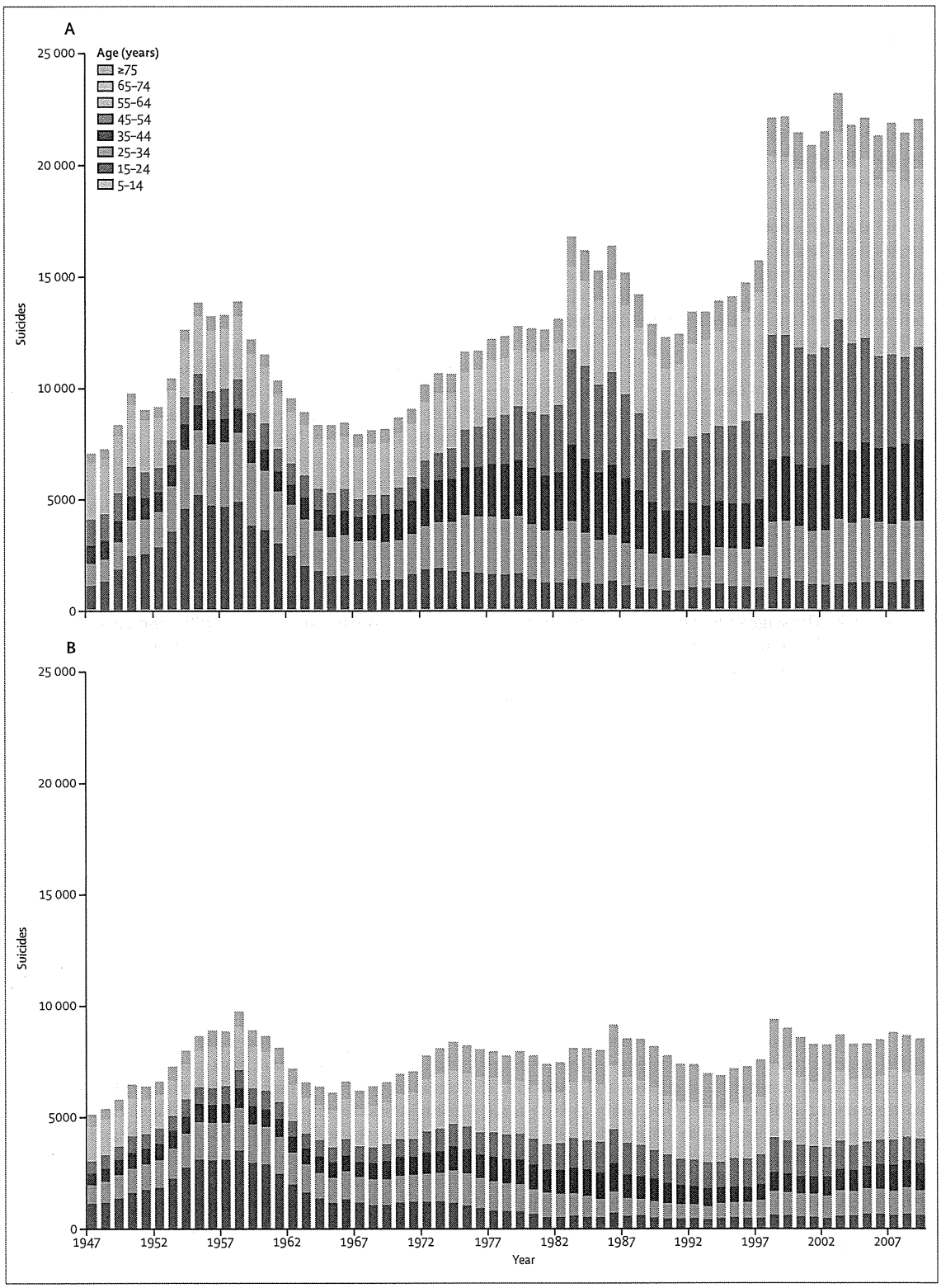

Figure 4: **Deaths from suicide by age in Japan, 1947–2009**
(A) Men. (B) Women. Data from the Ministry of Health, Labour and Welfare.[62]

population,[70] whereas increasing rates were reported for centenarians in other studies.[71,72] National health interview survey data have been used in studies to show that the functional health status of the Japanese people deteriorated during 1995–2004;[73] and morbidity rates decreased from 1984 until 1995, but the trend reversed in the late 1990s until 2004.[74] However, self-reported data were used for a few of the health domains in these studies. The survey questions and response categories are not detailed enough to obtain a reliable measure of the non-fatal health status of the population. Therefore, the national information infrastructure needs to be urgently improved to gather valid, reliable, and comparable data for the rates of disability and morbidity in the Japanese population.

Medical and long-term care

An unprecedented and unexpectedly steep reduction in mortality rates in older age groups[75] is contributing to the rapid increase in remaining life expectancy in Japan. The country has shown the most rapid increase in remaining life expectancy over the past six decades. For Japanese women, life expectancy at age 60 years increased from 16·4 years in 1950 to 28·1 years in 2007 (webappendix p 2), while life expectancy at age 80 years also increased substantially from 5·5 years to 11·4 years (webappendix p 2). The stagnating rate of increase in remaining life expectancy in other developed countries during the past two decades draws attention to Japan's exceptional improvement in life expectancy at older ages.

The nature of health care is also changing in this ageing society. The proportion of deaths resulting from illnesses that are no longer amenable to medical care, and Japanese society's concern about health have been increasing. A close link between medical care and long-term care should be further promoted to enhance population wellbeing and will be elaborated further in the fourth report in this Series.[76]

Global lessons

The experience of post-war Japan suggests that countries with low socioeconomic development can achieve progress in terms of their population health. Japan's national income was low in the beginning of the 1950s, when a tremendous increase in life expectancy at birth started largely as a result of the scale-up of the coverage of essential child survival interventions and provision of free treatment for tuberculosis. The main driving force for improved population health during this period was undoubtedly the strong stewardship of the new Japanese Government in implementing major structural reforms in the health sector and placing priority on investment in key interventions for public health in the early phase of economic growth.

The path towards universal coverage should be encouraged globally. Stroke mortality reduction was a major determinant of the sustained extension of the longevity of the Japanese population after the mid-1960s. The control of blood pressure improved with population-based interventions such as salt reduction campaigns and an increased availability of antihypertensive drugs through universal health insurance coverage. A reduction in mortality rates can be brought about by the interplay of improvements in both medical care and other societal factors (eg, income, education, nutrition, and sanitation). In turn, this reduction can vary by individual, place, and disease type.[77,78] A recent assessment of worldwide adult mortality rates[79] identified three important factors—socioeconomic development, increased access to health care and the progress in health technologies, and the diseases of affluence. Universal coverage is one of the most important factors and is essential in enhancing access to cost-effective health care at affordable prices that has indirectly contributed to the longevity through reduced cardiovascular-associated mortality rates in Japan. The lessons learned from the challenges and successes of population health in Japan lend support for the implementation of the current global health strategies to develop domestic health financing and risk-pooling mechanisms through health insurance and to scale up cost-effective interventions.[80]

Health disparities across regions and socioeconomic groups are quite small in this egalitarian society and have narrowed over time with increasing average population health. The establishment of free compulsory primary education early in the 20th century, a social insurance system before the war, and universal health insurance coverage in 1961 enabled the provision of equal opportunities for health promotion. These experiences confirm that working on population averages is not enough. Countries that have the least regional or socioeconomic disparity in longevity tend to be those in which the populations enjoy the longest life expectancies in the world.[69] Globalisation and rising economic disparity contribute to health inequalities and are increasingly causes for concern in many countries, and Japan is no exception. The goals of a health system include not only improvement of the averages but also reduction of health inequalities to a minimum.[81] By doing so, countries could accomplish what Japan has achieved.

Japan now has challenges for population health that many other countries will have soon. Further progress in terms of longevity in Japan is dependent on the prevention of major risk factors for non-communicable diseases such as tobacco smoking, high blood pressure, and metabolic syndrome. Prevention of premature mortality from suicide is another major issue requiring a comprehensive societal response that involves, for example, stabilisation of the labour market, and improvement of the promotion and provision of mental health services.[82] The rapidly ageing population as a result of improved survival also challenges financing and quality of care in Japan's health system.[30,54,76] The tsunami and nuclear crisis caused by the

magnitude 9·0 Great East Japan Earthquake on March 11, 2011, might also affect future population health, which will need to be monitored and assessed. How should Japan respond to these challenges? Policy options to tackle the challenges are addressed in the other five reports in this *Lancet* Series on Japan, which we hope will serve as a guide that will help other countries to develop policies that fit their specific circumstances. Indeed, this Series will draw attention to how Japan is unique in overcoming different and changing population health challenges in the past 50 years to achieve population longevity, and how the country's experience can be an important resource for the global health community and could transcend geographical, social, cultural, and political boundaries for understanding and helping enhance population health worldwide.

Contributors

All authors contributed to the study concept, design of the report, data analysis, and interpretation of the results. NI, ES, NK, and KS wrote the first draft. MI, HI, and ME did a systematic review. NI, ES, NK, HI, SI, TS, AS, and KS contributed to drafting and critical revision. All authors contributed to the discussion and have seen and approved the final version of the report.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgment

This work is in partly funded by the generous support from the Bill & Melinda Gates Foundation and the China Medical Board, a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare (H22-seisaku-shitei-033), and a Grant-in-Aid for Scientific Research (grant category B) from the Japan Society for the Promotion of Science (number 22390130). The views and opinions expressed by the authors are provided in their personal capacity and are their sole responsibility. We thank Hironori Imano and Zentaro Yamagata for their valuable contributions; Amarjargal Dagvadorji, Jinghua Li, Hatoko Sasaki, Mengru Wang, and Windy Mariane Virenia Wariki for their technical support; and Tomoko Suzuki and Tadashi Yamamoto for their administrative support.

