyen province in Northern Vietnam showed that the mortality of HIV-negative and HIV-positive persons were 41/1,000 PYs and 146/1,000 PYs respectively [16]. Based on this report, we assumed that the high proportion of IDUs—who are vulnerable for liver-related death, suicide, accidents or drug overdose [14, 15]—might lead to a high morality in our study population. Surprisingly, however, the overall mortality was 8.68/1,000 PYs and the mortality among IDUs was 18.9/1,000PYs, comparable to those in Western countries (range 10.4–20.1/1,000) [9]. A possible reason for the low mortality in our study may be the better immune status at baseline. Although acquiring ADIs was not rare despite being on ART, the fatality of acquired ADIs was low. In addition, plasma viral load monitoring was performed free of charge 6-monthly intervals and changes were made to ART regimens according to these results based on the national ART guideline. This may have contributed to the high proportion of virologic success of ART and may have led to a reduction in AIDS-related deaths. It may have also played some part inreduction in the frequency of non-AIDS deaths [30]. In addition,

Table 5. Factors Associated with All-Cause Mortality.

Variables	Univariate models, HR (95%CI), *p*						Multivariate models, HR (95%CI), *p*					
	All (n = 1197)			No prior ADIs at baseline (n = 951)			All (n = 1197)			No prior ADIs at baseline (n = 951)		
Gender male	3.61	(1.52–8.57)	0.004*	8.02	(1.89–34.1)	0.005*	1.14	(0.37–3.51)	0.82	5.52	(0.64–47.4)	0.12
Age 20–29	1			1			1			1		
Age 30–39	1.69	(0.74–3.83)	0.21	2.76	(0.77–9.92)	0.12	1.16	(0.43–3.13)	0.78	1.85	(0.36–9.60)	0.47
Age 40–49	2.5	(0.87–7.22)	0.09	5.23	(1.17–23.4)	0.03*	2.69	(0.85–8.55)	0.09	5.91	(1.06–32.9)	0.043*
Age over 50	4.53	(1.69–12.1)	0.003*	11.0	(2.74–43.9)	0.01*	7.39	(1.90–28.8)	0.004*	11.7	(1.75–77.6)	0.011*
Injection drug use	2.00	(1.09–3.67)	0.026*	1.38	(0.59–3.23)	0.46	1.76	(0.61–5.09)	0.30	0.93	(0.19–4.55)	0.93
HBs antigen positive	1.48	(0.69–3.20)	0.32	2.54	(1.05–6.12)	0.38	1.72	(0.69–4.26)	0.24	2.48	(0.81–7.60)	0.11
Anti-HCV antibody positive	1.80	(0.96–3.39)	0.07	1.24	(0.54–2.83)	0.61	1.99	(0.60–6.54)	0.26	1.79	(0.39–8.21)	0.46
CD4<200 at baseline	9.64	(1.31–70.8)	0.026*	5.95	(0.78–45.3)	0.85	6.12	(0.81–46.3)	0.08	5.12	(0.66–39.9)	0.12
Prior ADI at baseline	2.96	(1.60–5.46)	0.001*	-			2.03	(0.96–4.27)	0.06	-		

HR: hazard ratio; CI: confidential interval; ADI: AIDS-defining illness; HBs antigen: hepatitis B surface antigen; HCV: hepatitis C virus

Note:

* Statistically significant (P<0.05)

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most of our patients had taken cotrimoxazole as prophylaxis as indicated by the national guideline. This is widely acknowledged to reduce the incidence and mortality of AIDS-related conditions [31]. Another possible factor for better prognosis may be the characteristics of the study sites; both study sites were referral hospitals in urban settings, with more highly qualified staff and more advanced facilities than other clinics in Vietnam. Furthermore, the study sites made extensive efforts to actively trace of patients who had missed their scheduled visits, either by healthcare professionals or by peer supporters. We believe this led to the high rate of retention in care, 95.3% at 12 months of ART [32] and, as a result, the high proportion of virologic suppression (95.5%). Notably, having an IDU history was not statistically associated with increased mortality. We speculate that the extensive effort to keep patients retained in care might have facilitated communication between patients and healthcare professionals leading to an effective reduction in non-AIDS-related deaths, such as suicide, involvement to violence and accidents or drug overdose, all of which are common in IDUs.

This is the first study to describe causes of death in patients receiving ART in Vietnam. Although the accurate diagnosis of cause of death is always challenging in resource-limited settings, we collected as much clinical information as possible and successfully identified the underlying causes of death in all hospitalized cases. We found that 45% of deaths were attributable to AIDS, while non-AIDS illnesses were responsible for 33% of deaths. Although the size of our cohort was small, the proportion of AIDS-related deaths among all deaths was comparable to those reported by previous large studies (29% to 50%) [4, 7, 9]. Like other low- and middle-income countries, TB was the most common ADI seen in 8.2% in this study; this incidence is similar to previous studies [13, 33, 34]. However, the case-fatality rate of TB in our cohort (7.1%) is low compared with other resource-limited settings (7.0 to 27.4%) [33, 34]. Since all our TB cases were in the ART program, they may have been diagnosed and treated in the early stages of TB, which might have led to low fatality. On the other hand, although several studies have highlighted the increasing importance of liver-related deaths [35], non-AIDS malignancies and cardiovascular diseases [9], the rarity of these events in this study made it difficult to evaluate whether these conditions would have increased in this setting. Notably, there was only

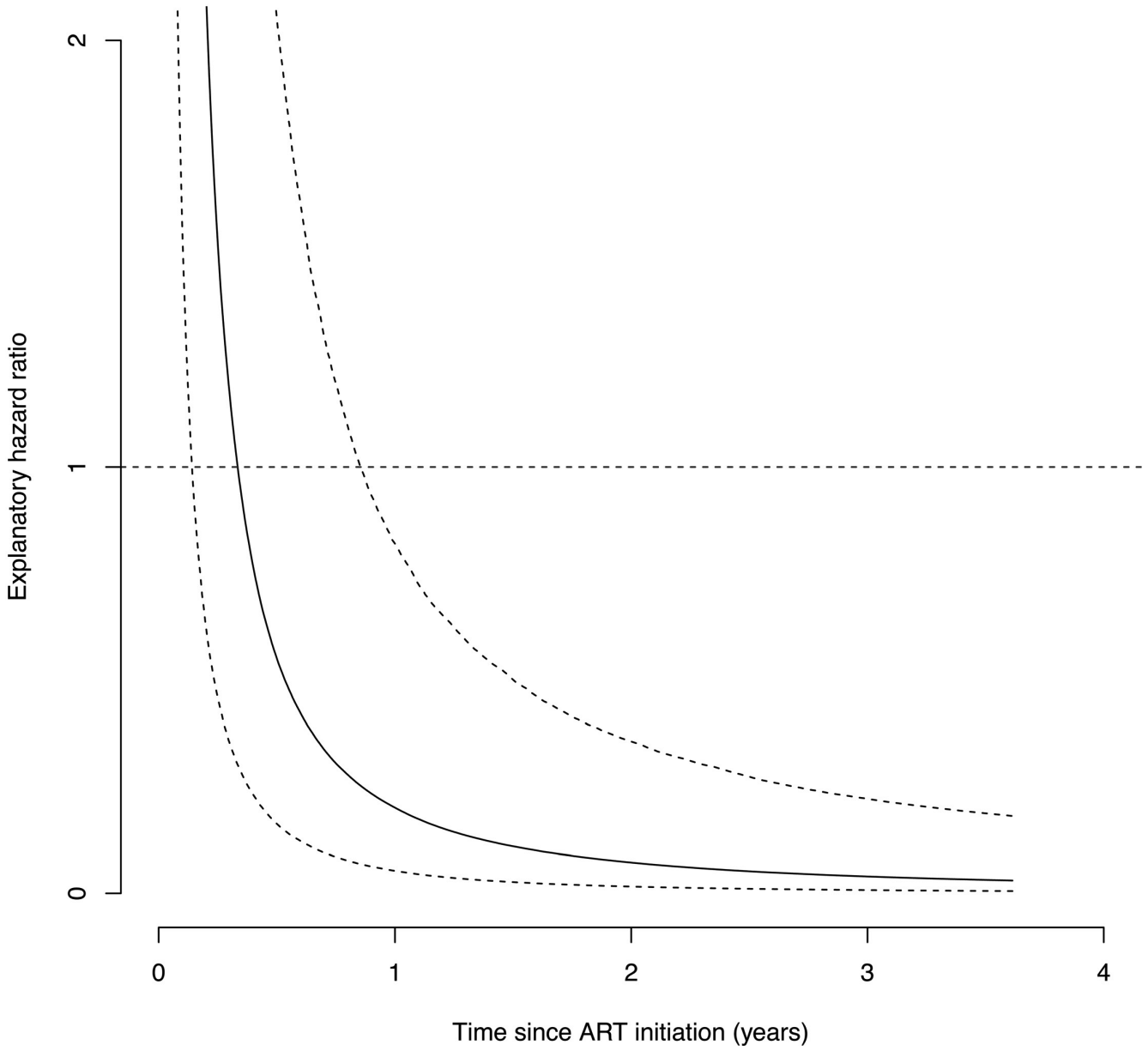

Fig 3. Explanatory Hazard Ratio in Semi-Competing Risks Analysis. The ratio of hazards for death after and before AIDS. If the risk of death is not influenced by the risk of AIDS (i.e., time to AIDS and death are independent) the ratio is equal to one (the blue horizontal line). Dotted lines indicate ranges of 95% CIs.

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one confirmed case of liver-related death, despite the high prevalence of hepatitis B or C co-infection. However, there were 9 cases (21.5%) whose causes of death were unidentifiable because they died at home. Although almost impossible to know if their deaths were related to injection drug use or other socially undesirable behaviors that could have prevented them from accessing care when dying, we need to view the distributions of causes of death in the context of such limitations of unidentifiable causes of deaths.

We found that age older than 50 years was associated with higher ADI incidence after controlling for CD4 count in 951 patients without ADI episodes prior to ART. Additionally, age older than 40 or 50 years was related to higher mortality during the observation period of up to 7.5 years. Estimated life expectancy in the Vietnamese general population is 73 years old [36], far beyond the maximum age of our patients, and half the deaths in our study were attributable to ADIs. Thus, the increased risk of shorter survival in older patients might not solely be explained by aging-associated conditions occurring independently of HIV infection. This finding is compatible with previous studies reporting that ART initiation at older ages is related to higher mortality, as a consequence of delayed HIV diagnosis and initiation of ART compared with younger patients [37]. Although not significant in multivariate models, the incidence of ADIs and mortality was higher in men than those in women in univariate models with high hazard ratios (2.23–8.02). Similarly, there have been a number of previous reports on gender and ART outcomes from resource-limited countries; most revealed higher mortality in men than women on ART [38–40]. It is usually speculated that such poorer survival in men is explained by late ART initiation due to their poorer health-seeking behavior and increased likelihood of their loss to follow up or treatment failure. Therefore, it is reasonable to view both older age and male gender as risk factors for shorter survival. Those having such factors should be carefully followed-up, as should IDUs.

To explore the impact of developing ADIs on survival, we evaluated whether experiencing an ADI prior to ART increased the risk of all-cause mortality after ART initiation. We found a significantly higher mortality after acquiring ADIs on ART in univariate—but not in multivariate—models. Patients without ADI events prior to ART were assigned to semi-competing risks analysis, where we found that death after acquiring ADIs was more likely to occur within six months of ART initiation, but rapidly decreased thereafter. Interestingly, however, survival time did not differ by cause of death (AIDS-deaths vs. non-AIDS deaths). Although this result was not conclusive and needs to be viewed within the limitations of statistical power due to the small sample size and the rarity of deaths, it implies that those who experienced ADIs might be more vulnerable to high mortality in the first six months of ART. Thus, early ART initiation, to prevent acquiring ADIs after starting ART, might be key to reducing the risk of shorter survival.

In addition to the small sample size and the rarity of fatal events, our study had several limitations. First, the duration of ART was diverse at study enrollment, particularly at the beginning of the study. Thus, we might have failed to include patients who died within the first 12 months of ART before 2007. This could make the survival time longer and the results of regression analysis could be biased toward null. Second, the accuracy of ADI diagnosis could have been affected by limitations of resource availability; ADI may have been underdiagnosed. In fact, examinations for opportunistic infections are not covered by ART programs, which may have discouraged patients to undergo investigation for financial reasons. Third, since our study clinics were both in large referential hospitals in urban Hanoi, it is unclear to what extent our results could be generalized. Strengths of our study include the prospective collection of events using structured reporting forms and the high retention rate in care by the active patient tracing system. Usually, ascertainment of deaths among people lost to follow-up is a critical problems in survival analysis in resource-limited settings. The active patient tracing system in our study sites enabled us to minimize censoring due to loss from the care.

In conclusion, the mortality after ART initiation in two large hospitals in urban Hanoi was comparable to that of Western countries, and AIDS-defining opportunistic infections were not rare while on ART. Almost half the deaths were attributable to AIDS, and age over 50 years at ART initiation was statistically associated with shorter survival, while IDU history was not associated with poor prognosis. This is the first in-depth data on survival of HIV-infected

patients receiving ART, and provides supportive evidence for the success of ART programs in urban Hanoi in the decade of rapid ART scale-up.