References

1 Ministry of Health, Labour and Welfare. Vital statistics of Japan 2007. Tokyo: Ministry of Health, Labour and Welfare, 2009. (in Japanese).
2 Hasegawa T. Japan: historical and current dimensions of health and health equity. In: Evans T, Whitehead M, Diderichsen F, Bhuiya A, Wirth M, eds. Challenging inequities in health: from ethics to action. New York: Oxford University Press, 2001: 90–103.
3 World Bank. World development indicators. 2010. http://data.worldbank.org/indicator/NY.GDP.MKTP.KD.ZG (accessed Dec 25, 2010).
4 UN Population Division. World population prospects: the 2010 revision population database. http://esa.un.org/unpd/wpp/index.htm (accessed Aug 1, 2011).
5 Ministry of Health, Labour and Welfare. Fact sheet of abridged life tables for Japan 2010. 2011. (in Japanese). http://www.mhlw.go.jp/toukei/saikin/hw/life/life10/index.html (accessed July 28, 2011).
6 OECD. OECD health data 2011: statistics and indicators. Paris: Organization for Economic Co-operation and Development, 2011.
7 University of California at Berkeley, Max Planck Institute for Demographic Research. Human mortality database. http://www.humanmortality.de (accessed Oct 25, 2010).
8 Ministry of Health, Labour and Welfare. The 20th life tables. Tokyo: Health and Welfare Statistics Association, 2007 (in Japanese).
9 WHO. World health statistics 2011. Geneva: World Health Organization, 2011.
10 WHO. World health statistics 2010. Geneva: World Health Organization, 2010.
11 Horiuchi S. Major causes of the rapid longevity extension in postwar Japan. *Jpn J Popul* 2011; **9**: 162–71.
12 Ohnuki-Tierney E. Illness and culture in contemporary Japan: an anthropological view. New York: Cambridge University Press, 1984.
13 Yamakado M. Taking stock of the Ningen Dock. *Highlighting Japan* 2010; **4**: 10–11.
14 Japan Brain Dock Society. Guidelines for the brain dock 2008. Sapporo: Kyobunsha, 2008 (in Japanese).
15 Ohno Y. Health development in Japan: determinants, implications and perspectives. *World Health Stat Q* 1985; **38**: 176–92.
16 Tominaga S, Kuroishi T. An ecological study on diet/nutrition and cancer in Japan. *Int J Cancer* 1997; **71** (suppl 10): 2–6.
17 Statistics Bureau, Ministry of Internal Affairs and Communications. Population of Japan: final report of the 2005 population census. Tokyo: Japan Statistical Association, 2010. (in Japanese).
18 Ministry of Health, Labour and Welfare. The national health and nutrition survey in Japan, 2007. Tokyo: Ministry of Health, Labour and Welfare, 2010 (in Japanese).
19 Ministry of Health, Labour and Welfare. Vital statistics of Japan 1950–2008. Tokyo: Ministry of Health, Labour and Welfare, 1952–2010 (in Japanese).
20 Institute for Health Metrics and Evaluation. Institute for Health Metrics and Evaluation database. Seattle, WA: Institute for Health Metrics and Evaluation, University of Washington, 2010.
21 Naghavi M, Makela S, Foreman K, O'Brien J, Pourmalek F, Lozano R. Algorithms for enhancing public health utility of national causes-of-death data. *Popul Health Metri* 2010; **8**: 9.
22 Nolte E, McKee M. Does health care save lives? Avoidable mortality revisited. London: Nuffield Trust; 2004.
23 Ministry of Health, Labour and Welfare. Municipal life table 1985–2005. Tokyo: Health and Welfare Statistics Association, 1989–2009 (in Japanese).
24 Statistics Bureau, Ministry of Internal Affairs and Communications. Population of Japan 1980–2000. Tokyo: Japan Statistical Association, 1985–2005 (in Japanese).
25 Sugiura Y, Ju YS, Yasuoka J, Jimba M. Rapid increase in Japanese life expectancy after World War II. *Biosci Trends* 2010; **4**: 9–16.
26 Reich MR, Ikegami N, Shibuya K, Takemi K. 50 years of pursuing a healthy society in Japan. *Lancet* 2011; published online Sept 1. DOI:10.1016/S0140-6736(11)60274-2.
27 Johansson SR, Mosk C. Exposure, resistance and life expectancy: disease and death during the economic development of Japan, 1900–1960. *Popul Stud (Camb)* 1987; **41**: 207–35.
28 Iwasaki T. The tuberculosis situation at the beginning of this century. *Bull Int Union Tuberc* 1974; **49**: 30–51.
29 Mori T. Recent trends in tuberculosis, Japan. *Emerg Infect Dis* 2000; **6**: 566–68.
30 Ikegami N, Yoo B-K, Hashimoto H, et al. Japanese universal health coverage: evolution, achievements, and challenges. *Lancet* 2011; published online Sept 1. DOI:10.1016/S0140-6736(11)60828-3.
31 Iso H. Changes in coronary heart disease risk among Japanese. *Circulation* 2008; **118**: 2725–29.
32 Robertson TL, Kato H, Gordon T, et al. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California. Coronary heart disease risk factors in Japan and Hawaii. *Am J Cardiol* 1977; **39**: 244–49.
33 Takeya Y, Popper J, Shimizu Y, Kato H, Rhoads G, Kagan A. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: incidence of stroke in Japan and Hawaii. *Stroke* 1984; **15**: 15–23.
34 Yano K, Reed DM, McGee DL. Ten-year incidence of coronary heart disease in the Honolulu Heart Program. Relationship to biologic and lifestyle characteristics. *Am J Epidemiol* 1984; **119**: 653–66.
35 Ueshima H, Tatara K, Asakura S, Okamoto M. Declining trends in blood pressure level and the prevalence of hypertension, and changes in related factors in Japan, 1956–1980. *J Chronic Dis* 1987; **40**: 137–47.
36 Okayama A, Ueshima H, Marmot MG, Nakamura M, Kita Y, Yamakawa M. Changes in Total Serum Cholesterol and Other Risk Factors for Cardiovascular Disease in Japan, 1980–1989. *Int J Epidemiol* 1993; **22**: 1038–47.

37 Shibuya K. Comprehensive assessment of risk factor interventions and prevention of disease burden in Japan. In: Shibuya K, ed. Comprehensive assessment of the health system in Japan. Report to the Ministry of Health, Labour and Welfare for 2010 grant-in-aid for scientific research (H22-seisaku-shitei-033), 2011: 5–13. (in Japanese)

38 Ikeda N, Gakidou E, Hasegawa T, Murray CJ. Understanding the decline of mean systolic blood pressure in Japan: an analysis of pooled data from the National Nutrition Survey, 1986–2002. *Bull World Health Organ* 2008; **86:** 978–88.

39 Iso H, Shimamoto T, Naito Y, et al. Effects of a long-term hypertension control program on stroke incidence and prevalence in a rural community in northeastern Japan. *Stroke* 1998; **29:** 1510–18.

40 Ministry of Health, Labour and Welfare. 2007 comprehensive survey of living conditions of the people on health and welfare. Tokyo: Health and Welfare Statistics Association, 2009 (in Japanese).

41 Shimamoto T, Komachi Y, Inada H, et al. Trends for coronary heart disease and stroke and their risk factors in Japan. *Circulation* 1989; **79:** 503–15.

42 Marmot MG, Smith GD. Why are the Japanese living longer? *BMJ* 1989; **299:** 1547–51.

43 Hamano T, Fujisawa Y, Ishida Y, Subramanian SV, Kawachi I, Shiwaku K. Social capital and mental health in Japan: a multilevel analysis. *PLoS One* 2010; **5:** e13214.

44 Nakaya T, Dorling D. Geographical inequalities of mortality by income in two developed island countries: a cross-national comparison of Britain and Japan. *Soc Sci Med* 2005; **60:** 2865–75.

45 Ezzati M, Friedman AB, Kulkarni SC, Murray CJL. The reversal of fortunes: trends in county mortality and cross-county mortality disparities in the United States. *PLoS Med* 2008; **5:** e66.

46 Cabinet Office, Government of Japan. Public Opinion Survey Concerning People's Lifestyles 2010. Tokyo: Cabinet Office, Government of Japan, 2011 (in Japanese).

47 OECD. Rights and translation unit. Growing unequal?: income distribution and poverty in OECD countries. Paris: Organization for Economic Co-operation and Development, 2008.

48 Kagamimori S, Gaina A, Nasermoaddeli A. Socioeconomic status and health in the Japanese population. *Soc Sci Med* 2009; **68:** 2152–60.

49 Murakami Y, Miura K, Okamura T, Ueshima H. Population attributable numbers and fractions of deaths due to smoking: a pooled analysis of 180,000 Japanese. *Prev Med* 2011; **52:** 60–65.

50 Shinmura H, Wakabayashi C, Kunisawa N, et al. A nationwide survey for implementation of Health Japan 21 anti-smoking countermeasures in municipalities throughout Japan. *Nippon Koshu Eisei Zasshi* 2008; **55:** 170–76 (in Japanese).

51 WHO. WHO report on the global tobacco epidemic, 2009: implementing smoke-free environments. Geneva: World Health Organization, 2009.

52 Honjo K, Kawachi I. Effects of market liberalisation on smoking in Japan. *Tob Control* 2000; **9:** 193–200.

53 Ogihara T, Kikuchi K, Matsuoka H, et al. The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2009). *Hypertens Res* 2009; **32:** 3–107.

54 Hashimoto H, Ikegami N, Shibuya K, et al. Cost containment and quality of care in Japan: is there a trade-off? *Lancet* 2011; published online Sept 1. DOI:10.1016/S0140-6736(11)60987-2.

55 Sakane N, Yoshida T, Umekawa T, Kondo M, Sakai Y, Takahashi T. Beta 3-adrenergic-receptor polymorphism: a genetic marker for visceral fat obesity and the insulin resistance syndrome. *Diabetologia* 1997; **40:** 200–04.

56 Yoshida T, Sakane N, Umekawa T, Sakai M, Takahashi T, Kondo M. Mutation of beta 3-adrenergic-receptor gene and response to treatment of obesity. *Lancet* 1995; **346:** 1433–34.

57 Ministry of Health, Labour and Welfare. Standard health examination and guidance program. Tokyo: Ministry of Health, Labour and Welfare, 2007 (in Japanese).

58 Parkin DM. The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 2006; **118:** 3030–44.

59 Tsukuma H, Tanaka H, Ajiki W, Oshima A. Liver cancer and its prevention. *Asian Pac J Cancer Prev* 2005; **6:** 244–50.

60 Kato M, Asaka M, Shimizu Y, et al. Relationship between *Helicobacter pylori* infection and the prevalence, site and histological type of gastric cancer. *Aliment Pharmacol Ther* 2004; **20** (suppl 1): 85–89.

61 Chen J, Choi Y, Mori K, Sawada Y, Sugano S. Those who are left behind: an estimate of the number of family members of suicide victims in Japan. *Soc Indic Res* 2009; **94:** 535–44.

62 Ministry of Health, Labour and Welfare. Vital statistics of Japan 1947–2009. Tokyo: Ministry of Health, Labour and Welfare, 1949–2011 (in Japanese).

63 Cabinet Office, Government of Japan. White paper on suicide 2010. Tokyo: Cabinet Office, Government of Japan, 2010 (in Japanese).

64 National Police Agency of Japan. Information on suicide in 2010. 2011. (in Japanese). http://www.npa.go.jp/safetylife/seianki/H22jisatsunogaiyou.pdf (accessed April 9, 2011).

65 Chang SS, Gunnell D, Sterne JAC, Lu TH, Cheng ATA. Was the economic crisis 1997–1998 responsible for rising suicide rates in East/Southeast Asia? A time-trend analysis for Japan, Hong Kong, South Korea, Taiwan, Singapore and Thailand. *Soc Sci Med* 2009; **68:** 1322–31.

66 Statistics Bureau, Ministry of Internal Affairs and Communications. Labor Force Survey. Tokyo: Ministry of Internal Affairs and Communications, 2010 (in Japanese).

67 Ministry of Health, Labour and Welfare. Act for securing the proper operation of worker dispatching undertakings and improved working conditions for dispatched workers. 2009 (in Japanese). http://law.e-gov.go.jp/htmldata/S60/S60HO088.html (accessed July 21, 2011).

68 Cabinet Office, Government of Japan. Basic act on suicide prevention. 2006 (in Japanese). http://law.e-gov.go.jp/announce/H18HO085.html (accessed July 21, 2011).

69 Christensen K, Doblhammer G, Rau R, Vaupel JW. Ageing populations: the challenges ahead. *Lancet* 2009; **374:** 1196–208.

70 Schoeni RF, Liang J, Bennett J, Sugisawa H, Fukaya T, Kobayashi E. Trends in old-age functioning and disability in Japan, 1993–2002. *Popul Stud* 2006; **60:** 39–53.

71 Gondo Y. Current research on centenarians in Japan. *Ronen Shakai Kagaku* 2007; **28:** 504–12 (in Japanese).

72 Suzuki M, Akisaka M, Ashitomi I, Higa K, Nozaki H. Chronological study concerning ADL among Okinawan centenarians. *Nippon Ronen Igakkai Zasshi* 1995; **32:** 416–23 (in Japanese).

73 Hashimoto S, Kawado M, Seko R, et al. Trends in disability-free life expectancy in Japan, 1995–2004. *J Epidemiol* 2010; **20:** 308–12.

74 Yong V, Saito Y. Trends in healthy life expectancy in Japan: 1986–2004. *Demogr Res* 2009; **20:** 467–94.

75 Olshansky SJ, Carnes BA, Desesquelles A. Demography. Prospects for human longevity. *Science* 2001; **291:** 1491–92.

76 Tamiya N, Noguchi H, Nishi A, et al. Population ageing and wellbeing: lessons from Japan's long-term care insurance policy. *Lancet* 2011; published online Sept 1. DOI:10.1016/S0140-6736(11)61176-8.

77 Oeppen J, Vaupel JW. Demography. Broken limits to life expectancy. *Science* 2002; **296:** 1029–31.

78 Riley JC. Rising life expectancy: a global history. Cambridge, UK: Cambridge University Press, 2001.

79 Rajaratnam JK, Marcus JR, Levin-Rector A, et al. Worldwide mortality in men and women aged 15–59 years from 1970 to 2010: a systematic analysis. *Lancet* 2010; **375:** 1704–20.

80 WHO. The world health report-health systems financing: the path to universal coverage. Geneva: World Health Organization, 2010.

81 WHO. The world health report 2002: reducing risks, promoting healthy life. Geneva: World Health Organization, 2002.

82 Kaga M, Takeshima T, Matsumoto T. Suicide and its prevention in Japan. *Leg Med (Tokyo)* 2009; **11:** S18–21.

Kantipong P, Yamada N, Nampaisan O, Moolphate S, Yanai H, Hamsuthivatchakul C. Predictors of antiretroviral therapy regimen changes in Northern Thailand. Bulletin of Department of Medical Services, 2012;36(7) *in press*.

Predictors of antiretroviral therapy regimen changes in Northern Thailand

Abstract:

Background: Chiang Rai province, Northern Thailand is a highly HIV prevalent area where nevirapine-based regimen is first line therapy. This study aimed to determine the rate, predictors, and reasons for highly active antiretroviral therapy (HAART) regimen change.

Methods: A total of 4981 HIV patients who attended 17 HIV clinics of Chiang Rai province were enrolled from 2006 to 2008. In the first year of follow up, we observed the HAART regimen changes rate and predictors of changes were investigated using Cox-proportional hazard model.

Result: Overall HAART regimen change rate within the first year was15.4% (95% Confidence Interval 14.4-16.4%) of 4981 The regimen change rate was higher in female than in male (17.6%vs.12.9%). Patients on efavirenz-based regimen underwent significantly higher rate of regimen change (20.3 %) than nevirapine-based (14.9%) and protease inhibitors-based regimen (11.9%). The regimen change rate became higher in 2007 and 2008 than in 2006 and higher at the provincial hospital than district community hospitals. Out of the 765 patients who underwent regimen change, the most common cause was adverse effect (83.5%; n=639)

Discussion; Strategy should be developed by the prospective trial to minimize the side effect, and reducing the regimen changes in order to improve the treatment outcomes.

Introduction:

HAART (Highly Active Antiretroviral Therapy) could prolong the life expectancy and quality of life of HIV-infected patients globally [1]. Sustainability of immunological and virological response achieved by a first-line regimen is important [2]. However, drug resistance, short-term and long-term side effects of antiretroviral drugs may lead to changes in first- line drugs regimen or switch to second-line regimens [3]. The recommendations on the timing of HAART regimen change differ by guidelines. The World Health Organization (WHO) HIV treatment guideline published in 2010 recommends reservation of second-line drugs unless there is strong reason to change the first-line regimen, while the European and British guidelines and Department of health and human service, U.S.A. (DHHS) guidelines favor more sensitive switch and change of HAART to prevent the accumulation of thymine analogue mutation (TAM) [2, 4-6].

Currently, ART (antiretroviral therapy) coverage in Thailand has extended to more than two thirds of those in need. Thailand has developed its own national HIV treatment guideline updated in 2007, 2008 and 2010 [7-9]. Until 2008, fixed combination of d4T/3TC/NVP (GPO-VIR S 30), fixed combination of AZT/3TC/NVP (GPO-VIR Z 250), and d4T+3TC+EFV were first line recommended HAART regimen by Thailand national treatment guideline [7-8].

Observational studies [10-12] have reported different regimen change rate at HIV cohort of different regions but mostly in developed countries using protease inhibitor (PI) based regimen to avoid the side effects of Non-Nucleic acid Reverse Transcriptase Inhibitor (NNRTI), in particular nevirapine (NVP). However, developing countries still use NVP due to its lower cost. So, it is important to know the nature of regimen changes, their cause of changes in a setting of limited resource, most of the patients were treated by NVP-based regimen.

We aimed to investigate the rate and predictors of ART regimen changes within first year of HAART initiation in newly treated HIV patients, and to compare the impact of regimen changes on treatment outcome between patients who underwent regimen changes within one year and those who did not.

Materials and methods

Study design and data collection

A retrospective cohort was constructed enrolling HIV patients treated in HIV clinics of 17 hospitals in Chiang Rai province between 2006 and 2008. We used part of the provincial AIDS program database at Chiang Rai Provincial Health Office with the supplemental information and validation of data with the hospital medical records. Inclusion criteria to comprise the study cohort were HIV patients receiving HAART of first line regimens, and treated for the first time. Those aged less than 15 years, lost to follow up or died before one year follow up were excluded from the study. Those who were receiving second line therapy at the start of NAP (National AIDS Program) were also excluded.

Total follow-up period was two years. Within the first year of follow-up, study cohort was observed for event of regimen changes. Based on whether a patient's HAART regimen has been changed within the first year of observation, the cohort was divided into RCG (regimen change group) and NCG (non regimen change group) groups. These two groups were followed into the second year to compare the treatment outcome by immunological status and virological response.

Definitions of HAART regimen change:

Regimen change was defined as change in at least one drug within one year of treatment. Dose modifications were not considered as change. .

Laboratory monitoring tools:

HIV RNA viral load (VL) was checked once a year and CD4 counts were checked every six months for patients who were provided HAART through the National Health Security Office (NHSO). Under this scheme, VL testing was provided only after one year of treatment initiation and thus baseline VL was not available.