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Author Contributions

Conceived and designed the experiments: JT KL SH DTHN TTP KVN SO. Analyzed the data: JT KL SH. Contributed reagents/materials/analysis tools: KL SH. Wrote the paper: JT KL SH. Collected data: JT SM DTHN DTN CDD.

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ORIGINAL ARTICLE

Influence of the polymorphism of the *DUSP14* gene on the expression of immune-related genes and development of pulmonary tuberculosis

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Recently, a genome-wide screening identified a functional single-nucleotide polymorphism in dual-specificity phosphatase 14 gene (*DUSP14*), which was associated with pulmonary tuberculosis (TB) in a West African study. DUSP14 regulates T-cell proliferation and cytokine production in a negative way via dephosphorylation and inactivation of key signaling molecules. The aim of this study is to further explore the possible significance of the *DUSP14* polymorphism. Total RNA was extracted from the whole blood of 109 healthcare workers (HCWs) in Vietnam and subjected to quantitative reverse-transcription PCR for *DUSP14* and 20 immune-related genes. *DUSP14* rs1051838 was genotyped in 502 new pulmonary TB patients and 506 healthy controls. Among disease-free individuals (HCWs), T-helper type-1 (Th1)-related genes, interferon-gamma receptor 2 (*IFNGR2*) and signal transducer and activator of transcription-1 (*STAT1*) mRNA levels significantly increased as the number of A alleles of rs1051838 increased, whereas the *DUSP14* mRNA level tended to decrease. The AA genotype was associated with protection against active TB in younger patients (⩽45 years old, OR = 0.63, 95% CI 0.44–0.90). Our results suggest that a low-expression genotype of *DUSP14* accompanied by high transcript levels of Th1 immune-related genes may confer protection against early TB development.

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INTRODUCTION

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), infects an estimated one-third of the world's population and establishes a persistent infection in which innate and adaptive immune cells are involved.[1] Approximately 5% of infected individuals develop active TB in the first 12–24 months, with another 5% developing the disease later. Others can limit the infection for a long time via the formation of a granuloma, in which the Th1 immune system appears essential to maintain the containment of *Mtb*.[2] Host risk factors that determine the progression of latent TB infection (LTBI) to active disease have been widely investigated.[3] Although the molecular pathogenesis is still largely unknown, attenuation of cellular immunity has a role in disease development,[4,5] and components that regulate the Th1 immune response, such as involvement of regulatory T cells or signaling pathways, including coinhibitory molecules, may influence the outcome of LTBI.[6]

Human genetic variants with a strong clinical effect on TB have not been identified thus far, except for mutations of interferon-gamma (IFN-γ)-dependent immunity-related genes involved in Mendelian susceptibility to mycobacterial disease.[7] Recently, Barreiro *et al.*[8] mapped the expression quantitative trait loci in the genome using human primary monocyte-derived dendritic cells (DCs) with or without *Mtb* infection. A single-nucleotide polymorphism (SNP) rs712039 was identified in intron 1 of the dual-specificity phosphatase 14 gene (*DUSP14*), which affected the transcript level of *DUSP14* in the DCs. Furthermore, this was significantly associated with pulmonary TB when information from a genome-wide association study in Ghana and Gambia was integrated. Their report suggests a novel functional role of DUSP14 in TB development.

A family of DUSPs functions to remove phosphates from phosphoserine, phosphothreonine and phosphotyrosine residues in activated kinases to render them inactive.[9] One group of the DUSP family, called MAPK phosphatases (MKPs), controls the levels of proinflammatory cytokines, limiting the activities of dynamic MAPK pathways, and thus, has an important role in the negative regulation of cellular immune responses. Of these, DUSP14, also known as MKP6, is reported to dephosphorylate JNK, ERK,[10] transforming growth factor-α-activated kinase 1 (TAK1 or mitogen-activated protein kinase kinase kinase 7, MAP3K7),[11] and TAK1-binding protein 1 (TAB1) to negatively regulate T-cell proliferation and cytokine production.[12] Hence, to explore the possible significance of *DUSP14* polymorphisms on host immune balance, we analyzed the transcript levels of *DUSP14* and a variety of immune-related genes in whole peripheral blood collected from disease-free individuals and further investigated whether the *DUSP14* SNP is associated with newly diagnosed pulmonary TB in Asians.

RESULTS

SNPs in the 5′ region and exons of *DUSP14* in the Vietnamese

After screening of *DUSP14* polymorphisms in the 109 disease-free healthcare workers (HCWs), three SNPs, rs853196, rs853197 and

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rs62076700, were found in the 5′ region up to 1300 bp from the transcription start site of the reference mRNA sequence (NM_007026.3), and a G/A of rs1051838 was located in the 5′ untranslated region of exon 2 (Table 1). Three low-frequency variants with minor allele frequency between 1% and 5% were also found in the region analyzed, whereas no polymorphism was found in the coding region. The degree of linkage disequilibrium (LD) was analyzed with the intron 1 C/T of rs712039, which was previously reported to be associated with *DUSP14* transcript levels.[8] Of the four SNPs, rs853196, rs853197 and rs1051838 were in perfect LD with rs712039 (minor allele frequency = 0.48; $r^2 = 1$), whereas rs62076700 was in weak LD with the other SNPs (minor allele frequency = 0.21; $r^2 = 0.24$). In this study, rs1051838 was thereafter used as a representative of all four SNPs, including rs712039.

DUSP14 mRNA expression in the whole-blood and SNP genotypes among disease-free individuals

Because of the perfect LD shown above, the A allele of rs1051838 was equivalent to the T allele of rs712039, which was strongly associated with low expression of *DUSP14* mRNA in unstimulated DCs in a previous report.[8] As shown in Figure 1a, when the number of A alleles of rs1051838 increased, the *DUSP14* mRNA level assayed by quantitative reverse-transcription PCR also tended to decrease in the whole blood of disease-free individuals

Table 1. *DUSP14* polymorphisms and low-frequency variants found in the region analyzed

rs number	*Position*	*Major/minor allele*	*Minor allele frequency*	*LD with rs712039, r^2*
rs853196	5′ near gene (−1292[a])	A/T	0.482	1
rs853197	5′ near gene (−1235[a])	T/C	0.482	1
rs853198	5′ near gene (−609[a])	C/A	0.037	0.03
rs62076700	5′ near gene (−229[a])	C/G	0.211	0.24
rs373703160	exon 1 (5′-UTR)	—/CCGCG	0.037	0.03
rs712039	intron 1	C/T	0.482	
rs574325598	intron 1	—/T	0.243	0.17
rs1051838	exon 2 (5′-UTR)	G/A	0.482	1
rs117551799	intron 2 (near exon 3 boundary)	T/C	0.037	0.11

Abbreviations: *DUSP14*, dual-specificity phosphatase 14 gene; LD, linkage disequilibrium; UTR, untranslated region. [a]Nucleotide position from the transcription start site of reference mRNA sequence (NM_007026.3).

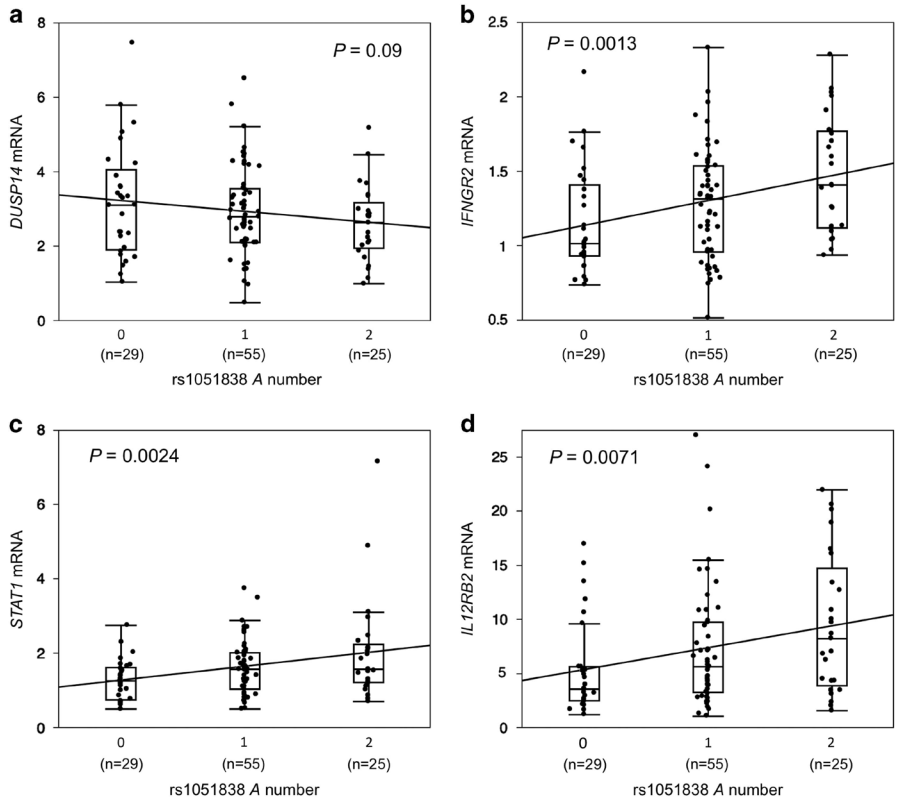

Figure 1. Expression levels of *DUSP14*, *IFNGR2*, *STAT1* and *IL12RB2* transcripts by the number of A alleles of rs1051838 among disease-free individuals. A linear regression model was used to assess the allele number-dependent change of gene expression levels, which are displayed using box and whisker plots. (**a**) *DUSP14*; (**b**) *IFNGR2*; (**c**) *STAT1*; and (**d**) *IL12RB2*.

(HCWs), although this was not significant when assessed using a linear regression model ($P = 0.09$). The expression level of *DUSP14* was hardly affected by rs62076700 or rs574325598 (data not shown). Frequencies of the other variants were too low to be analyzed further. The state of LTBI was estimated by positive results of commercially available IFN-γ release assay (IGRA). *DUSP14* transcript levels were not significantly different between IGRA-negative ($n = 68$) and -positive ($n = 41$) groups (data not shown).

Immune gene expressions and *DUSP14* SNP genotypes among disease-free individuals

Of the 20 immune-related genes analyzed in the 109 disease-free individuals (HCWs), transcript levels of six genes, *IFNGR2*, *STAT1*, *IL12RB2*, *TGFB1*, *FOXP3* and *TNF* were elevated in the whole blood as the number of A alleles of rs1051838 increased ($P < 0.05$, Supplementary Table 1). When expression levels of the six genes were further analyzed together with IGRA results, effects of an interaction term between A allele number and IGRA results were not statistically significant (Supplementary Table 2). In addition, for each of the six genes, the A allele dependency of mRNA levels in the IGRA-negative and -positive subgroups are shown with *P*-values in Supplementary Table 1. When the A allele-dependent gene expression was adjusted for age, sex and IGRA results in a multiple regression model, *TNF* expression was associated with both age ($P = 0.0278$) and with the number of A alleles ($P = 0.0387$). *P*-values showing associations between expression of the above six genes and the number of A alleles were all < 0.05, even after adjustment for the above factors (Table 2).

The top three *P*-values demonstrating A allele-dependent gene expression were those of *IFNGR2*, *STAT1* and *IL12RB2*. Differences in expression levels of these three genes by the number of A alleles are shown in Figures 1b, c, and d. When Bonferroni's corrections for multiple comparisons were applied, based on the number of all genes analyzed, *IFNGR2* and *STAT1* levels remained significant ($P < 0.0025$) (Table 2). Average expression levels of the genes, *IFNGR2*, *STAT1*, *TGFB1*, *FOXP3* and *TNF*, were not significantly different between IGRA-negative and -positive groups (data not shown), but the average mRNA level of *IL12RB2* was slightly higher in the IGRA-positive group than in the IGRA-negative group ($P = 0.03$). Because *DUSP14* mRNA levels tended to decrease and immune-related gene expression levels increased as the number of A alleles increased, we expected to observe negative correlations between *DUSP14* mRNA level and these six immune gene expression levels. However, it was not the case (Table 3). On the contrary, positive correlations were observed between *DUSP14* mRNA and expression of two immune genes, *IL12RB2* and *TNF*. Of whole-blood cell populations, *DUSP14* has been reported to be expressed more in Th1 and Th2 cells.[13] Considering a possibility that some of the immune genes analyzed in the whole blood are highly influenced by the proportions of individual cell populations, we further calculated correlation coefficients between *DUSP14* and transcription factors that may be associated with such cell populations. As a result, *DUSP14* mRNA level was positively correlated ($P < 0.0001$, $r = 0.468$) with the mRNA level of T-bet gene (*TBX21*), which is a key transcriptional activator of Th1 cell differentiation.[14] When partial correlation coefficients for *TBX21* mRNA levels were calculated, the positive correlations between expression of *DUSP14* and the two genes, *IL12RB2* and *TNF*, disappeared (Table 3).

Table 2. Associations of the immune gene expression with the number of dual-specificity phosphatase 14 gene (*DUSP14*) rs1051838 A alleles, age, sex and interferon-γ release assay (IGRA) results using multivariate linear regression analysis

Gene	*All healthcare workers* (n = *109*) P-*value*[a]			
	DUSP14 A allele number (0, 1, 2)	*Age (⩽45, >45)*	*Sex (male, female)*	*IGRA (negative, positive)*
IFNGR2	**0.0019**	0.5449	0.1783	0.2675
STAT1	**0.0023**	0.8916	0.1454	0.0759
IL12RB2	0.0081	0.6831	0.8279	0.1248
TGFB1	0.0115	0.5069	0.8561	0.2236
FOXP3	0.0165	0.8110	0.8926	0.2077
TNF	0.0387	0.0278	0.5933	0.1670

P-values < 0.0025 are shown in bold type, applying Bonferroni's correction for multiple comparisons. [a]*P*-values < 0.05 for the *DUSP14* allele number are shown in the table.

Association between AA genotype of rs1051838 and protection against TB in younger patients

We analyzed rs1051838 genotypes in 502 Vietnamese TB patients and 506 controls, and the genotype distribution in the controls did not deviate from the Hardy–Weinberg equilibrium. When the genotype frequencies were compared between all patients and controls, only the AA versus non-AA model demonstrated a statistically significant association, and the genotype AA was associated with the protection against TB ($P = 0.019$, odds ratio (OR) = 0.71 95% confidence interval (CI) 0.53–0.94; Table 4a). Association between TB disease and the AA genotype remained significant when adjusted for sex and age (adjusted OR = 0.71 95% CI 0.52–0.95).

Considering the relationship between the genotype and age at onset of the disease, we divided the patients and the controls into two subgroups by age (⩽45 and >45). A significant protective

Table 3. Correlations between dual-specificity phosphatase 14 gene (*DUSP14*) mRNA level and transcript level of the immune-related gene

	P-*value*	r	$r_{partial}$[a]
IFNGR2	0.9701	0.0036	−0.0867
STAT1	0.4153	0.0788	0.0378
IL12RB2	0.0003	0.3431	0.0342
TGFB1	0.2564	0.1096	−0.2714
FOXP3	0.0961	0.1602	−0.0685
TNF	0.0089	0.2494	−0.0939

[a]Partial correlation coefficient for *TBX21* mRNA expression.

Table 4a. Genotype and allele frequencies of *DUSP14* rs1051838 in controls and TB patients in the Vietnamese population

rs1051838	*Control* (n = *506*)		*TB* (n = *502*)		
Genotype					
AA	152	30.0%	117	23.3%	*P* = 0.019, OR 0.71 95% CI 0.53–0.94[a]
GA	232	45.8%	256	51.0%	
GG	122	24.1%	129	25.7%	
		HW*P* = 0.07			
Allele					
A	536	0.53	490	0.488	*P* = 0.068[b]
G	476	0.47	514	0.512	

Abbreviations: CI, confidence interval; *DUSP14*, dual-specificity phosphatase 14 gene; HW*P*, Hardy–Weinberg *P*-value; TB, tuberculosis. [a]TB development associated with AA was assessed by odds ratios (OR) with non-AA as a reference. [b]*P*-values for 2 × 2 Fisher's exact test.

association was observed between the proportion of individuals having TB and the AA genotype only in the younger subgroup ($P = 0.011$, OR 0.63 95% CI 0.44–0.90; Table 4b) and not in the older subgroup ($P = 0.52$, OR 0.85 95% CI 0.51–1.39; Table 4c). Furthermore, using a logistic regression model, OR per one A allele increase was 0.79 (95% CI 0.64–0.98) in the younger group. We further analyzed a possible relationship between *DUSP14* alleles and genetic lineages of *Mtb* strains that were clinically isolated from 429 TB patients, but the *DUSP14* genotype and allele frequencies were not significantly different between groups infected with Beijing genotype strains ($n = 250$) or those not infected with Beijing genotype strains (data not shown). The genotype and allele frequencies of rs1051838 were not significantly different between the IGRA-negative and -positive HCWs (Table 5).

Table 4b. Genotype and allele frequencies of *DUSP14* rs1051838 in controls and patients with TB (age ⩽45 years) in the Vietnamese population

rs1051838	*Control ⩽45 years* (n = *386*)		*TB ⩽45 years* (n = *289*)		
Genotype					
AA	117	30.3%	62	21.5%	$P = 0.011$, OR 0.63, 95%CI 0.44–0.90[a]
GA	175	45.3%	147	50.9%	
GG	94	24.4%	80	27.7%	
	HW$P = 0.08$				
Allele					
A	409	0.53	271	0.469	$P = 0.028$[b]
G	363	0.47	307	0.531	

Abbreviations: CI, confidence interval; *DUSP14*, dual-specificity phosphatase 14 gene; HW*P*, Hardy–Weinberg *P*-value; TB, tuberculosis. [a]TB development associated with AA was assessed by odds ratios (OR) with non-AA as a reference. [b]*P*-values for 2×2 Fisher's exact test.

Table 4c. Genotype and allele frequencies of *DUSP14* rs1051838 in controls and patients with TB (age >45 years) in the Vietnamese population

rs1051838	*Control >45 years* (n = *120*)		*TB >45 years* (n = *213*)		
Genotype					
AA	35	29.2%	55	25.8%	$P = 0.52$, OR 0.85, 95%CI 0.51–1.39[a]
GA	57	47.5%	109	51.2%	
GG	28	23.3%	49	23.0%	
	HW$P = 0.61$				
Allele					
A	127	0.53	271	0.51	$P = 0.12$[b]
G	113	0.47	307	0.49	

Abbreviations: CI, confidence interval; *DUSP14*, dual-specificity phosphatase 14 gene; HW*P*, Hardy–Weinberg *P*-value; TB, tuberculosis. [a]TB development associated with AA was assessed by odds ratios (OR) with non-AA as a reference. [b]*P*-values for 2×2 Fisher's exact test.

DISCUSSION

In the present study, we found that Th1 immune gene expression levels were significantly elevated in the whole blood of disease-free individuals when the number of A allele of rs1051838 in *DUSP14* increased and that the SNP genotype AA was less frequently observed in young Vietnamese patients who developed active TB.

DUSP14 was originally cloned as MKP6, which dephosphorylates JNK and ERK signaling molecules in T cells.[10] Recently, TAK1 and TAB1 were identified as novel direct targets of DUSP14 dephosphorylation.[11,12] By generating DUSP14-deficient mice, it has also been shown that DUSP14 leads to TAB1–TAK1 complex inactivation, and DUSP14-deficiency in T cells results in enhanced T-cell proliferation and increased IL-2, IFN-γ, and IL-4 production upon T-cell activation.[12] However, DUSP14 is widely distributed[9] and is also expressed in immune cells other than T cells.[13] Because activation of TAK1 and TAB1 as well as MAPKs regulates immune and inflammatory responses in many types of cells, including macrophages and DCs, which have an important role in innate and adaptive immunity,[15] it is conceivable that DUSP14-mediated negative feedback to limit activated MAPKs could be acting on more than one type of immune cell. Barreiro *et al.*[8] found that a *DUSP14* SNP was associated with the secretion levels of TNF-α and IFN-γ from monocyte-derived DCs. Considering these reports, the original West African quantitative trait loci association study,[8] and our current findings, we can postulate that the low-expression genotype of *DUSP14* may confer resistance to TB development by enhancing the T-cell response against *Mtb*, directly acting on human T cells or indirectly through other immune cells.

We observed significantly higher expression of *IFNGR2* and *STAT1* in the whole blood of HCWs when the number of A allele of rs1051838 increased. *IL12RB2* did not reach a statistically significant level after adjusting *P*-values for multiple comparisons and thus, we need to carefully interpret the results. However, the top three *P*-values showing higher expression of Th1-related genes (*IFNGR2*, *STAT1* and *IL12RB2*) with the A allele of rs1051838, which was equivalent to the low-expression allele in a previous report.[8] This finding is in agreement with the known Th1-suppressing function of intact DUSP14 through the inhibition of

Table 5. Genotype and allele frequencies of *DUSP14* rs1051838 in Vietnamese healthcare workers

rs1051838	*All* (n = *109*)		*IGRA negative* (n = *68*)		*IGRA positive* (n = *41*)		
Genotype							
AA	25	22.9%	15	22.1%	10	24.4%	$P = 0.82$, OR 1.14 95% CI 0.45–2.83[a]
GA	55	50.5%	35	51.5%	20	48.8%	
GG	29	26.6%	18	26.5%	11	26.8%	
	HW$P = 0.91$						
Allele							
A	105	0.482	65	0.478	40	0.488	$P = 0.89$[b]
G	113	0.518	71	0.522	42	0.512	

Abbreviations: CI, confidence interval; HW*P*, Hardy–Weinberg *P*-value. [a]The genotype AA association with interferon-γ release assay (IGRA)-positive group compared with IGRA-negative group was assessed by odds ratio (OR) with non-AA as a reference. [b]*P*-value for 2×2 Fisher's exact test.

ERK activation.[12] This suggests that the *DUSP14* SNP influences the immune system against mycobacterial infection or other intracellular pathogens,[16] although it remains to be determined which variant in the same LD region is functionally important. Considering that Th1 immune gene expression did not show direct negative correlations with *DUSP14* mRNA levels in the peripheral blood but did show negative associations with the number of *DUSP14* rs1051838 A alleles in the genome, activation of other immune cells, such as DCs, may be controlled by their own DUSP14 expression and indirectly regulate Th1 immune gene expression in blood cells. One can assume that functional changes of immune cells in the infected tissue may also influence mRNA levels in the peripheral blood of patients with LTBI. However, differences in *DUSP14* genotype-dependent patterns of *IFNGR2*, *STAT1* and *IL12RB2* expression were not clearly distinguished between the IGRA-negative and -positive groups, as indicated by no significant effect of an interaction term between A allele number and IGRA results in the multiple regression model of our study. The lack of LTBI-specific patterns implies that baseline *DUSP14* expression in the whole peripheral blood samples may regulate the expression of immune-related genes irrespective of the LTBI status. In addition, in the present study, as we used whole-blood cells in RNA stabilizing solution for gene expression analysis, cell type-specific expression patterns were not separately obtained, and it is possible that a subpopulation of the whole-blood cells also influenced transcript levels of cell-specific gene expression. This may partly explain why we did not observe direct negative correlations between the expression levels of *DUSP14* and immune-related genes in the whole blood. Aging also appears to act as a confounder, partly affecting cytokine gene expression, including *TNF*. The exact mechanism by which *DUSP14* SNP changes expression levels of Th1-related genes in the peripheral blood should be investigated in future.

Associations of host genetic variations with TB are often age dependent, as previously reported by us and other researchers.[17–20] This tendency was also observed in our study; probably the development of the disease at a younger age represents a weaker protective immunity against TB. In Hanoi, patients with TB caused by the Beijing genotype *Mtb* strains were significantly younger than those with TB caused by the non-Beijing genotype,[21] but in the present study, *DUSP14* genotype did not show direct associations with Beijing or non-Beijing *Mtb* lineage. The host–pathogen interaction should be analyzed further because host responses could be different among *Mtb* lineages and sublineages,[22] and some proteins derived from *Mtb* are known to directly target members of MAPKs and MKPs.[15]

The *DUSP14* genotype frequencies and expression levels were not different between IGRA-negative and -positive HCWs. The protective role of *DUSP14* is presumably involved in disease development from LTBI rather than establishment and maintenance of *Mtb* infection, although a large prospective study would be necessary to draw a definite conclusion. This is a limitation of our study. Nevertheless, we minimized the bias of *Mtb* exposure levels between cases and controls in our disease association study, recruiting all individuals from the same area during the same period and compared the immunogenetic effect on individuals who developed TB and those who were not infected or infected with *Mtb*. If DUSP14 affects disease progression, then reduction of DUSP14 activity by its inhibitors may provide opportunities to inhibit active TB development from LTBI because the DUSP family proteins are promising drug targets for manipulating MAPK-dependent immune responses and have been extensively studied.[9,13]

In conclusion, our findings confirmed the results of a previous African genome-wide study and further indicated that a low-expression genotype of *DUSP14* may confer protection against early TB development, accompanied by high transcript levels of Th1 immune-related genes.

MATERIALS AND METHODS

Study population

To investigate a genotype–phenotype relationship of *DUSP14*, 109 HCWs (age 34 ± 10.1, males 23.9%) were recruited as disease-free individuals in Hanoi, Vietnam. The state of LTBI was estimated by positive results of the ELISA-based IGRA, QuantiFERON-TB Gold In-Tube (Cellestis, Carnegie, VIC, Australia).[23] A half of the participants had a history of BCG vaccination (data not shown).