CD4 count: CD4 count was checked by the dual-platform flow cytometry at the provincial hospital and one major district hospital.

HIV RNA Viral load: HIV RNA viral load test were done by real-time PCR. The minimum detection limit was 40 copies /ml. Undetectable viral load was considered as HIV RNA viral load less than 50 copies ml according to current guidelines [2, 4].

Statistical analysis: The predictors of ART regimen changes were analyzed using Cox-proportional hazard model to determine the crude and adjusted hazard ratio (AHR) and its 95% confidence interval (CI). Variable which have statistical significance of P-value less than 0.2 in univariate analysis were included in the multivariate models. Mann Whitney's test was used to analyze the baseline quantitative variables. All tests were two tailed. Statistical significance was considered as P value less than 0.05. STATA release 10 was used for data analysis.

Ethical consideration

Ethical approval to conduct this study was obtained from the Chiang Rai Prachanukroh Hospital ethical committee, Ministry of Public Health, Thailand

Results

The study cohort comprised 4891 patients. Median age was 36 years (IQR: interquantile range 31-41) and 42% of the patients were female. All patients were Thai citizens. 96.6% of patients were supported by the health security system including NHSO (89.0%),

social security for employee/employer (4.4%), and government officer (0.4%). HAART was started in HIV patients with CD4 count less than 200 cells/μl according to national HIV treatment guidelines at that time [7, 9].

765 patients (15.4%, 95%CI 14.4-16.4%) of 4891 underwent ART changes within one year of first time HAART initiation in health service system. (Table 1) Regimen change rate was significantly higher in female patients compared to male patients, and those treated in provincial hospital compared to district hospitals. In addition, a secular trend was observed where regimen change was significantly more frequent in 2008 compared to 2006. Patients receiving EFV-based regimen had significantly higher change rate (20.3%, 95/469) than those on NVP-based regimen (14.9%, 662/4445). Patients receiving d4T+3TC backbone underwent higher rate of regimen change compared to AZT+3TC.

With multivariate analysis by Cox-proportional hazard model, female sex (AHR 1.48), EFV based regimen (AHR 1.48), d4T+3TC backbone (AHR 1.44), treatment at provincial hospital (AHR 1.41), ART started in 2008 (AHR 2.28) were significant predictors of the ART regimen change within first year of HAART initiation. Cumulative Percentage of HAART regimen change by initial type based on third agent were shown in figure 1.

Out of the 765 patients who underwent regimen change, the most common cause reported was adverse effect (83.5%; n=639) followed by treatment failure (6.0%; n=46), clinician`s decision to prevent side effect (3.9%; n=30), drug interaction (3.1%; n=24), hepatitis B co- infection (2.1%; n=16), and pregnancy (1.3%; n=10) (Table 3).

Based on presence of regimen change in the first year after treatment initiation, the cohort was divided in to RCG (regimen changed group) and NCG (non regimen changed group). These two groups were followed into the second year and immunological and virological outcome were compared. Overall 87.8% of the entire cohort was having CD4 higher than 200cells/ul at two years after initiation of HAART. The proportions patients in different CD4 count categories were not different statistically, i.e., 12.1% in RCG vs. 12.3% in NCG had CD4 count under 200 cells/ul (P=0.89). Overall, 87.8% of cohort achieved undetectable viral load at one year after regimen change. 84.7% of RCG and 89.2% of NCG had undetectable viral load in follow-up after changes.

Discussion

The regimen change rate within first year of HAART initiation in Chiang Rai was 15.4%. The regimen change rate was higher at the provincial hospital compared to district hospitals. Difference in laboratory facility for identification of drug resistance and immunological failure and difference in level of care by specialist physicians may have contributed to the difference observed. Female patients were more prone to regimen change compared to male.

HAART regimen change rate in first years were reported by different cohorts in the existing literature; 37% in Swiss cohort from 2000-2005[10], 18.4% in USA [11], 36.1% in Italian cohort from 1997-2007[13], and 28% throughout the Caribbean and Latin America from 1996-2007 [14]. Treat Asia cohort reported 29% regimen change rate among 1846 patients on median follow up for 3.2 years [15]. Compared to these figures, HAART regimen change rate in our study was relatively low.

Reported reasons for changes are variably contributed by adverse effect and treatment failure among cohorts of developed and developing countries [11, 13]. Adverse effect proportion is larger in developing setting and failure proportion is larger in developed probably because of earlier detection [10-11, 13].

Chiang Rai is a resource-limited area with high burden of HIV infection. D4T/3TC/NVP regimen which is the first-line regimen in this setting is well known for side effects. Thailand began a national ARV treatment program in 2000 as the Access to Care program and then expanded this program in 2004 as the National Access to ARVs for People Living with HIV/AIDS (NAPHA). In these Thai national programs in 2000-2007 report 66.9% of the regimen changes were attributable to adverse effects and 21.3% to treatment failure [16]. In our study, the most common reason for regimen change was adverse effects and few were attributable to treatment failure.

The majority of the patients in our study were provided HAART under the NHSO which aims for universal coverage. This scheme only allows viral load testing at six months after HAART initiation and yearly thereafter. Good adherence to ART of patients which is strongly reinforced by network of PLWH in Chiang Rai [17] might also contibuted. These factors could have lead to the relatively low rate of regimen change observed

Our study result showed that EFV-based regimen is more likely to undergo regimen change within first year (AOR= 1.63). Although many previous study results are more favorable for EFV compared to NVP in term of efficacy and safety profile [18-19], similar finding of higher regimen change rate with EFV based regimen was reported by a secondary analysis of a cohort study in South Africa [20]. Treat Asia study [15] also reported 3 or more ART with NNRTI, no PI (other than d4T/3TC/NVP), which EFV-based regimen is 86.7% (686/791), has higher regimen change rate compared with d4T/3TC/NVP as multivariate rate ratio (RR)= 1.64 (95%CI=1.38-1.96).

The d4T+3TC back bone was also a significant predictor for regimen change. Adverse effects of d4T are reported and d4T is expected to be withdrawn from the Thai national program in the coming two years [8].

Swiss cohort study [10] reported difference of regimen change in patients with CD4+ cell counts more than 350 /mm^3 compared with 200-350, it was not different between <200 and 200-350. The other studies [11, 13, 14,] did not have association of CD4+ T cell counts level and regimen change even 350 cells/mm^3 cut-off. Thai guideline recommended to start HAART in asymptomatic HIV-infected patients at CD4+ T cell counts <200 cells/mm^3 in 2008 recommendation [8] which has increased <350 cells/mm^3 in 2010 [9], thus we do not have association with CD4 + T cell counts level is consistent.

In Thailand there are several pharmacogenetic study to investigate the biomarker for side effect of Nevirapine [21] and Stavudine (d4T) [22]. This information should be used for the prospective trial to minimize the side effect, reducing the regimen changes, and improve the treatment outcomes [23].

Acknowledgements
This study is supported by Ministry of Health, Labor, and Welfare of Japan (H21-HIV/AIDS-012). Assistant Professor Dr. Ai Koyanagi of Department of Global Health Policy, Graduate School of Medicine, University of Tokyo gave kind suggestions and advices for this study.

References:

1. Fang CT, Chang YY, Hsu HM, Twu SJ, Chen KT, Lin CC, *et al.* Life expectancy of patients with newly-diagnosed HIV infection in the era of highly active antiretroviral therapy. Q J Med. 2007; 100(2):97-105.
2. World Health Organization. Antiretroviral therapy for HIV infection in adults and adolescents; Recommendations for a public health approach, Recommendations for a public health approach, 2010 revision. 2010; Available from: URL:http://www.who.int/hiv/pub/arv/adult2010/en/index.html.
3. Mandalia S, Parmar D, Fisher M, Poziak A, Tang A, Youle M, *et al.* Naively changing HAART. HIV Medicine. 2002; 3(4):254-62.
4. Thompson MA, Aberg JA, Cahn P, Montaner JSG, Rizzardini G, Telenti A, *et al.* Antiretroviral Treatment of Adult HIV Infection: 2010 Recommendations of the International AIDS Society-USA Panel. JAMA. 2010; 304(3):321-33.
5. Gazzard B, on behalf of the BTGWG. British HIV Association guidelines for the treatment of HIV-1-infected adults with antiretroviral therapy 2008. HIV Medicine. 2008; 9(8):563-608.
6. Cozzi-Lepri A, Phillips AN, Ruiz L, Clotet B, Loveday C, Kjaer J, *et al.* Evolution of drug resistance in HIV-infected patients remaining on a virologically failing combination antiretroviral therapy regimen. AIDS. 2007; 21(6):721-32.
7. Department of Disease Control MoPH. Thailand National Antiretroviral Treatment Guideline (2006/2007)2007.
8. Sungkanuparph S, Anekthananon T, Hiransuthikul N, Bowonwatanuwong C, Supparatpinyo K, Mootsikapun N, *et al.* Guidelines for antiretroviral therapy in HIV-1 infected adults and adolescents: the recommendations of the Thai AIDS Society (TAS) 2008. J Med Assoc Thai. 2008; 91(12):1925-35.
9. Sungkanuparpha S, Techasathit W, Utaipiboonc C, Chasombat S, Bhakeecheep S, Leechawengwongs M, *et al.* Thai national guidelines for antiretroviral therapy in HIV-1 infected adults and adolescents 2010. Asian Biomedicine. 2010; 4(4):515-28.
10. Vo Thi Tuyet N, Ledergerber B, Keiser O, Hirschel B, Furrer H, Battegay M, *et al.* Durability and Outcome of Initial Antiretroviral Treatments Received during 2000–2005 by Patients in the Swiss HIV Cohort Study. The Journal of Infectious Diseases. 2008; 197(12):1685-94.
11. Yuan Y, L'Italien G, Mukherjee J,Iloeje U. Determinants of discontinuation of initial highly active antiretroviral therapy regimens in a US HIV-infected patient cohort. HIV Medicine. 2006; 7(3):156-62.
12. Hart E, Curtis H, Wilkins E,Johnson M. National review of first treatment change after starting highly active antiretroviral therapy in antiretroviral-naïve patients. HIV Medicine. 2007; 8(3):186-91.
13. Cicconi P, Cozzi-Lepri A, Castagna A, Trecarichi EM, Antinori A, Gatti F, *et al.* Insights into reasons for discontinuation according to year of starting first regimen of highly active antiretroviral therapy in a cohort of antiretroviral-naïve patients. HIV Medicine. 2010; 11(2):104-13.
14. Cesar C, Shepherd BE, Krolewiecki AJ, Fink VI, Schechter M, Tuboi SH, *et al.* Rates and Reasons for Early Change of First HAART in HIV-1-Infected Patients in 7 Sites throughout the Caribbean and Latin America. PLoS One. 2010;5(6):e10490.
15. Srasuebkul P, Calmy A, Zhou J, Kumarrasamy N, Law M, Lim PL for The TREAT Asia HIV Observational Database. Impact of drug classes and treatment availability on the rate of antiretroviral treatment change in the TREAT Asia HIV Observational Database (TAHOD). AIDS Research and Therapy. 2007; 4(1):18.
16. Chasombat S, McConnell MS, Siangphoe U, Yuktanont P, Jirawattanapisal T, Fox K, *et al.* National expansion of antiretroviral treatment in Thailand, 2000-2007: program scale-up and patient outcomes. J Acquir Immune Defic Syndr. 2009; 50(5):506-12.