For a genetic association analysis, 555 active pulmonary TB patients (age 41 ± 14.8, males 79.8%) and 506 healthy volunteers (non-HCWs; age 37 ± 10.3, males 50.0%) participated in the study. All were unrelated Vietnamese recruited in the Hanoi area.[24,25] All TB patients were diagnosed as sputum smear-positive pulmonary TB without previous TB episode and treated with anti-TB therapies following the guidelines of the national TB program. They were immediately recruited after the diagnosis was made. Forty-nine HIV-positive TB patients and four patients with no information about their HIV status were excluded from further analysis. Beijing genotype of *Mtb* isolates was distinguished from non-Beijing genotype in 429 TB patients with no HIV infection using a SNP-based *Mtb* genotyping method described previously.[26] Written informed consent was obtained from all participants. The study protocol was approved by the ethics committees of the Ministry of Health, Vietnam (4481/QD-BYT, 2529/QD-SYT), the National Center for Global Health and Medicine (NCGM-A-000185-00, 63) and the Research Institute of Tuberculosis (RIT/IRB25-1, 25-2), Japan.

Screening of polymorphisms in the 5′ and exon regions of *DUSP14*

Genomic DNA samples from 109 HCWs were extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and subjected to PCR amplification of the 5′ region of the *DUSP14* gene with the primer set 5′-GTACAGGCATGTATAAGCCCGCACTTCCAC-3′ and 5′-CGGAGCGCAGGC GGGACACGGGCATTTGAG-3′ or with the primer set 5′-ACTCTTAGGTGCCT TGACTCACGGCAGAGC-3′ and 5′-GCTCCAGAGCGGGTGGATGCCAGCGAG-3′, using KOD FX neo (Toyobo, Osaka, Japan). The amplified products (1148 and 1367 bp) were purified and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the 3130xl Genetic Analyzer (Applied Biosystems). Intron 1 SNP rs712039 and exon 2 SNP rs1051838 were also detected by PCR and the direct sequencing method. A coding region of *DUSP14* in exon 3 was amplified with primers 5′-TTTTGGACAATCACCAGAGAGCTG-3′ and 5′-GCCCCGCTCG GGCTGCTGACGCAG-3′ (953 bp) and sequenced.

Quantitative reverse-transcription PCR of mRNA

From HCWs, 2.5 ml of peripheral whole blood was collected in a PAXgene Blood RNA Tube (PreAnalytiX, Hombrechtikon, Switzerland) and stored at − 80 °C prior to processing. Total RNA was extracted using the PAXgene Blood RNA Kit (Qiagen) and was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random nonamer (TaKaRa, Shiga, Japan). Transcript levels of *DUSP14* and 20 immune-related genes (*IFNG*, *IL2*, *STAT1*, *STAT4*, *TBX21*, *IL12A*, *IFNGR1*, *IFNGR2*, *IL12RB1*, *IL12RB2*, *IL23A*, *RORC*, *GATA3*, *IL4*, *STAT6*, *FOXP3*, *TGFB1*, *LTA*, *TNF* and *IL10*; official full names of all genes are listed in Supplementary Table 1) were analyzed by TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) using StepOne Plus Real-Time PCR System (Applied Biosystems) as recommended by the manufacturer. *GAPDH* was used to normalize the values of the target gene expression by ΔΔCt method, and relative expression level of each gene was calculated to a fixed control complementary DNA throughout the study.

Genotyping of rs1051838 in TB patients and controls

Genomic DNA was extracted from the peripheral blood as describe above, and G/A SNP in exon 2 (rs1051838) was amplified by PCR using primers 5′-CCGGTGTTTCTTACTGGTGCAGCC-3′ and 5′-CAGTGATTTCAGTCAGACAA GAGC-3′. An amplified product of 320 bp was digested with *Msp* I (New England Biolabs, Ipswich, MA, USA) and was electrophoresed on 2% agarose gels with ethidium bromide. Genotypes were determined by the length of PCR products after digestion (A allele with 288 bp and G allele with 220 bp).

Statistical analysis

Frequencies of haplotypes containing multiple polymorphic sites and LD values were estimated by Haploview ver. 4.2.[27] Univariate and multivariate linear regression models were used to assess the allele number-dependent change of gene expression levels. Bonferroni's correction was used when necessary. Correlation and partial correlation coefficients were calculated to analyze possible correlations between the immune gene and *DUSP14* mRNA expression. Gene expression levels between two groups were also compared using Wilcoxon rank-sum test. Disease associations with SNP alleles and genotypes were assessed by Fisher's exact test, and *P*-values < 0.05 were considered significant. TB disease associated with the SNP was further assessed by an OR with or without adjustment for sex and age using a logistic regression model. Subgroup analysis by age at onset ($\leqslant 45$ and > 45) was also applied.[20] Statistical analysis was performed using JMP 8.0.2 package (SAS institute Japan Ltd, Tokyo, JAPAN).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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RESEARCH ARTICLE Open Access

Circulating granulysin levels in healthcare workers and latent tuberculosis infection estimated using interferon-gamma release assays

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Abstract

Background: Granulysin (GNLY) is produced by human lymphocyte subpopulations and exhibits antimicrobial activity against *Mycobacterium tuberculosis*. We examined the association between GNLY levels in blood and latent tuberculosis (TB) infection.

Methods: Latency of TB infection among Vietnamese healthcare workers was estimated using interferon-gamma release assays (IGRA), and serum GNLY concentrations were measured using enzyme-linked immunosorbent assays. The levels of *GNLY* expression in whole blood and the presence of *GNLY* alleles with the exon-4 polymorphism rs11127 were also determined using PCR-based methods.

Results: Among 109 study participants, 41 (37.6 %) were IGRA positive and had significantly lower serum GNLY concentrations compared with IGRA-negative participants (adjusted mean, 95 % confidence interval; 2.03, 1.72–2.44 vs. 2.48, 2.10–2.92 ng/ml, $P = 0.0127$; analysis of covariance). Serum GNLY concentrations and TB antigen-stimulated interferon-gamma values were weakly inversely correlated ($r = -0.20$, $P = 0.0333$). Serum GNLY concentrations varied with *GNLY* genotypes even after adjustment for gender and age (adjusted $P = 0.0015$) and were moderately correlated with *GNLY* expression in blood cells ($r = 0.40$, $P < 0.0001$). In subsequent analyses, low serum GNLY concentrations were significantly associated with IGRA status (adjusted odds ratio and 95 % confidence interval, 0.55 and 0.31–0.98, respectively), although *GNLY* genotype and mRNA levels were not.

Conclusions: Decreased GNLY, presumably at the protein level, is linked to the immunological condition of latent TB infection.

Keywords: Granulysin, Serum concentration, Latent tuberculosis infection, Gene expression, Genotype, Biomarker

Background

According to global estimation, latent *Mycobacterium tuberculosis* (MTB) infection is present in approximately one third of the human population (~2 billion people) [1]. Latent infection follows prolonged survival of MTB despite containment by host defense mechanisms [2] and contributes significantly to the risk of overt disease following reactivation of MTB under conditions of compromised immunity [3], which presents a major obstacle to achieving global tuberculosis (TB) control.

Interferon-gamma release assays (IGRA) are diagnostic of TB infection and operate on the principle that MTB-specific antigens provoke immune responses in whole blood after the establishment of infection [4]. IGRA offer more specific detection of latent TB infection (LTBI) than tuberculin skin test in many circumstances [5]. Accordingly, QuantiFERON-TB Gold In-tube enzyme-linked immunosorbent assay (ELISA)-based IGRA are recommended in multiple current guidelines, including those of the United States Center for Disease Control

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and Prevention for testing LTBI among people who are at risk of TB infection (i.e., healthcare workers) [6].

As an effector molecule, the saposin family protein granulysin (GNLY) is involved in protective immunity, and is released from natural killer (NK) cells, NKT cells, γδ T cells, and cytotoxic T lymphocytes (CTLs). This molecule directly eliminates extracellular MTB and intracellular bacteria in the presence of perforin by causing osmotic shock and inducing apoptosis [7]. Recently Walch et al. [8] suggested that GNLY initially penetrates infected cells and delivers bactericidal granzymes to intracytoplasmic bacteria. Hence, in the presence of GNLY, granzymes eliminate bacteria independently of host cell death.

Precursor and mature GNLY proteins of 15 and 9 kDa, respectively, have been identified, and mature GNLY is found with perforin and granzymes in cytotoxic granules. Moreover, a previous report has shown that the 9-kDa mature protein is mainly secreted from activated CTLs, whereas the 15-kDa precursor protein is found in plasma under physiological conditions [9]. Although the precursor protein may have more potent chemotactic and inflammatory activities, its physiological function remains poorly understood [10].

Circulating GNLY levels may be a useful indicator of overall host cellular immunity [11]. Moreover, GNLY expression has been identified as a marker for outcomes in cancer and organ transplantation patients and is an important mediator of damage in skin diseases [10]. In TB, plasma GNLY levels were significantly lower in patients with active disease than in healthy individuals and increased after successful anti-TB treatment [12]. In an animal study, GNLY expression was associated with protection after vaccination against TB in cattle [13]. However, it is not clear whether GNLY levels are associated with human LTBI. In the present study, we investigated the relationship between serum GNLY concentrations and LTBI status as diagnosed using IGRA, determined *GNLY* mRNA expression in blood cells, and analyzed *GNLY* genetic polymorphisms.

Methods

Study population and data collection

Healthcare workers were recruited from ten district TB-centers in Hanoi city. These TB centers are responsible for implementing directly observed treatment short-course (DOTS) programs for half of the city's population under the management of a municipal TB hospital (Hanoi Lung Hospital). Staff members who did not participate in the 2007 LTBI survey in this hospital [14] were also recruited and those who were employed for less than one year were excluded.

All study participants were interviewed and demographic information and factors associated with TB exposure were recorded using a structured questionnaire. Histories of Bacillus Calmette-Guérin (BCG) vaccination were obtained and confirmed according to the presence of BCG scars. Peripheral blood was collected for IGRA and for analyses of serum concentrations, mRNA expression, and polymorphisms of *GNLY*. Participants were encouraged to take chest X-rays and sputum tests, when IGRA positive status was identified. The participants were also given the chances of LTBI treatment after consultation with TB specialists.

Interferon-gamma release assays

IGRA for TB are used to estimate interferon-gamma induction by MTB-specific antigens (TB antigens). In this study, ELISA-based IGRA were performed using the third version assay QuantiFERON-TB Gold In-Tube™ (Cellestis, Victoria, Australia). Briefly, whole blood was collected in separate tubes that were not pre-coated with any stimulants for the negative control, and pre-coated with mitogen for the positive control or TB antigens. After 18 h incubation at 37 °C, concentrations of interferon-gamma in plasma supernatants were measured using the ELISA method. Interferon-gamma concentrations were calculated by subtracting negative control values from TB antigen-stimulated values, and the cut-off value was 0.35 IU/ml. The testing procedure was carefully monitored [15] and quality control was included in each run according to the manufacturer's instructions.

Serum granulysin assays

Both 15- and 9-kDa forms of serum GNLY were measured using a sandwich ELISA system with anti-GNLY (RB1) mouse IgG1κ as the capturing antibody and anti-GNLY-biotin (RC8) mouse IgG1κ as the detecting antibody as described previously [11]. Recombinant human (rh)-GNLY was used as a standard and culture supernatants from *GNLY* (15 kDa)-expressing Cos-7 cells were used as positive controls. GNLY concentrations were calculated from a standard curve of serially diluted rh-GNLY standards. This ELISA system is specific for GNLY and has a detection limit of approximately 20 pg/ml [11].

Quantitative real-time polymerase chain reaction (PCR) of granulysin mRNA in whole blood

Whole blood samples of 2.5 ml were collected into PAXgene™ Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and stored as recommended by the manufacturer. Total RNA was then extracted using PAXgene™ Blood RNA Kits (QIAGEN, Hilden, Germany) and reverse transcribed using random nonamers (TaKaRa, Shiga, Japan) and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was

performed using the TaqMan® Gene Expression Assay Hs00246266_m1 for *GNLY* (Applied Biosystems, Foster City, CA, USA) with a CFX96 real-time PCR system (BioRad, Hercules, CA, USA). *GNLY* expression was normalized to that of *GAPDH* using the ΔΔCt method [16] and mRNA expression levels of *GNLY* were expressed relative to control cDNA.

Single nucleotide polymorphism analysis

Genomic DNA samples were extracted from blood cells using QIAamp DNA Blood Mini Kits (QIAGEN) and were subjected to PCR amplification for analysis of single nucleotide polymorphism (SNP) of the *GNLY*. Among the Kinh Vietnamese people, using the 1000 Genomes Database, twenty-one SNPs were identified in *GNLY* and the 5′ region up to 1000 bp from the transcription start site of the reference mRNA sequence (NM_006433.4) [17]. All SNPs were in strong linkage disequilibrium (D' = 1, $r^2 > 0.85$) with each other, except for the SNP rs2043760 in intron 1 (D' = 1, $r^2 = 0.7$). A non-synonymous SNP rs11127 (C/T) of the exon 4 (NM_006433.4) was selected and genotyped as a representative SNP. Genomic DNA was amplified using the primers 5′-GGAGGTATCAGTCTAGAG**G**TA-3′ and 5′-GCTAAAGTCCATCTGCTCAA-3′, and a mismatch nucleotide (bold) was introduced in the sense primer to generate a *Kpn* I restriction enzyme site when the rs11127 allele was C. Genotype was determined according to the length of PCR products after digestion with *Kpn* I (TaKaRa) and C and T alleles gave 207- and 229-bp fragments, respectively.