17. Tsunekawa K, Moolphate S, Yanai H, Yamada N, Summanapan S, Ngamvithayapong J. Care for people living with HIV/AIDS: an assessment of day care centers in northern Thailand. AIDS Patient Care STDS. 2004;18(5):305-14.
18. van Leth F, Phanuphak P, Ruxrungtham K, Baraldi E, Miller S, Gazzard B, *et al.* Comparison of first-line antiretroviral therapy with regimens including nevirapine, efavirenz, or both drugs, plus stavudine and lamivudine: a randomised open-label trial, the 2NN Study. The Lancet. 2004;363(9417):1253-63.
19. Wester CW, Thomas AM, Bussmann H, Moyo S, Makhema JM, Gaolathe T, *et al.* Non-nucleoside reverse transcriptase inhibitor outcomes among combination antiretroviral therapy-treated adults in Botswana. AIDS. 2010; 24:S27-S36 .
20. Nachega JB, Hislop M, Dowdy DW, Gallant JE, Chaisson RE, Regensberg L, *et al.* Efavirenz versus nevirapine-based initial treatment of HIV infection: clinical and virological outcomes in Southern African adults. AIDS. 2008; 22(16):2117-25.
21. Chantarangsu S, Mushiroda T, Mahasirimongkol S, Kiertiburanakul S, Sungkanuparph S, Manosuthi W, *et al.* HLA-B*3505 allele is a strong predictor for nevirapine-induced skin adverse drug reactions in HIV-infected Thai patients. Pharmacogenet Genomics. 2009 Feb;19(2):139-46.
22. Wangsomboonsiri W, Mahasirimongkol S, Chantarangsu S, Kiertiburanakul S, Charoenyingwattana A, Komindr S, *et al.* Association between HLA-B*4001 and lipodystrophy among HIV-infected patients from Thailand who received a stavudine-containing antiretroviral regimen. Clin Infect Dis. 2010 Feb 15;50(4):597-604.
23. Hosono N, Chantarangsu S, Kiyotani K, Takata S, Tsuchiya Y, Mahasirimongkol S, *et al.* Development of new HLA-B*3505 genotyping method using Invader assay. Pharmacogenet Genomics. 2010 Oct;20(10):630-3.
24. Kiertiburanakul S, Sungkanuparph S, Malathum K, Watcharananan S, Sathapatayavongs B, Charoenyingwattana A, *et al.* A model and risk score for predicting nevirapine-associated rash among HIV-infected patients: in settings of low CD4 cell counts and resource limitation. Open AIDS J. 2009 Jul 8;3:24-30.
25. Kiertiburanakul S, Sungkanuparph S, Charoenyingwattana A, Mahasirimongkol S, Sura T, Chantratita W. Risk factors for nevirapine-associated rash among HIV-infected patients with low CD4 cell counts in resource-limited settings. Curr HIV Res. 2008 Jan;6(1):65-9.

Table 1: HAART regimen change rate within first year of initiation by baseline patient characteristic

Characteristic N=4981	Regimen change rate (%)	Change /total number	P value [1]
Overall	15.4%	765/4981	
Age (years)			
>50	19.4%	49/253	0.07
15-50	15.1%	716/4728	
Base line CD4 (cells/ul)			
>50	15.6%	665/4260	0.54
0-50	16.7%	70/418	
Missing	9.9%	30/303	
Gender			
Male	12.9%	308/2389	<0.001
Female	17.6%	457/2592	
HAART regimen[2]			
NVP- based	14.9%	662/4445	<0.01
EFV-based	20.3%	95/469	
PI-based	11.9%	8/67	
NRTI Backbone			
Stavudine (d4T)+3TC	15.9%	652/4095	0.02
AZT+3TC	12.8%	113/886	
Presence of OI[3] at HAART initiation			
Yes	16.3%	17/104	0.82
No	15.5%	748/4820	
Missing	0.0%	0/57	
Type of hospital			
Provincial	19.1%	174/913	<0.01
District	14.5%	591/4068	
Year of HAART initiation			
2006	11.0%	24/218	<0.01
2007	14.7%	539/3659	
2008	18.3%	202/1104	

[1]P-value : calculated by chi-square test. [2]Types of HAART regimen based on the third agent. [3]OI: WHO stage 3 and 4 opportunistic infection

Table 2: Cox proportional hazards model analyses for predictors of HAART regimen changes within one year of treatment initiation.

Characteristic N=4981	Unadjusted Hazards ratio (95%CI[1])	Adjusted[2] Hazards ratio (95%CI)	P
Age (years)			
>50	1	1	
15-50	1.35 (1.01-1.8)	1.28 (0.96-1.71)	0.10
Base line CD4 (cells/ul)			
>50	1		
0-50	1.2 (0.94-1.54)		
Gender			
Male	1	1	
Female	1.4 (1.22-1.62)	1.48 (1.28-1.72)	<0.001
HAART regimen[3]			
NVP-based	1	1	
EFV-based	1.4 (1.13-1.74)	1.48 (1.19-1.84)	<0.001
PI-based	0.79 (0.4-1.59)	0.77 (0.38-1.55)	0.46
NRTI Backbone			
AZT+3TC	1	1	
D4T+3TC	1.29 (1.06-1.58)	1.44 (1.17-1.77)	<0.01
Presence of OI[4] at HAART initiation			
Yes	1		
No	1.18 (0.73-1.91)		
Type of hospital			
Provincial	1	1	
District	0.74 (0.63-0.88)	0.71 (0.6-0.84)	<0.001
Year of HAART initiation			
2006	1	1	
2007	1.44 (0.96-2.17)	1.72 (1.14-2.6)	0.01
2008	1.96 (1.28-2.99)	2.28 (1.49-3.49)	<0.001

[1]CI: Confidence Interval [2]Adjusted for all other covariates in the model. [3]Types of HAART regimen based on third main agent. [4]OI: Who stage 3 and 4 opportunistic infection.

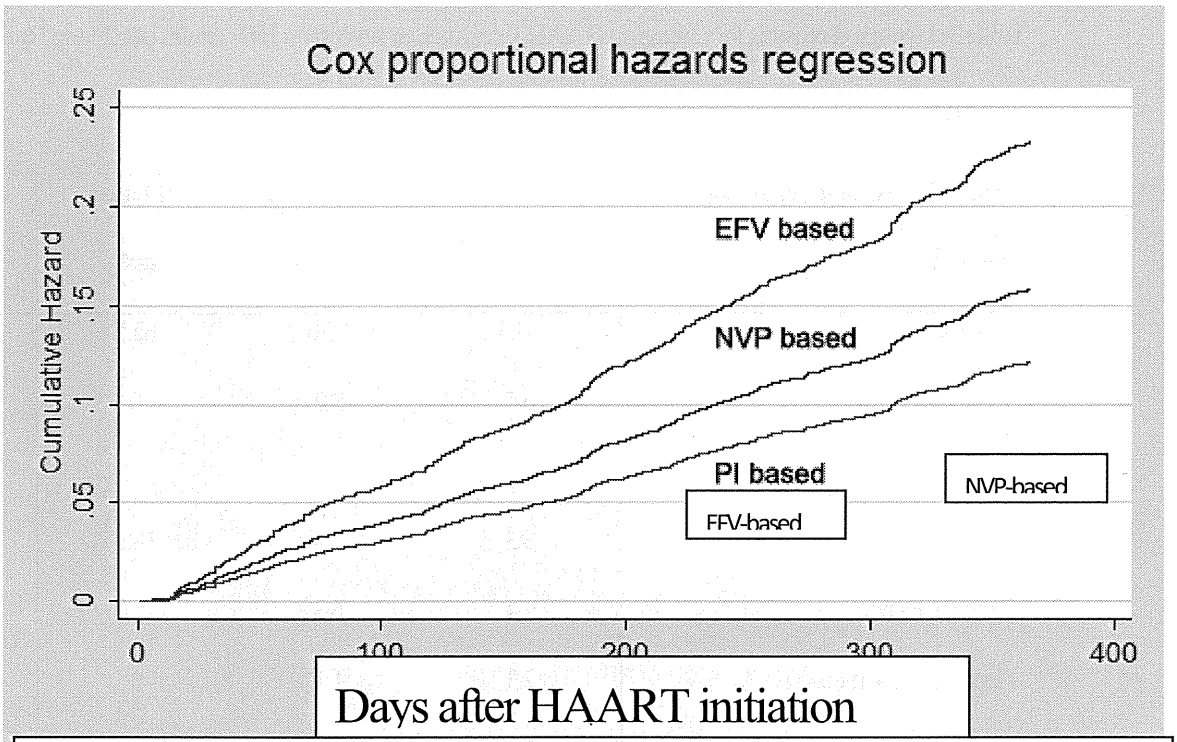

Figure 1. Cumulative Percentage of HAART regimen change by initial type based on third agent