Statistical analysis

Proportions between two study groups were compared using the chi-squared test. First, the serum GNLY concentrations measured by ELISA were used to check their distribution, and Wilcoxon rank-sum test was used to compare the distribution between groups. Then serum GNLY concentrations and *GNLY* mRNA levels were used for further analyses after logarithmic transformation of the values to approximate normal distribution. Since GNLY levels are influenced by gender and age, measurements of GNLY in the two groups were compared using analysis of covariance (ANCOVA) to adjust for covariates after performing unpaired t-tests. Relationships between GNLY levels and other parameters were assessed using Pearson's correlation coefficients and logistic regression models were used to assess risk factors for positive IGRA status. Allele-number dependent changes in gene expression were identified using univariate and multivariate linear regression models. Variables with biological meaning and with P values < 0.2 in univariate analyses were included in multivariate models. Differences and correlations were considered significant when $P < 0.05$, unless otherwise specified. Statistical analyses were performed using STATA version 11 (StataCorp, College Station, TX, USA).

Results

Characteristics of the study population

Among 109 healthcare workers, 53 (46.8 %) were recruited from the 225 staff members working in a municipal hospital that specializes in TB, and 150 of these members participated in the 2007 TB infection survey. This hospital has 260 inpatient beds, receives approximately 5000 inpatients per year, performs 120 consultation per day in the hospital outpatient department, and performs 7000 examinations per year in the community. In addition, 56 (51.4 %) healthcare workers were recruited from TB units that are located in district centers of preventive medicine, which function to implement DOTS at the grass roots level.

All participants answered questionnaires and provided blood samples. No participants showed physical signs of active TB. Almost half of the participants were less than 30 years old and 76.2 % of participants were female. Approximately 10 % of participants were obese with a body mass index (BMI) ≥ 25.0 and 50 % of participants had histories of BCG vaccination, as indicated by the presence of BCG scars. Although 78.0 % of participants declared frequent exposure to TB in their working places, only 42 (38.5 %) wore masks frequently (Table 1).

Serum granulysin levels and interferon-gamma levels after stimulation

Forty-one individuals were IGRA positive (37.6 %; 28.5–47.4 %). IGRA-positive status did not differ significantly between subjects from the TB hospital and district TB centers (data not shown). Distribution of GNLY levels was significantly different between IGRA-positive and negative groups (median, interquartile range; 2.14, 1.70–2.82 vs. 2.60, 2.01–3.23 ng/ml, $P = 0.0190$ by Wilcoxon rank-sum test) (Fig. 1). After logarithmic transformation of GNLY values and adjustment for age and gender using ANCOVA, GNLY concentrations were significantly lower in the IGRA-positive group than in the IGRA-negative group (adjusted mean, 95 % confidence intervals (95 % CI); 2.03, 1.72–2.44 vs. 2.48, 2.10–2.92 ng/ml; $P = 0.0127$; Table 2). Moreover, GNLY concentrations were negatively correlated with interferon-gamma levels after stimulation with the TB antigens used in IGRA ($r = -0.20$, $P = 0.0333$; Fig. 2).

The levels of TB exposure assessed by the questionnaire [14] were not different between IGRA-positive and negative groups (data not shown).

Table 1 Characteristics of the study population (n = 109)

	Number	Percent
Age (in years)		
20–29	54	49.5
30–39	26	23.9
40–49	15	13.8
≥ 50	14	12.8
Sex		
Men	26	23.8
Women	83	76.2
Body mass index		
< 18.5	7	6.4
18.5–24.9	92	84.4
≥ 25.0	10	9.2
Education level		
High school	3	2.8
Primary, secondary, pre-university	76	69.7
University and higher	23	21.1
Others	7	6.4
BCG vaccination scar		
No	51	46.8
Yes	54	49.5
NA	4	3.7
Ever diagnosed as TB		
No	107	98.2
Yes	2	1.8
Ever treated for TB		
No	107	98.2
Yes	2	1.8
Years served in healthcare profession		
< 2	26	23.9
2–4.99	37	33.9
5–9.99	21	19.3
≥ 10	25	22.9
Job		
Medical doctor	19	17.4
Nurse	55	50.5
Laboratory Technician	11	10.1
X-ray technician	2	1.8
Other	22	20.2
Current working place		
Lung hospital	53	48.6
District TB center	56	51.4
Current working area		
Outpatient department	9	8.3
TB ward	57	52.3
Non-TB ward	11	10.1
TB bacteriology laboratory	11	10.1
Non-TB bacteriology laboratory	1	0.9
Non-bacteriology laboratory	2	1.8
Administration	11	10.1
Other	5	4.6
NA	2	1.8
Current TB exposure in working place		
Never	8	7.3
Rare	3	2.8
Occasionally	12	11.0
Frequently	85	78.0
Do not know	1	0.9
Mask use		
Never	11	10.1
Rare	3	2.8
Occasionally	52	47.7
Frequently	42	38.5
NA	1	0.9

TB tuberculosis, *NA* not available, *BCG* Bacillus Calmette–Guérin

Serum granulysin concentrations, granulysin mRNA expression levels in blood, and polymorphisms of rs11127 SNP in exon 4 of the granulysin gene

A positive correlation was identified between serum GNLY concentrations and *GNLY* mRNA expression levels in the blood (r = 0.40, P < 0.0001; table not shown). In the

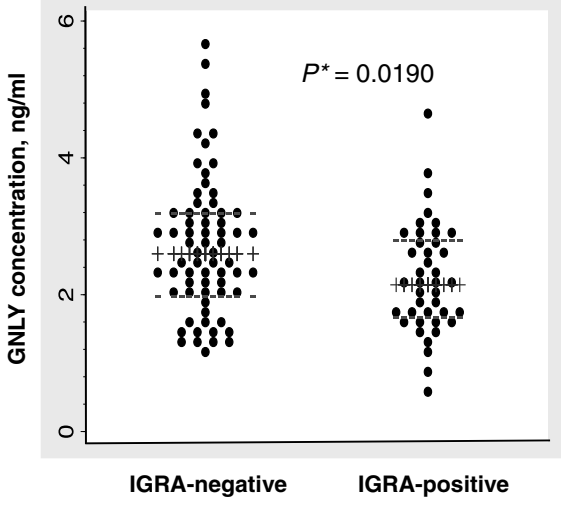

Fig. 1 Distribution of serum GNLY concentrations in IGRA-negative and IGRA-positive groups. Blue horizontal lines (+++), median values; green line (—), interquartile range; *by Wilcoxon rank-sum test; GNLY, granulysin; IGRA, Interferon-gamma release assays

Table 2 GNLY concentrations and IGRA results

	IGRA negative (*n* = 68)	IGRA positive (*n* = 41)
Before adjustment[a]		
Mean value[b] of GNLY concentrations	2.53	2.10
95 % CI	2.32–2.77	1.84–2.36
P value[c]	0.0116	
After adjustment[a]		
Mean value[b] of GNLY concentrations	2.48	2.03
95 % CI	2.10–2.92	1.72–2.44
P value[d]	0.0127	

GNLY granulysin, *IGRA* interferon-gamma release assays, *95 % CI* 95 % confidence intervals
[a]adjustment for age and sex
[b]GNLY concentrations with the original unit (ng/ml) are shown after transforming back
[c]unpaired t-test after logarithmic transformation of GNLY concentrations
[d]ANCOVA after logarithmic transformation of GNLY concentrations

subgroup analysis, serum GNLY concentrations correlated with *GNLY* mRNA, as the number of C allele was increased (r = 0.24, P = 0.1057 for TT genotype; r = 0.44, P = 0.0012 for CT; and r = 0.80, P = 0.001 for CC; Fig. 3). Serum GNLY levels varied significantly with genotype after adjustment for gender and age (P = 0.0015) and were low in participants with the CC genotype (adjusted mean, 95 % CI; 1.60, 1.25–2.05 ng/ml), intermediate in participants with the CT genotype (2.23, 1.8–2.64), and high in participants with the TT genotype (2.48, 2.12–2.92). Moreover, numbers of C alleles in these individuals were inversely associated with serum GNLY levels in univariate and multivariate regression models (Table 3). However, no clear differences in mRNA expression were found between participants with CC, CT, and TT genotypes either before or after adjustment for gender and age (adjusted mean, 95 % CI; 9.21, 6.11–13.87; 12.43, 9.30–16.61; and 9.78, 7.46–12.68 arbitrary units, respectively; P = 0.0994). Moreover, univariate and multivariate regression models did not show any signification associations between numbers of C alleles and *GNLY* mRNA expression levels (Table 3).

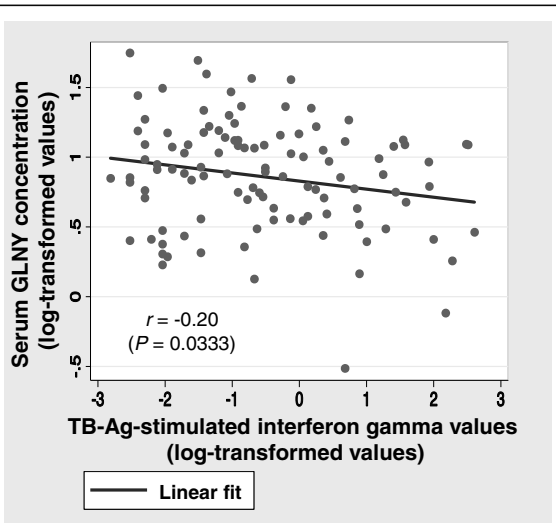

Fig. 2 Serum GNLY concentrations and interferon-gamma levels after stimulation with the TB antigens in IGRA. GNLY, granulysin; TB-Ag-stimulated interferon-gamma values, tuberculosis antigen values minus negative-control values in the blood. The scales of x- and y- axes are based on the values after logarithmic transformation of the original units; IU/ml for IGRA values and ng/ml for serum GNLY concentrations

Factors associated with latent tuberculosis infection estimated using interferon-gamma release assays

In both univariate and multivariate regression models, significant reverse associations were shown between IGRA-positive status and serum GNLY concentrations [unadjusted odds ratio (OR), 95 % CI; 0.55, 0.35–0.89; and adjusted OR, 95 % CI; 0.55, 0.31–0.98], whereas the number of C alleles and *GNLY* mRNA levels were not associated with IGRA-positive status. High BMI was independently associated with IGRA-positive status (Table 4). In addition, GNLY concentrations were significantly lower in IGRA-positive group than in IGRA-negative group, even after the effect of C alleles was considered (ANCOVA; P = 0.006, data not shown).

Discussion

In the present study, we investigated GNLY in LTBI as identified using IGRA among healthcare workers in Hanoi, Vietnam. Serum GNLY concentrations in the IGRA-positive group were significantly lower than those in the IGRA-negative group. Moreover, serum GNLY concentrations were significantly positively correlated with *GNLY* mRNA expression levels in blood and were dependent on the number of C alleles of the rs11127 SNP in exon 4 of *GNLY*, whereas *GNLY* mRNA expression levels were not dependent on the number of C alleles of the rs11127 SNP in exon 4 of *GNLY*. Of the three parameters (SNP genotype, mRNA expression, and concentrations of GNLY in blood), only circulating levels of GNLY protein were significantly negatively associated with IGRA-positive status.

GNLY, a major antimicrobial molecule, has been shown to preferentially bind and disrupt cholesterol-poor membranes of microbes [10]. However, no homolog has been found in rodents, and investigations using human samples are required to elucidate the significance of GNLY in vivo, as shown in the present study. In a recent study [8], bactericidal mechanisms involving cooperation of GLNY and granzymes have been clarified. Specifically, GLNY delivers granzymes to intracellular bacteria leading to oxidative damage through cleavage of bacterial antioxidants. Thus,

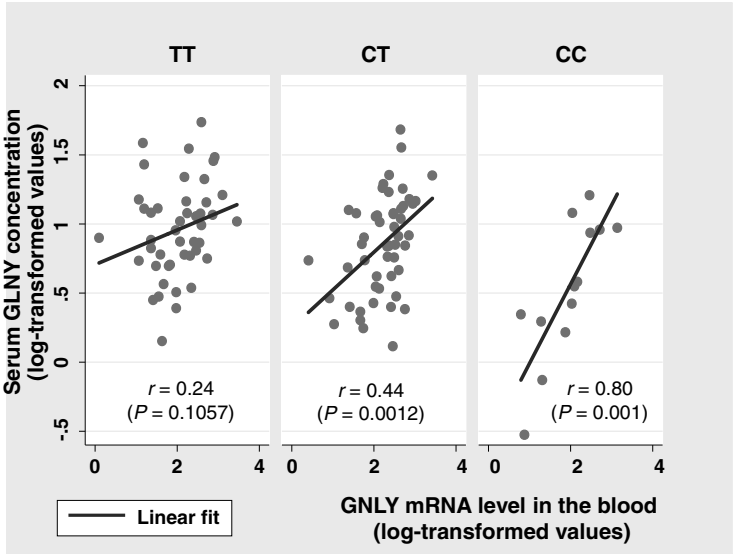

Fig. 3 Serum GNLY concentrations and *GNLY* mRNA gene expression levels in the blood by different genotypes of rs11127 SNP of the exon 4 of *GNLY* gene. GNLY, granulysin; mRNA, messenger RNA; SNP: single nucleotide polymorphism. The scales of x- and y- axes are based on the values after logarithmic transformation of the original units; arbitrary unit for *GNLY* mRNA levels, and ng/ml for serum GNLY concentrations

GNLY shows both cytolytic and bactericidal activities with other effector molecules such as granzymes and perforin, although GNLY acts independently at micromolar concentrations and under hypotonic or acidic conditions [18, 19].