Table 3: Reported reasons for changing HAART regimen at seventeen HIV clinics of Chiang Rai Province

Reported reason for changing regimen	NVP-based (n=662)	EFV-based (n=95)	PI-based (n=8)	Total (N=765)
Adverse effect	84.3	80.0	62.5	83.5
Treatment failure	6.5	3.2	0	6.0
Clinicians' decision to prevent side effect	3.3	7.4	12.5	3.9
Drug interaction	2.4	6.3	25.0	3.1
Hepatitis B co-infection	2.3	1.1	0	2.1
Pregnancy	1.2	2.1	0	1.3
Total	100.00	100.00	100.00	100.0

Microbiology and Immunology

Microbiol Immunol 2011; **55**: 565–573
doi:10.1111/j.1348-0421.2011.00348.x

ORIGINAL ARTICLE

Decreased plasma granulysin and increased interferon-gamma concentrations in patients with newly diagnosed and relapsed tuberculosis

Nada Pitabut[1], Surakameth Mahasirimongkol[2], Hideki Yanai[3], Chutharut Ridruechai[1], Shinsaku Sakurada[4], Panadda Dhepakson[5], Pacharee Kantipong[6], Surachai Piyaworawong[7], Saiyud Moolphate[3], Chamnarn Hansudewechakul[8], Norio Yamada[9], Naoto Keicho[4], Masaji Okada[10], and Srisin Khusmith[1]

[1]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, [2]Medical Genetic Section, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, [3]TB/HIV Research Project, Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Chiang Rai, [4]Department of Respiratory Diseases, Research Institute, National Center Global Health and Medicine, Kiyose, Tokyo, [5]Medical Biotechnology Center, National Institute of Health, Department of Medical Science, Ministry of Public Health, Nonthaburi, [6]Chiang Rai Hospital, Chiang Rai, [7]Mae Chan Hospital, Chiang Rai, [8]Chiang Rai Provincial Health Office, Chiang Rai, Thailand, [9]Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Kiyose, Tokyo, and [10]Clinical Research Center, National Hospital Organization, Kinki-Chuo Chest Medical Center, Sakai, Osaka, Japan

ABSTRACT

Granulysin and interferon-gamma (IFN-γ) have broad antimicrobial activity which controls *Mycobacterium tuberculosis* (*M. tuberculosis*) infection. Circulating granulysin and IFN-γ concentrations were measured and correlated with clinical disease in Thai patients with newly diagnosed, relapsed and chronic tuberculosis (TB). Compared to controls, patients with newly diagnosed, relapsed and chronic TB had lower circulating granulysin concentrations, these differences being significant only in newly diagnosed and relapsed TB ($P < 0.001$ and 0.004, respectively). Granulysin concentrations in patients with newly diagnosed and relapsed TB were significantly lower than in those with chronic TB ($P = 0.003$ and $P = 0.022$, respectively). In contrast, significantly higher circulating IFN-γ concentrations were found in patients with newly diagnosed and relapsed TB compared to controls ($P < 0.001$). The IFN-γ concentrations in newly diagnosed and relapsed patients were not significantly different from those of patients with chronic TB. However, *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) from patients with newly diagnosed, relapsed and chronic TB with purified protein derivative (PPD) or heat killed *M. tuberculosis* (H37Ra) enhanced production of granulysin by PBMCs. *In vitro*, stimulation of PBMCs of newly diagnosed TB patients with PPD produced greater amounts of IFN-γ than did controls, while those stimulated with H37Ra did not. The results demonstrate that patients with active pulmonary TB have low circulating granulysin but high IFN-γ concentrations, suggesting possible roles in host defense against *M. tuberculosis* for these agents.

Key words clinical disease, granulysin, IFN-γ, tuberculosis.

Correspondence
Srisin Khusmith, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand.
Tel: +66 2 3549100-13 ext. 1594; fax: +66 2 6435583; email: tmskm@mahidol.ac.th

Received 2 February 2011; revised 19 April 2011; accepted 21 April 2011.

List of Abbreviations: APC, antigen presenting cell; BCG, Bacillus Calmette-Guérin; CTL, cytotoxic T lymphocyte; E, ethambutol; H, isoniazid; IFN-γ, interferon gamma; IGRA, interferon-γ release assay; IL, interleukin; MDR, multi-drugs resistance; MHC, major histocompatibility complex; *Mtb*, *Mycobacterium tuberculosis*, *M. tuberculosis*, *Mycobacterium tuberculosis*; NK, natural killer; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; R, rifampicin; S, streptomycin; TB, tuberculosis; Th1, T-helper type 1; TMB, tetramethylbenzidine; TNF, tumor necrosis factor; TST, tuberculin skin test; XDR, extensively drug resistant; Z, pyrazinamide.

Tuberculosis is a major health problem worldwide, with one third of the world population being infected and approximately 1.1–1.7 million deaths annually (1). Most individuals infected with *Mtb* are asymptomatic. However, 5–10% will progress to active TB during their lifetime, the remainder being resistant to active TB, but remaining infected. Relapse of TB, which is defined as an episode of infection occurring after a previous episode has been treated and considered cured, is possibly due to endogenous reactivation when it occurs in geographical areas with a low incidence of TB infection (2). However, generally the risk of relapse depends on the intensity of exposure to *Mtb*. Other factors that directly affect the clinical course of TB are host factors, including age, immune status, genetic factors and coinfection with HIV, and bacterial factors, including degree of exposure, virulence of strain, MDR and XDR.

Protective immunity against *Mtb* infection involves activated macrophages, antigen-specific T cells and type-1 cytokines such as IL-12, IFN-γ and TNF (3, 4). Inherited defects of the IL-12/IFN-γ pathway appear to result in a variety of changes in mycobacterial susceptibility. People with genetic deficiencies in the type-1 cytokine (IL-12/IL-23/IFN-γ) axis, and those with neutralizing autoantibody against IFN-γ, have been found to be highly susceptible to mycobacterial infections including TB (5–8). In active pulmonary TB, these effectors of the immune response are activated, as evidenced by observation of high circulating IFN-γ concentrations that decrease significantly following two months of therapy (9, 10).

Granulysin can kill extracellular *Mtb* directly, or intracellular bacteria in the presence of perforin (11), expression of granulysin in CD8+T cells being induced upon activation. It has recently been reported that granulysin is strongly associated with diverse activities of NK cells and CTLs in physiological and pathological settings, and might be a useful novel serum marker for evaluating the overall status of host cellular immunity (12). In patients with cutaneous leprosy, the frequency of granulysin-expressing T cells lesions is 6-fold greater than in those with the disseminated lepromatous form of the disease (13). In contrast, adults with active pulmonary TB in a highly TB endemic area in Indonesia had significantly lower plasma granulysin concentrations than did controls, these concentrations increasing after 2 months of anti-TB therapy to values similar to those of controls, and having increased even further after completion of anti-TB therapy. These changes in granulysin concentrations occurred predominantly in patients in whom IFN-γ negative T cells were expressed, suggesting that in TB the cellular sources of IFN-γ and granulysin are partly non-overlapping (14). Similar findings have been reported for Italian children, the lowest concentrations having been found in TB patients who were PPD negative at the time of diagnosis (15), indicating the involvement of granulysin and IFN-γ in curative immune responses against *Mtb*. In chronic pulmonary TB, lung tissue biopsy has shown reduction in amounts of perforin and granulysin in relation to granzyme A, while higher per cell expression of perforin and granulysin is associated with bacteriological control, suggesting that perforin and granulysin could be used as markers or correlates of immune protection in human TB (16). However, effective host mechanisms against *Mtb* infection are not well understood, this lack of understanding being a problem in regard to vaccine development and immunotherapy for TB. Moreover, so far there is limited information regarding the roles of IFN-γ and granulysin in recurrent TB. Therefore, the present study aimed to investigate whether granulysin and IFN-γ responses are associated with clinical disease in patients with newly diagnosed, relapsed and chronic pulmonary TB in northern Thailand, where TB is endemic.

MATERIALS AND METHODS

Subjects

One hundred and fifty-five pulmonary TB patients (aged 9 to 88 years) were recruited from the outpatient and inpatient clinics of Chiang Rai Hospital and Mae Chan Hospital, in the north of Thailand. These included 102 male and 53 female patients with newly diagnosed and previously treated pulmonary TB. Patients with extrapulmonary TB and pulmonary TB/HIV seropositive were excluded. All patients with pulmonary TB had clinical symptoms and a confirmed diagnosis on the basis of presence of acid-fast bacilli in sputum on microscopic examination, positive cultures of *Mtb*, medical history and chest radiographic findings. Patients were categorized according to World Health Organization criteria (1), which include ascertaining whether the patient has previously received TB treatment. The TB drug regimens were based on the recommendations of the National Tuberculosis Program, Ministry of Public Health, Thailand. Standard TB treatment drugs consist of streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). In this study, patients with newly diagnosed TB were defined as those who had never received treatment for TB or had taken anti-TB drugs for less than 1 month prior to enrollment ($n = 84$). Patients with relapsed TB were defined as those previously treated for TB and declared "cured" or "treatment completed", and currently diagnosed as *Mtb* positive by smears and cultures ($n = 35$). Patients with chronic TB were defined as those who had started on a retreatment regimen after having failed previous treatment ($n = 36$). No patients had been reported to be MDR or

XDR cases on the basis of drug sensitivity tests at the time of enrollment in this study.