Since CTLs play crucial roles in the containment of MTB in TB granulomas [2, 19], GNLY appears to be an important effector molecule in human TB infection, alone and in cooperation with granzymes and perforin. In a previous study in which the same ELISA system was used [11], GNLY concentrations of 244 presumably TB-unexposed healthy Japanese individuals were 3.7 ± 3.2 ng/ml (mean ± standard deviation), and these values were even higher than those in IGRA-negative Vietnamese individuals presumably resistant to TB infection in our study. It suggests that TB exposure may decrease serum GNLY levels, and that LTBI state may further suppress the level by some unknown mechanism, though effects of ethnicity and other background such as comorbidity and nutritional status should be taken into consideration and it is difficult to draw any conclusions from direct comparison between different studies. Moreover, previous studies of active TB disease have shown that GNLY levels in the blood vary with clinical stage [12, 20]. Although serum GNLY concentrations were lower in the LTBI group than in the non-LTBI group, this GNLY concentration (median, interquartile range; 2.144, 1.703–2.824 ng/ml) was higher than that shown previously in newly diagnosed active TB (median ± standard error, 1.511 ± 0.287 ng/ml) and relapsed TB (1.458 ± 0.329 ng/ml) patients in our

Table 3 *GNLY* polymorphisms and other factors associated with serum GNLY concentrations or *GNLY* mRNA expression levels in blood in univariate and multivariate regression models (n = 109)

	Univariate			Multivariate		
	Coefficient	95 % CI	*P* value	Coefficient	95 % CI	*P* value
a) Polymorphism of rs11127 SNP in exon 4 of the *GNLY* was inversely associated with serum GNLY concentrations (log-transformed values)						
Age (increased by one year)	0.00	−0.01 to 0.01	0.985	0.00	−0.01 to 0.01	0.666
Gender (female vs. male)	0.04	−0.13 to 0.22	0.628	0.08	−0.09 to 0.25	0.359
Genotype (increased by one C allele)	−0.18	−0.29 to −0.07	0.001	−0.19	−0.30 to −0.08	0.001
b) Polymorphism of rs11127 SNP in exon 4 of the *GNLY* was not associated with *GNLY* mRNA expression levels (log-transformed values) in blood						
Age (increased by one year)	0.00	−0.01 to 0.01	0.870	0.00	−0.01 to 0.01	0.883
Gender (female vs. male)	−0.33	−0.61 to −0.05	0.021	−0.34	−0.62 to −0.06	0.019
Genotype (increased by one C allele)	0.03	−0.15 to 0.21	0.725	0.06	−0.12 to 0.24	0.532

GNLY granulysin, *SNP* single nucleotide polymorphism, *95 % CI* 95 % confidence interval

Table 4 Serum GNLY concentrations and other factors associated with IGRA-positive status in univariate and multivariate logistic regression models (n = 109)

	No (%)	Univariate		Multivariate[a]	
		OR	95 % CI	OR	95 % CI
Age (by year)		1.02	0.98–1.06	1.03	0.98–1.09
Sex					
Male	11/26 (42.3)	Reference		Reference	
Female	30/83 (36.1)	0.77	0.31–1.89	0.83	0.26–2.65
BMI					
18.5–24.9	31/92 (33.7)	Reference		Reference	
< 18.5	3/7 (42.9)	1.48	0.31–7.01	3.99	0.53–29.92
≥ 25.0	7/10 (70.0)	**4.59**	**1.11–18.99**	**8.43**	**1.37–51.75**
Job					
Others	4/22 (18.2)	Reference		Reference	
Doctor	10/19 (52.6)	5.00	1.22–20.46	3.37	0.69–16.52
Nurse	22/55 (40.0)	3.00	0.89–10.06	2.51	0.56–11.18
Technician	5/13 (38.5)	2.81	0.59–13.34	1.48	0.22–10.03
Working place					
Non-TB	8/30 (26.7)	Reference		Reference	
TB	32/77 (41.6)	1.96	0.77–4.94	1.70	0.55–5.22
GNLY concentration (by ng/ml)		**0.55**	**0.35–0.89**	**0.55**	**0.31–0.98**
GNLY mRNA expression (by one unit)		0.95	0.88–1.02	0.98	0.88–1.08
Genotype (by number of C allele)		0.84	0.47–1.51	-	-

GNLY granulysin, *IGRA* interferon-gamma release assays, *OR* odds ratio, *95 % CI* 95 % confidence interval, *BMI* body mass index, *TB* tuberculosis; significant associations are presented in bold

[a]Multivariate analyses included age, sex, BMI, job category, working place, GNLY concentration, and *GNLY* mRNA expression levels

collaborative study using the same ELISA system [20]. Potential interpretations of low GNLY levels in TB infection are as follows. First, the present data may reflect sequestration of GNLY-producing cells: Lymphocytes with high GNLY expression accumulate at the sites of latent infection. At these sites, continuous cross-talk between the host immune system and the pathogen [21] may facilitate GNLY turnover, and accumulation of these GNLY-producing lymphocytes potentially results in a relative decrease in circulating GNLY in infected individuals. Moreover, it is well known that MTB-specific CTLs accumulate in areas surrounding infected epithelioid cells of TB granulomas [19, 22] and may also occur in individuals with LTBI. This condition may differ from that of chronic TB, which is characterized by persistent inflammation and active clinical manifestations, and is associated with diminished GNLY in granuloma-associated CTLs, resulting in impaired CTL activities [23]. Second, protein levels of GNLY were negatively associated with interferon gamma release in the present study, whereas *GNLY* expression levels and *GNLY* polymorphisms were not, suggesting that GNLY may lack stability in TB infection, and its rates of metabolism or degradation may be affected by chronic inflammation and immune reactions in TB. Finally, during the very early stages of TB infection, NK and NKT cells may destroy pathogens and infected cells [24], and as innate and intermediate immune cells, they express both GNLY and granzymes. Hence, limited expression of GNLY in these cells may lead to failure to eliminate MTB and it may cause IGRA-positive status. However, although frequencies of the C allele of the rs11127 *GNLY* polymorphism may lead to low serum GNLY concentrations, these inherent genotypes were not associated with IGRA-positive status, indicating that low GNLY concentrations may be a consequence rather than a cause of LTBI.

Sputum smear-positive cases of TB have long been targeted in TB control programs [25] and preventive therapy for LTBI in the general population has not been feasible in most countries with high TB burdens, including in Vietnam. However, identification of subgroups of individuals who are at high risk of developing active disease may provide a useful and cost-effective strategy to control TB [26]. Thus, future investigations are required to explore the roles of GNLY and other antimicrobial molecules as biomarkers, for example, in prospective cohort studies with follow up of blood levels during the first two years after infection.

Our study has some limitations. We could not recruit individuals with no TB exposure. We compared our results with those obtained from previous studies and discussed possible interpretations of our findings. Because previous studies of granzymes [8] and perforin [7] reveal that GNLY works in cooperation with these molecules to eliminate MTB, it may be of further value to investigate the co-localization of these effector molecules using flow cytometry or cell population analyzers to offer greater certainty of this assertion. Moreover, the 15-kDa precursor form of GNLY is better known as a potent chemotactic factor during inflammation than as a bactericidal molecule. Since the present sandwich ELISA system captures both forms of GNLY, further studies are required to determine which form of GNLY is predominant in blood from patients with LTBI and active TB. Lastly, the SNP rs11127 (C/T) of exon 4 leads to an amino acid substitution (Ile104Thr) in the GNLY protein and may accelerate consumption or enhance stability of GNLY. Since *GNLY* genotype was associated with GNLY concentration, but not with mRNA expression level, we can assume that *GNLY* polymorphism may have affected GNLY concentrations at the protein level; and *GNLY* may have been expressed in the way partly independent of *GNLY* genotype. A genetic variation that directly affects GNLY concentrations was not clearly determined in this study because of strong linkage disequilibrium around the *GNLY* locus. Nevertheless, the SNP rs11127 has been reported to have an important role in clearance of hepatitis B virus [27, 28], and further study on GNLY genetic polymorphism is necessary whether it may contribute to optimize granulysin vaccines against TB [29, 30].

Conclusion

Serum GNLY concentrations in IGRA-positive participants were significantly lower than in those with IGRA-negative status. Therefore, changes in circulating GNLY, presumably at the protein level, may be involved in the immunological condition of LTBI.

Abbreviations

BCG: Bacillus Calmette-Guérin; CI: Confidence interval; CTL: Cytotoxic T lymphocytes; DOTS: Directly observed treatment short-course; ELISA: Enzyme-linked immunosorbent assay; GNLY: Granulysin; IGRA: Interferon-gamma release assays; LTBI: Latent tuberculosis infection; MTB: *Mycobacterium tuberculosis*; NK: Natural killer; OR: Odds ratio; PCR: Polymerase chain reaction; SNP: Single nucleotide polymorphism; TB: Tuberculosis

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Availability of data and materials

All the data will be made available by the corresponding author upon request. Identifying/confidential patient data however will not be shared.

Authors' contributions

PHT, SS participated in the conception, design, supervision of the study, and drafting of the paper. DBT, LTH carried out the immunoassays. NTLH participated in supervision of on-site implementation, analysis and interpretation of data, drafting the paper, and substantially revising it. MH conducted PCR and SNP analysis. PTMN, PTA, VCC participated in on-site implementation of the study. IM participated in technical transfer and supervision. LTL participated in conception, design and supervision of the study. NK participated in the conception and design of the study, analysis and interpretation of data, drafting the paper and substantially revising it. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All study participants gave written informed consent, and the study was approved by the ethics committees of the Hanoi Department of Health, Vietnam, National Center for Global Health and Medicine, Japan and the Research Institute of Tuberculosis Japan Anti-TB Association.

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Multidrug-Resistant Sequence Type 235 *Pseudomonas aeruginosa* Clinical Isolates Producing IMP-26 with Increased Carbapenem-Hydrolyzing Activities in Vietnam

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Forty clinical isolates of multidrug-resistant *Pseudomonas aeruginosa* were obtained in a medical setting in Hanoi, Vietnam. Whole genomes of all 40 isolates were sequenced by MiSeq (Illumina), and phylogenic trees were constructed from the single nucleotide polymorphism concatemers. Of these 40 isolates, 24 (60.0%) harbored metallo-β-lactamase-encoding genes, including bla_{IMP-15}, bla_{IMP-26}, bla_{IMP-51}, and/or bla_{NDM-1}. Of these 24 isolates, 12 harbored bla_{IMP-26} and belonged to sequence type 235 (ST235). *Escherichia coli* expressing bla_{IMP-26} was significantly more resistant to doripenem and meropenem than *E. coli* expressing bla_{IMP-1} and bla_{IMP-15}. IMP-26 showed higher catalytic activity against doripenem and meropenem than IMP-1 and against all carbapenems tested, including doripenem, imipenem, meropenem, and panipenem, than did IMP-15. These data suggest that clinical isolates of multidrug-resistant ST235 *P. aeruginosa* producing IMP-26 with increased carbapenem-hydrolyzing activities are spreading in medical settings in Vietnam.

Pseudomonas aeruginosa isolates producing IMP- or VIM-type metallo-β-lactamases (MBLs) have been detected in various Asian countries (1). For example, isolates producing IMP-type MBLs have been observed in China, Japan, South Korea, Malaysia, Singapore, Thailand, and Vietnam, and isolates producing VIM-type MBLs have been identified in China, India, Indonesia, South Korea, Malaysia, Saudi Arabia, and Taiwan (2, 3). In addition, a *P. aeruginosa* isolate producing an NDM-type MBL has been reported in India (4).

To date, more than 58 IMP-type MBL variants have been reported (ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Allele.tab). IMP-15 was first identified in a *P. aeruginosa* clinical isolate from Mexico (5). Since then, five reports have described the detection of IMP-15 in clinical isolates, including *P. aeruginosa* isolates in Mexico, Germany, and Lebanon (6–10). IMP-26 was first identified in a *P. aeruginosa* clinical isolate in Singapore (11). Since then, one report has described an IMP-26-producing *P. aeruginosa* isolate in Malaysia (3), and five reports have described the detection of IMP-26 in clinical isolates of other species, including *Enterobacter cloacae* (12–14) and *Klebsiella oxytoca* (15), in China and *Klebsiella pneumoniae* in the Philippines (16). IMP-51 was recently identified in a *P. aeruginosa* clinical isolate in Vietnam and showed increased doripenem- and meropenem-hydrolyzing activities (17). The enzymatic properties of IMP-15 and IMP-26, however, have not yet been determined.

This is the first report of *P. aeruginosa* producing IMP-26 and IMP-15 in Vietnam, and it describes the biochemical characterization of these IMP-type MBLs.

MATERIALS AND METHODS

Bacterial strains. Multidrug-resistant *P. aeruginosa* was defined as described previously (18). Forty isolates of multidrug-resistant *P. aeruginosa* were obtained from March 2013 to December 2014 in a medical setting in Hanoi, Vietnam. Of these 40 isolates, 33 were isolated from respiratory tracts, and 7 were isolated from urinary tracts. The samples of these isolates were from intensive care units (19 samples), a gyniatrics ward (7 samples), a cardiology ward (3 samples), a respiratory ward (3 samples), an emergency room (2 samples), a nephrology ward (2 samples), a rehabilitation center (3 samples), and a rheumatology ward (1 sample). The MICs for these isolates of various antibiotics were determined using the microdilution method, according to the guidelines of the Clinical and Laboratory Standards Institute (19).

Whole-genome sequencing. Whole genomes of the 40 isolates were extracted using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced by MiSeq (Illumina, San Diego, CA). We achieved >30.6-fold coverage for each isolate. Raw reads of each isolate were assembled using CLC Genomic Workbench version 8.0.2, and drug resistance genes were identified using ResFinder 2.1 (https://cge.cbs.dtu.dk//services/ResFinder/). Multilocus sequence typing (MLST) was deduced, as described by the protocols of the PubMLST (http://pubmlst.org/paeruginosa/) databases.

Phylogenetic analysis. To identify single nucleotide polymorphisms (SNPs) among all 40 whole genomes, all reads of each isolate were aligned against the *P. aeruginosa* PAO1 sequence (accession no. AE004091) using CLC genomics workbench version 8.0.2 (CLC bio, Tokyo, Japan). To analyze the relation among sequence type 235 (ST235) *P. aeruginosa* isolates tested, the complete genome of NCGM2.S1 (accession no. AP012280) was used as a reference (20). SNP concatenated sequences were aligned by MAFFT (http://mafft.cbrc.jp/alignment/server/). Models and

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parameters used for the phylogenetic analyses were computed using j-Model Test-2.1.4. A maximum-likelihood phylogenetic tree was constructed from SNP alignment with PhyML 3.0 (21).

***Escherichia coli* transformants expressing bla_{IMP-1}, bla_{IMP-15}, and bla_{IMP-26}.** The open reading frames (ORFs) of bla_{IMP-1}, bla_{IMP-15}, and bla_{IMP-26} were PCR amplified using the following primer sets: EcoRI-IMP-1-F (5′-GGGGAATTCATGAGCAAGTTATCTGTATTC-3′) and PstI-IMP-1-R (5′-AAACTGCAGTTAGTTGCTTGGTTTTGATGG-3′), EcoRI-IMP-15-F (5′-ATGAATTCATGAACAAGTTATCTGTATTCT-3′) and PstI-IMP-15-R (5′-ATCTGCAGTTAGTTACTTGGCAGTGAT GGT-3′), and EcoRI-IMP-26-F (5′-ATGAATTCATGAGCAAGTTATCT GTATTCT-3′) and PstI-IMP-26-R (5′-ATCTGCAGTTAGTTGCTTAG TTTTGATGGT-3′), respectively. The *E. coli* transformants expressing bla_{IMP-1}, bla_{IMP-15}, and bla_{IMP-26} were produced as previously described (22). The PCR products of each were digested with EcoRI and PstI and ligated into pHSG398 (TaKaRa Bio, Shiga, Japan). The plasmids were used to transform DH5α, the transformants were selected on Luria-Bertani agar containing chloramphenicol at 30 μg/ml, and their susceptibility to various β-lactams was assayed. The promoter regions of each bla_{IMP} were TTCACA (−35 sequence) and CATGAT (−10 sequence), which were located at the upstream region of EcoRI recognition site and separated by a space of 17 bp.

Purification of IMP-1, IMP-15, and IMP-26. Recombinant IMP-1, IMP-15, and IMP-26 proteins were purified as described previously (22). The β-lactamase activities were monitored during the purification process using nitrocefin (Oxoid, Ltd., Basingstoke, United Kingdom). The initial rates of hydrolysis were determined at 37°C in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl and 5 μM $Zn(NO_3)_2$ using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The reaction was initiated by direct addition of substrate into the cuvettes of the spectrophotometer, allowing the UV absorption of the reaction mixture to be determined during the initial phase of the reaction. The K_m, k_{cat}, and k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis using Lineweaver-Burk plots. Previously reported wavelengths and extinction coefficients were used for the analysis of β-lactam substrates (23–25). K_m and k_{cat} values were determined using triplicate analyses.

Pulsed-field gel electrophoresis and Southern blotting. DNA plugs of IMP-26, IMP-15, and IMP-51 producers, digested with S1 nuclease, were prepared, separated by pulsed-field gel electrophoresis, and subjected to Southern hybridization (26) using 16S rRNA and bla_{IMP} probes (27, 28).

Accession number(s). The whole-genome sequences of all 40 isolates have been deposited at GenBank as accession numbers DRA003741 (DRX036168 to DRX036207 and DRR039942 to DRR039981).

RESULTS AND DISCUSSION

Antimicrobial susceptibility. Most of the 40 *P. aeruginosa* clinical isolates were resistant to amikacin, ceftazidime, imipenem, and meropenem but were sensitive to colistin (see Table S1 in the supplemental material). All isolates were resistant to ciprofloxacin (see Table S1 in the supplemental material). Of the 40 isolates, 12 (30.0%), 15 (37.5%), 20 (50.0%), and 13 (32.5%) were highly resistant to imipenem, meropenem, amikacin, and arbekacin, respectively, with MICs of ≥512 mg/liter (data not shown). One isolate was resistant to colistin, with an MIC of 128 mg/liter.

MLST and phylogenetic analysis. The 40 *P. aeruginosa* isolates belonged to 15 different MLSTs, including two new sequence types, ST2165 and ST2166 (Table 1). The new sequence types, ST2165 and ST2166, had different sequences of all seven alleles of MLST from those of ST235. Fifteen isolates belonged to ST235, which has been recognized as one of three high-risk clones, i.e., ST235, ST111, and ST175 (29). Phylogenetic analysis revealed that the isolates belonging to ST235 formed the largest clade among the 40 isolates (see Fig. S1A in the supplemental material). The 15 isolates belonging to ST235 each harbored one or two of three genes encoding carbapenemases, including bla_{IMP-26}, bla_{IMP-51}, and bla_{NDM-1}, and genes encoding aminoglycoside resistance factors, including *aac(6′)-Ib*, *aacA37*, and *aadA11* (Table 1). Of the 15 isolates belonging to ST235, 12 harbored bla_{IMP-26}, *aac(6′)-Ib*, *aacA37*, and *aadA11* (Table 1). Of the remaining three isolates, two harbored bla_{IMP-51} and *aac(6′)-Ib*, and one harbored bla_{IMP-51}, bla_{NDM-1}, and *aac(6′)-Ib* (Table 1). The phylogenetic tree among ST235 isolates showed two clades. Twelve isolates in clade 1 harbored bla_{IMP-26}, *aac(6′)-Ib*, *aacA37*, and *aadA11*; two isolates in clade 2 harbored bla_{IMP-51} and *aac(6′)-Ib*; and one isolate harbored bla_{IMP-51}, bla_{NDM-1}, and *aac(6′)-Ib* (see Fig. S1B in the supplemental material). Isolates belonging to other STs harbored a gene encoding a carbapenemase, bla_{IMP-15}, or genes encoding extended-spectrum β-lactamases, bla_{PSE-1} or bla_{VEB-1}, as well as genes encoding aminoglycoside resistance factors: *rmtB*, *aac(6′)-Ib*, *aac(3″)-Ia*, *aadA1*, *aadA11*, *aadB*, or *aph(3′)-VIa* (Table 1). The colistin-resistant *P. aeruginosa* isolate NCGM3006 belonged to ST235 (see Fig. S1B in the supplemental material) and did not have *mcr-1* or any mutation in *cprRS*, *parRS*, *pmrAB*, and *phoPQ* associated with colistin resistance (30).

Multidrug-resistant *P. aeruginosa* belonging to ST235 may have been disseminated among medical settings in Vietnam. *P. aeruginosa* belonging to ST235 has spread in Asian countries, including India, South Korea, Malaysia, Sri Lanka, Thailand, and Taiwan (3). In particular, 88.3% of multidrug-resistant *P. aeruginosa* strains obtained in medical settings in Japan belonged to ST235 (31).

Our study indicates that IMP-26-producing *P. aeruginosa* clinical isolates spread in a medical setting in Vietnam. IMP-15 was initially identified in a *P. aeruginosa* clinical isolate obtained in 2003 from Mexico, and IMP-26 was initially identified in a *P. aeruginosa* isolate obtained in 2008 from Singapore. The IMP-26 producer in Singapore belonged to ST654, whereas all IMP-26 producers in Vietnam belonged to ST235 (Table 1). ST654 is probably unrelated to ST235 because of the different sequences for all seven alleles, as determined by MLST. ST235 is a worldwide clone, whereas ST654 may not be, although it has been reported

TABLE 1 MLST and drug resistance genes in 40 *P. aeruginosa* isolates

MLST	No. of isolates[a]	Carbapenemase- and ESBL-encoding gene(s)	Aminoglycoside resistance gene(s)
ST235	15	bla_{IMP-26} (12/15)[b], bla_{IMP-51} (3/15), bla_{NDM-1} (1/15)	*aac(6′)-Ib*, *aacA37* (12/15), *aadA11* (12/15)
ST277	2		*rmtB*, *aadA11*
ST308	1	bla_{IMP-15}, bla_{PSE-1}	*rmtB*, *aadA11*
ST310	4	bla_{IMP-15}, bla_{PSE-1}	*rmtB*, *aadA11*
ST357	2	bla_{PER-1}	*aph(3′)-VIa*, *aadA11* (1/2)
ST360	1	bla_{IMP-15}, bla_{PSE-1}	*rmtB*, *aadA11*
ST644	2	bla_{PSE-1}	*aac(6′)-Ib*, *aac(3″)-Ia*, *aadA1*, *aadB*
ST664	3		*aac(6′)-Ib*
ST773	4	bla_{VEB-1}	*aac(3″)-Ia*, *aadA1*, *aadB*
ST1021	1	bla_{VEB-1}	*aac(3″)-Ia*, *aadA1*, *aadA11*
ST1420	1	bla_{IMP-15}, bla_{PSE-1}	*rmtB*, *aadA11*
ST2069	2	bla_{IMP-15}, bla_{PSE-1}	*rmtB*, *aadA11*
ST2165	1		*rmtB*, *aadA11*
ST2166	1	bla_{IMP-15}, bla_{PSE-1}	*rmtB*, *aadA11*

[a] That is, the total no. of isolates belonging to the same sequence type.
[b] The numbers in parentheses indicate the number of isolates harboring the gene or belonging to the same sequence type/the total number of isolates.

TABLE 2 MICs of β-lactams for *E. coli* transformants containing bla_{IMP-1}, bla_{IMP-15}, bla_{IMP-26}, and bla_{IMP-51}

Antibiotic(s)[a]	MIC (μg/ml) for *E. coli* DH5α carrying:				
	pHSG398/ IMP-1	pHSG398/ IMP-15	pHSG398/ IMP-26	pHSG398/ IMP-51[b]	pHSG398
Ampicillin	256	256	32	32	8
Ampicillin-sulbactam	64	64	8	8	4
Penicillin G	128	256	64	32	32
Aztreonam	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125
Cefepime	32	8	32	8	≤0.125
Cefotaxime	32	32	64	64	≤0.125
Cefoxitin	>1,024	512	256	>1,024	16
Cefozopran	32	8	16	8	≤0.125
Cefpirome	1	1	1	0.5	≤0.125
Ceftazidime	512	256	512	128	0.5
Ceftriaxone	128	32	256	128	≤0.25
Cephradine	>1,024	512	256	64	16
Doripenem	4	1	16	4	≤0.125
Imipenem	2	0.5	2	0.5	≤0.125
Meropenem	2	2	16	4	≤0.125
Panipenem	4	1	4	0.5	≤0.125
Moxalactam	1,024	128	1,024	1,024	≤0.125

[a] The ratio of ampicillin to sulbactam was 2:1. The ratio of piperacillin to tazobactam was 4:1. The ratio of ticarcillin to clavulanic acid was 15:1.
[b] From Tada et al. (17).

from several countries, including Argentina (PubMLST ID 1610), France (32), Poland (33), Nigeria (PubMLST ID 2010), Sweden (PubMLST ID 679), Singapore (11), and Tunisia (34).