Thirty three healthy individuals (aged 21 to 54 years old, median = 36 years) recruited from the Blood Bank of Chiang Rai Hospital, Mae Chan Hospital and Phan Hospital were used as controls. They had no history suggestive of TB or other acute infectious diseases or diabetes at the time of enrollment. However, they were not subject to chest X-rays, TSTs or testing for latent TB infection and infection manifesting as active TB by IGRA upon enrollment.

The ethical aspects of this study were approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand (Ref. No.3/2550) as part of a project studying multiple factors in recurrent TB, and written informed consent was obtained from all subjects.

Blood samples

Before instituting anti-TB therapy, blood was collected aseptically in EDTA Vacutainers. Plasma and packed cells were separated by centrifugation and stored at −80°C.

HIV screening

HIV positive cases were excluded from the study by screening with the particle agglutination assay (Serodia-HIV-1/2, Fujirebio, Tokyo, Japan) and/or immunochromatographic rapid test (Determine HIV-1/2, Abbott Laboratories, Champaign, IL, USA) or by ELISA (Enzygnost Anti-HIV 1/2 plus ELISA, Dade Behring, Marburg, Germany).

Peripheral blood mononuclear cells isolation and stimulation

Peripheral blood mononuclear cells from 75 pulmonary TB patients and 4 healthy controls were isolated by Ficoll-Hypaque density gradient centrifugation. In brief, 3 mL of whole blood in K_3EDTA (Greiner Bio-One, Bangkok, Thailand) was diluted with an equal volume of PBS, mixed gently and layered carefully over 3 mL Ficoll-paque PLUS (Amersham Biosciences, Uppsala, Sweden). After centrifugation at 1000 g for 20 min at room temperature, the PBMCs were harvested. The supernatant was removed after centrifugation at 700 g for 10 min at 4°C and the pellet adjusted with RPMI 1640 containing 10% FBS. The viable PBMCs were counted in 0.2% Trypan blue. Approximately 1×10^6 PBMCs/mL in RPMI 1640 medium containing 10% FBS and 2-mercapto ethanol were added to each well of a 24 well plate, stimulated either with 20 μg/mL of PPD (Japan BCG laboratory, Kiyose, Japan) or heat killed *Mtb* (H37Ra) (Difco, Detroit, MI, USA) and incubated at 37°C in 5% CO_2. The supernatants were harvested after 40 hr of stimulation, centrifuged at 1200 g for 3 min at 4°C and kept at −80°C. PMBCs stimulated with 20 μg/mL of PPD and not stimulated were used as positive and negative controls, respectively.

Determination of circulating granulysin and granulysin production by peripheral blood mononuclear cell stimulation assay

The granulysin concentrations in plasma and stimulated PBMC supernatant were determined by ELISA according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). The tests were done in duplicate. Briefly, a microtiter plate (Costar, Cambridge, MA, USA) was coated with 100 μL/well of 5 μg/mL monoclonal mouse anti-human granulysin (clone RB1) (MBL International, Nagoya, Japan) in 0.05 M carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with buffered protein solution with ProClin-150 at room temperature for 1 hr. After being washed, the undiluted plasma was added and incubated for 2 hr at room temperature. The bound antigens were detected with 0.1 μg/mL of monoclonal mouse anti-human granulysin biotin (RC8) (MBL International) and avidin-horseradish peroxidase (Av-HRP) conjugate (BD Biosciences Pharmingen) diluted to 1:1000. After incubation for 1 hr, the reactions were developed by coloring with TMB substrate (BD Biosciences Pharmingen) for 20 min in the dark. The reaction was stopped by 2N H_2SO_4 solution (BD Biosciences Pharmingen). Optical densities were measured at 450 nm wavelength by an ELISA reader (ELx808 IU ultra microplate reader, Bio-Tek instruments, Winooski, VT, USA). Granulysin concentrations were calculated from a standard curve using granulysin containing culture supernatant obtaining from Cos7 cell transfected with gene encoding 15K granulysin. The lower detection limit for granulysin was 0.047 ng/mL.

Determination of circulating interferon-γ concentrations and interferon-γ production from stimulated mononuclear cells *in vitro*

Interferon-γ concentrations in plasma and stimulated PBMC supernatant were determined by ELISA according to the manufacturer's instruction (BD Biosciences Pharmingen). The tests were done in duplicate. Briefly, a microplate (Costar) was coated with 100 μL/well of anti-human IFN-γ (diluted to 1:250 in 0.1 M sodium carbonate) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20, blocked with 200 μL/well of buffered protein solution with ProClin-150 and incubated at room temperature for 1 hr. After being washed, 100 μL of undiluted sample was added and incubated for 2 hr at room temperature. The bound

antigen were detected with biotinylated anti-human IFN-γ monoclonal antibody and streptavidin-horseradish peroxidase conjugate (diluted to 1:250 with 10% FBS in PBS) and incubated for 1 hr at room temperature. Then, 100 μL of TMB substrate solution was added and incubated for 30 min at room temperature in the dark. The reaction was stopped by 2N H_2SO_4 solution. Samples were analyzed at 450/550 nm wavelength with a microplate ELISA reader (ELx808 IU ultra microplate reader) and IFN-γ concentrations were calculated from a standard curve using recombinant human IFN-γ. The lower detection limit was 4.7 pg/mL.

Statistical analyses

Statistical analyses were performed by SPSS software version 17.0. IFN-γ and granulysin concentrations in different independent subject groups were compared by Mann-Whitney U test. A P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of subjects

The clinical characteristics of the patients in the study with newly diagnosed, relapsed and chronic TB are summarized in Table 1. Infiltrates without cavitation were found on the chest radiographs of the majority of patients with newly diagnosed (57.1%) and relapsed TB (51.4%). Most patients with newly diagnosed TB (63.1%) were treated with category 1 drug regimens (2HRZE(S)/4HR) whereas relapsed (60%) and chronic TB patients (52.8%) were treated with category 2 drug regimens (2HRZES/1HRZE/5HRE). Treatment success ("cure" or "treatment completed") was achieved in 66.7%, 57.1% and 47.2% of patients with newly diagnosed, relapsed and chronic TB, respectively. Nine chronic TB patients (25.0%) had microscopically positive sputum smears at the end of their treatment course, indicating treatment failure. The median treatment duration was 7 months in patients with newly diagnosed and relapsed TB and 9 months in those with chronic TB.

Table 1. Characteristics and clinical profile of study subjects

Characteristic	Newly diagnosed TB $N = 84$	Relapsed TB $N = 35$	Chronic TB $N = 36$
Sex			
Male	60	27	15
Female	24	8	21
Age (years)			
Median	44	48	49
Range	9–85	28–88	14–82
Chest X-ray findings			
Infiltrate/Non-cavitating	48	18	15
Cavitating	14	8	6
Not documented	22	9	15
Treatment regimens			
2HRZE(S)/4HR[a] (CAT1)	53	10	
2HRZES/1HRZE/5HRE[a] (CAT2)	19	21	19
2HRZ/2HR[a] (CAT3)			
Second line drug (CAT4)	12	4	17
Duration of treatment (months)			
Median	7	7	9
Range	0–26	0–14	5–20
Treatment outcomes			
Cure	51	18	14
Completed	5	2	3
Default	10	5	7
Died	4	6	3
Failure	7	3	9
Not documented	7	1	

[a]The standard code for TB treatment regimens, each anti-TB drug has an abbreviation: streptomycin (S), isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol (E). CAT, category.

Circulating granulysin concentrations in clinical tuberculosis before anti-tuberculosis therapy

The concentrations of circulating granulysin in patients with newly diagnosed TB (median ± SE = 1.511 ± 0.287 ng/mL, range 0.560–15.600 ng/mL) and relapsed TB (median ± SE = 1.458 ± 0.329 ng/mL, range 0.403–8.110 ng/mL) were significantly lower than those of healthy controls (median ± SE = 2.470 ± 0.186 ng/mL, range 0.662–5.055 ng/mL) ($P < 0.001$, $r = -3.816$ and $P = 0.004$, $r = -2.853$, respectively). Patients with chronic TB (median ± SE = 1.917 ± 0.264 ng/mL, range 0.549–6.970 ng/mL) had lower granulysin concentrations than controls, this difference not being significant ($P = 0.442$, $r = -0.769$). Median concentrations of granulysin were similar in patients with newly diagnosed and relapsed TB, but both were significantly lower than in chronic TB ($P = 0.003$, $r = -2.967$ and $P = 0.022$, $r = -2.294$, respectively) (Fig. 1).

Granulysin production in peripheral blood mononuclear cell stimulation assay

Granulysin production in PBMCs stimulated *in vitro* with PPD and H37Ra were measured in 46 patients with newly diagnosed, 21 with relapsed and 8 with chronic TB. Granulysin production by newly diagnosed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.796 ± 0.071 ng/mL, range 0.208–2.196 ng/mL) and H37Ra (median ± SE = 0.976 ± 0.065 ng/mL, range 0.246–1.823 ng/ml) were significantly higher than those of

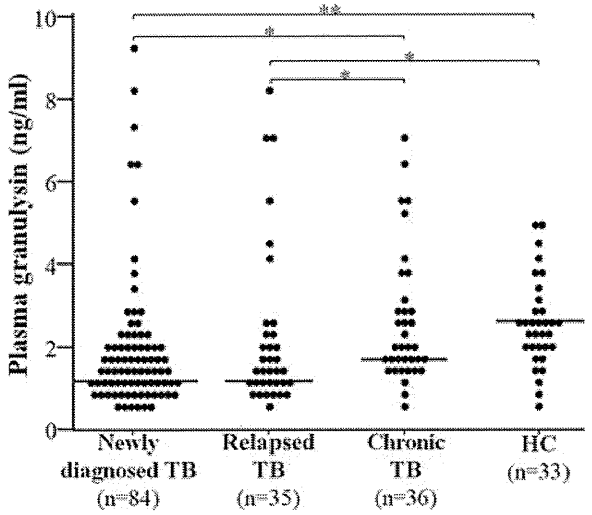

Fig. 1. Circulating granulysin concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represented one individual. The horizontal bars indicate the median of each group. *, $P < 0.05$; **, $P < 0.001$; HC, healthy control.