Drug susceptibility of *E. coli* DH5α transformants expressing IMP-1, IMP-15, and IMP-26. All *E. coli* DH5α expressing bla_{IMP-1} (DH5α/IMP-1), bla_{IMP-15} (DH5α/IMP-15), and *E. coli* DH5α expressing bla_{IMP-26} (DH5α/IMP-26) were significantly more resistant to all β-lactams tested, except for aztreonam, than DH5α expressing a vector control (Table 2). A comparison of drug susceptibilities among DH5α/IMP-1, DH5α/IMP-15, and DH5α/IMP-26 showed that DH5α/IMP-15 was less resistant to doripenem, imipenem, and panipenem than was DH5α/IMP-1 (Table 2). DH5α/IMP-26 was more resistant to doripenem and meropenem than was DH5α/IMP-1 (Table 2). That is, the MICs of cefepime, cefozopran, ceftriaxone, cephradine, doripenem, imipenem, and panipenem for DH5α/IMP-15 were <4-fold lower than those for DH5α/IMP-1 (Table 2). The MICs of doripenem and meropenem for DH5α/IMP-26 were >4-fold higher than those for DH5α/IMP-1, whereas the MICs of ampicillin, ampicillin-sulbactam, cefoxitin, and cephradine were <4-fold lower than those for DH5α/IMP-1 (Table 2). We recently identified IMP-51 with increased doripenem- and meropenem-hydrolyzing activities in a *P. aeruginosa* isolate in Vietnam (17). Comparisons of the drug susceptibilities of DH5α/IMP-26 and DH5α/IMP-51 showed that DH5α/IMP-26 was more resistant to carbapenems, ceftriaxone, and moxalactam than DH5α/IMP-51 (Table 2). That is, the MICs of ceftazidime, cephradine, doripenem, imipenem, meropenem, and panipenem for DH5α/IMP-26 were >4-fold higher than those for DH5α/IMP-51, whereas the MICs of cefoxitin for DH5α/IMP-26 were >4-fold lower than that for DH5α/IMP-51 (Table 2). Collectively, DH5α/IMP-26 was significantly resistant to all of the carbapenems tested, including doripenem, imipenem, meropenem, and panipenem (Table 2).

Catalytic activities of IMP-1, IMP-15, and IMP-26. Recombinant IMP-1, IMP-15, and IMP-26 hydrolyzed all tested β-lactams, except for aztreonam (Table 3). IMP-15, compared to IMP-1, showed significantly higher k_{cat}/K_m ratios for penicillin G but lower ratios for ampicillin, cefepime, cefoxitin, ceftazidime, cephradine, imipenem, panipenem, and moxalactam (Table 3). IMP-26, compared to IMP-1, showed significantly higher k_{cat}/K_m ratios for cefotaxime and meropenem but lower ratios for ampicillin, penicillin G, cefoxitin, and cephradine (Table 3). IMP-26, compared to IMP-15, showed significantly higher k_{cat}/K_m ratios for cefepime, ceftazidime, doripenem, imipenem, meropenem, moxalactam, and panipenem but lower k_{cat}/K_m ratios for ampicillin and penicillin G (Table 3). The lower K_m values for IMP-26 than for IMP-15 for carbapenems, including doripenem, imipenem, meropenem, and panipenem, resulted in higher k_{cat}/K_m ratios for IMP-26 (Table 3). The enzymatic activities of IMP-26 against cefotaxime, cefoxitin, cefpirome, and cephradine were similar to those of IMP-15 (Table 3). Compared to IMP-51, IMP-26 showed >2-fold-higher catalytic activity against penicillin G, cefepime, cefpirome, ceftazidime, cephradine, imipenem, meropenem, and panipenem, whereas IMP-26 showed >2-fold-

TABLE 3 Kinetic parameters of IMP-1, IMP-15, and IMP-26 enzymes[a]

β-Lactam	IMP-1			IMP-15			IMP-26		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Ampicillin	192 ± 12	34 ± 2	0.18	178 ± 20	8.4 ± 0.5	0.047	238 ± 37	1.4 ± 0.1	0.0058
Penicillin G	414 ± 97	81 ± 15	0.2	28 ± 4	11.2 ± 0.4	0.41	284 ± 37	6.5 ± 0.5	0.023
Aztreonam	NH	NH	NH	NH	NH	NH	NH	NH	NH
Cefepime	33 ± 4	3.1 ± 0.3	0.093	59 ± 13	1.2 ± 0.1	0.020	56 ± 2	3.0 ± 0.1	0.052
Cefotaxime	21 ± 2	5.2 ± 0.2	0.24	6.9 ± 1.1	2.2 ± 0.1	0.32	13 ± 1	7.1 ± 0.1	0.53
Cefoxitin	20 ± 2	5.6 ± 0.1	0.28	10 ± 1	1.2 ± 0.1	0.12	7.4 ± 1.0	0.52 ± 0.01	0.07
Cefpirome	294 ± 45	17 ± 2	0.057	48 ± 11	2.3 ± 0.2	0.048	73 ± 1	4.0 ± 0.1	0.054
Ceftazidime	42 ± 4	1.24 ± 0.03	0.030	46 ± 4	0.55 ± 0.02	0.012	17 ± 3	0.93 ± 0.03	0.058
Cephradine	95 ± 10	53 ± 3	0.56	101 ± 8	22 ± 1	0.22	81 ± 2	10.3 ± 0.1	0.13
Doripenem	105 ± 32	11 ± 2	0.11	60 ± 3	3.5 ± 0.1	0.058	22 ± 1	3.8 ± 0.1	0.18
Imipenem	91 ± 7	10.3 ± 0.2	0.11	302 ± 26	4.9 ± 0.3	0.016	180 ± 5	11.7 ± 0.3	0.065
Meropenem	142 ± 10	4.4 ± 0.2	0.031	80 ± 1	1.8 ± 0.1	0.022	20 ± 2	2.9 ± 0.1	0.14
Panipenem	234 ± 39	35 ± 5	0.15	74 ± 6	4.4 ± 0.1	0.059	36 ± 2	4.7 ± 0.1	0.13
Moxalactam	39 ± 2	15 ± 1	0.38	27 ± 4	4.1 ± 0.3	0.15	15 ± 1	6.5 ± 0.1	0.43

[a] The proteins were initially modified by a His tag, which was removed after purification. K_m and k_{cat} values are presented as means ± the standard deviations from three independent experiments. NH, no hydrolysis was detected with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

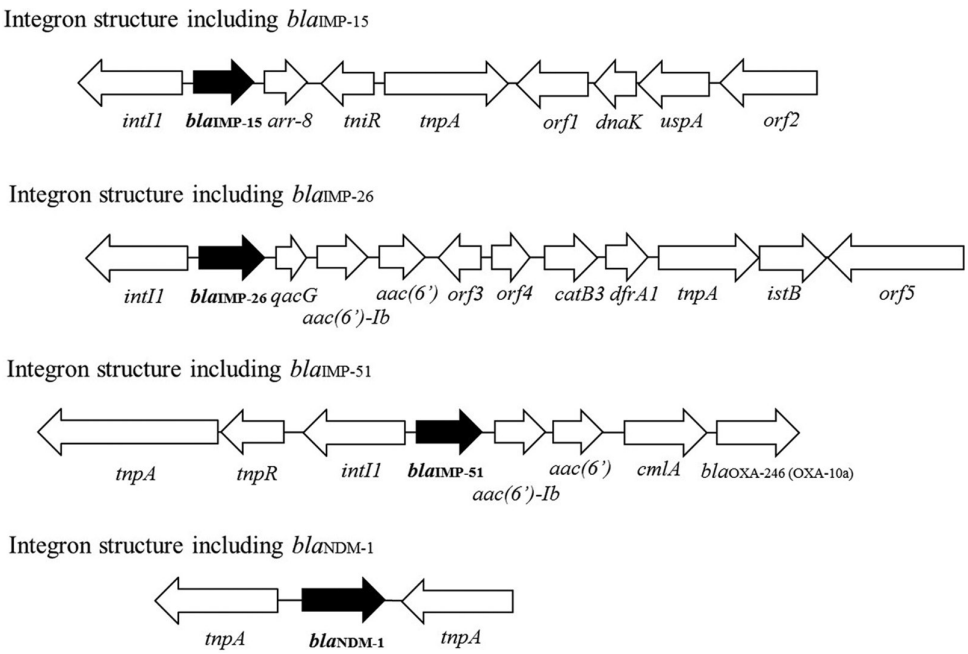

FIG 1 Structures of the variable regions of the integrons including bla_{IMP-15}, bla_{IMP-26}, bla_{IMP-51}, and bla_{NDM-1}. *orf1*, SdiA-regulated motif containing a protein-encoding gene; *orf2*, a putative sulfate permease-encoding gene; *orf3* and *orf4*, hypothetical protein-encoding genes; *orf5*, a restriction endonuclease subunit R-encoding gene.

lower catalytic activities against cefoxitin (see Tables 3 and 2 in reference 17).

A dendrogram of IMP-type enzymes (see Fig. S2 in the supplemental material) showed that IMP-15 does not belong to any cluster, whereas IMP-26 belonged to a cluster that contained IMP-4 and IMP-38. The sequence of IMP-26 was 99% identical to those of IMP-4 from *Acinetobacter baumannii* in Hong Kong (35) and IMP-38 from *K. pneumoniae* KP9 in China (accession no. HQ875573). In addition, the sequence of IMP-26 was 95.5% identical to that of IMP-1 from *Serratia marcescens* (36) and 84.6% identical to that of IMP-2 from *A. baumannii* (37). Recombinant IMP-4 was active against imipenem and meropenem, with V_{max} values of 53 and 8%, respectively, relative to that for penicillin G (35).

IMP-26 has 2 amino acid substitutions each compared to IMP-4 (Ser3Pro and Val67Phe) and IMP-38 (Val67Phe and Gly196Ser) (see Fig. S2 in the supplemental material). In IMP-43 and IMP-44, the amino acid substitution Val67Phe has been associated with enzymatic activities against carbapenems (see Fig. S2 in the supplemental material) (2). This amino acid substitution differentiates IMP-43 from IMP-7, whereas both this substitution and Phe87Ser differentiate IMP-44 from IMP-11 (see Fig. S2 in the supplemental material). Compared to IMP-7 and IMP-11, IMP-43 and IMP-44, respectively, showed more-efficient catalytic activities against carbapenems (2). Therefore, the Val67Phe amino acid substitution in IMP-26 may affect its catalytic efficiency against all carbapenems tested, including doripenem, imipenem, meropenem, and panipenem. Amino acid residue 67 in IMP-1 is located at the end of an enzymatic active-site loop consisting of residues 60 to 66 (38). This loop may be a major determinant for the tight binding of substrates in the active site (38). The active-site loop of MBL BcII has been reported to contribute to substrate binding by providing hydrophobic interactions between the phenyl and methyl groups of penicillin G and residues located at both ends of the loop (Phe61 and Val67) (38).

Genetic environments surrounding bla_{IMP-15} and bla_{IMP-26}. bla_{IMP-15} and bla_{IMP-26} are present in class I integrons, which differ in structure from each other (Fig. 1). The genetic structure surrounding bla_{IMP-15} was identical in all isolates harboring bla_{IMP-15} tested in this study. The sequence from nucleotides (nt) 135 to 2184, which contained *intI1* and bla_{IMP-15} (Fig. 1), was >99% identical to that of *In589*, which was detected in an IMP-15-producing *P. aeruginosa* isolated in Spain (accession no. KC310496) (8). The sequence from nt 6037 to 10882, which contained *tnpA-orf1-dnaK-uspA-orf2* (Fig. 1), was 93% identical to that in an IMP-9-producing *P. aeruginosa* PA96 plasmid pOZ176 (accession no. KC543497; nt 409845 to 414689), which was isolated in 2000 in China (39).

The genetic structure surrounding bla_{IMP-26} was also identical in all isolates harboring bla_{IMP-26} tested in this study. The sequence from nt 1 to 3321, which contained *intI1-*bla_{IMP-26}*-qacG-aac(6′)-Ib* (Fig. 1), was >99% identical to that of the IMP-4-producing *Enterobacter cloacae* strain El1573 (nt 54849 to 58169 of accession no. JX101693), which was isolated in 2004 in Australia (40).

The genetic structure surrounding bla_{IMP-51} was different from that surrounding bla_{IMP-15} and bla_{IMP-26} (Fig. 1). The genetic structure surrounding bla_{IMP-51} was located in a class1 integron on the chromosome (17). The sequence of the *tnpA-tnpR* (nt 1 to 5059) (Fig. 1) was identical to that of Tn*1403*-like transposon in a plasmid pOZ176 from *P. aeruginosa* PA96 isolated in China (28). The *cmlA1-*$bla_{OXA-246}$ (nt 7321 to 9786) (Fig. 1) was similar to part

of DK45-2 class 1 integron (nt 669 to 3134) in *P. aeruginosa* DK45 isolated in South Korea (GQ853420).

The genetic structure surrounding bla_{NDM-1} (Fig. 1) showed >99% identity with the chromosome of *P. aeruginosa* strain N15-01092 (nt 4093958 to 4098533), which was isolated in the United States. bla_{IMP-15}, bla_{IMP-26}, bla_{IMP-51}, and bla_{NDM-1} are probably all located on chromosomes because these genes were nor present in plasmids (data not shown).

P. aeruginosa clinical isolates producing the metallo-β-lactamases IMP-15, IMP-26, IMP-51, and/or NDM-1 were isolated in a medical setting in Vietnam. It is necessary to survey MBLs producing *P. aeruginosa* in medical settings in Vietnam.

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