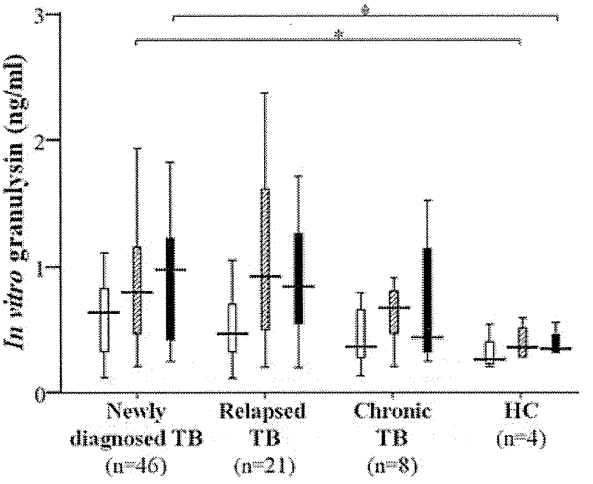

Fig. 2. *In vitro* granulysin production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and heat killed *Mycobacterium tuberculosis* (H37Ra) (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of each group. *, $P < 0.05$.

healthy controls stimulated *in vitro* with PPD (median ± SE = 0.359 ± 0.073 ng/mL, range 0.283–0.591 ng/mL), and H37Ra (median ± SE = 0.348 ± 0.056 ng/mL, range 0.320–0.559 ng/mL) ($P = 0.022$, $r = -2.289$ and $P = 0.032$, $r = -2.146$, respectively). Controls were PBMC supernatants from healthy controls without stimulation (median ± SE = 0.262 ± 0.076 ng/mL, range 0.206–0.542 ng/mL) and PBMC supernatants from newly diagnosed TB patients without stimulation (median ± SE = 0.636 ± 0.051 ng/mL, ranged 0.117–1.665 ng/mL). Although granulysin production by relapsed TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 0.922 ± 0.146 ng/mL, range 0.205–2.374 ng/mL) and H37Ra (median ± SE = 0.841 ± 0.123 ng/mL, range 0.197–2.324 ng/mL) were higher than those of healthy controls, these differences were not significant ($P = 0.054$, $r = -1.927$ and $P = 0.081$, $r = -1.742$, respectively). PBMCs of patients with chronic TB stimulated *in vitro* with PPD (median ± SE = 0.674 ± 0.120 ng/mL, range 0.475–1.345 ng/mL) and H37Ra (median ± SE = 0.435 ± 0.173 ng/mL, range 0.408–1.521 ng/mL) produced greater amounts of granulysin than did healthy controls, the difference not being significant ($P = 0.089$, $r = -1.698$ and $P = 0.497$, $r = -0.679$, respectively). Similar median amounts of granulysin were produced by PBMCs of newly diagnosed and relapsed TB stimulated *in vitro* with PPD and H37Ra but higher amounts by PBMCs of chronic TB, the difference not being significant (newly diagnosed and chronic TB: $P = 0.330$, $r = -0.974$ for PPD and $P = 0.242$, $r = -1.169$ for H37Ra; relapsed and chronic TB: $P = 0.232$, $r = -1.196$ for PPD and $P = 0.380$, $r = -0.878$ for H37Ra) (Fig. 2).

Circulating interferon-γ concentrations in clinical tuberculosis before anti-TB therapy

In contrast to granulysin, the circulating IFN-γ concentrations in patients with newly diagnosed TB (median ± SE = 6.15 ± 4.58 pg/mL, range < 4.7–300 pg/mL) and relapsed TB (median ± SE = 7.93 ± 8.86 pg/mL, range <4.7–310.73 pg/mL) were significantly higher than those of healthy controls (median ± SE = <4.7 ± 0.20 pg/mL, range <4.7–10.13 pg/mL) ($P < 0.001$, $r = -3.923$ and $P < 0.001$, $r = -4.325$, respectively). Circulating IFN-γ concentrations in most chronic TB patients were similar to those of healthy individuals (median ± SE = <4.7 ± 3.76 pg/mL, range <4.7–123.69 pg/mL) ($P = 0.051$, $r = -3.486$). The median concentrations of IFN-γ were similar in patients with newly diagnosed and relapsed TB, but both were higher than in chronic TB, the difference not being significant ($P = 0.395$, $r = -0.851$ and $P = 0.333$, $r = -0.968$, respectively) (Fig. 3).

Interferon-γ production in peripheral blood mononuclear cell stimulation assay

The median IFN-γ production by PBMCs of newly diagnosed TB patients stimulated *in vitro* with PPD (median ± SE = 535 ± 94 pg/mL, range <4.7–2400 pg/mL) was higher than that of healthy controls (median ± SE = 434 ±

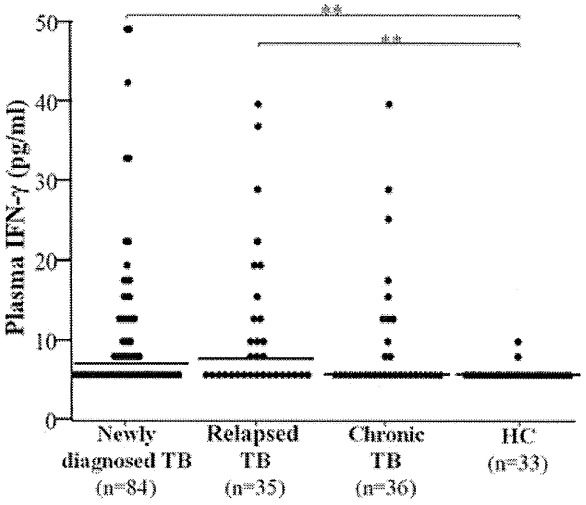

Fig. 3. Circulating IFN-γ concentrations in patients with newly diagnosed, relapsed and chronic TB in comparison with healthy controls. Each dot represents one individual. The horizontal bars indicate the median of each group. **, $P < 0.001$; HC, healthy control.

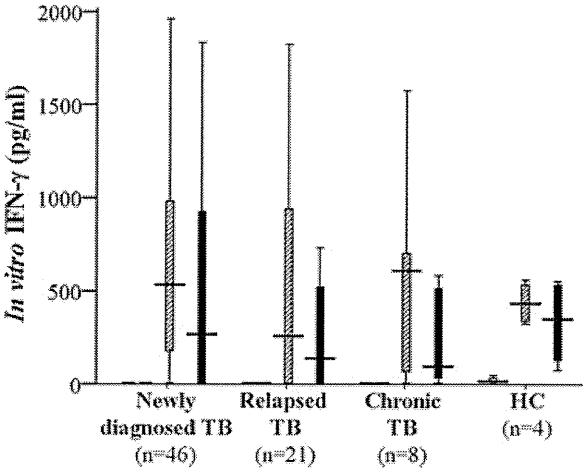

Fig. 4. *In vitro* IFN-γ production by PBMCs from patients with newly diagnosed, relapsed and chronic TB and healthy individuals stimulated with PPD (diagonal shading) and H37Ra (black). Supernatant from PBMCs without stimulation was used as controls (clear). The horizontal bars indicate the median of each group.

57 pg/mL, range 326–562 pg/mL) ($P = 0.591$, $r = -0.537$). However, most newly diagnosed TB-PBMCs stimulated *in vitro* with H37Ra produced higher IFN-γ concentrations (range <4.7–8025 pg/mL), but the median was similar (median ± SE = 270 ± 260 pg/mL) to that of healthy controls (median ± SE = 351 ± 120 pg/mL, range 76–556 pg/mL) ($P = 0.914$, $r = -0.107$). Supernatant from PBMCs without stimulation was used as a cell control (median ± SE = 14.29 ± 8.88 pg/mL, range 9.85–48.06 pg/mL), while supernatant from newly diagnosed TB-PBMCs without stimulation was used as a control for IFN-γ production (median ± SE = <4.7 ± 5.08 pg/mL, range <4.7–231 pg/mL). IFN-γ production by PBMCs from half the patients with relapsed TB stimulated either with PPD (range <4.7–4225 pg/mL) or H37Ra (range <4.7–2575 pg/mL) was higher than that of normal controls. However, their medians (median ± SE = 260 ± 258 pg/mL for PPD, and median ± SE = 138 ± 136 pg/mL for H37Ra) were lower than those of healthy controls; these differences were not significant ($P = 0.823$, $r = -0.223$ and $P = 0.412$, $r = -0.821$, respectively). Chronic TB-PBMCs stimulated *in vitro* with PPD (median ± SE = 610 ± 166 pg/mL, range <4.7–1575 pg/mL) produced higher IFN-γ concentrations than did healthy controls, and some PBMCs stimulated *in vitro* with H37Ra also produced higher IFN-γ concentrations (range <4.7–1835 pg/mL) although the median was lower (median ± SE = 95 ± 198 pg/mL) than that of healthy controls ($P = 0.758$, $r = -0.309$ and $P = 0.354$, $r = -0.927$, respectively). Similar median amounts of IFN-γ production by PBMCs of newly diagnosed and chronic TB stimulated *in vitro* with PPD were found, and these were higher than for relapsed TB, the difference not being significant ($P = 0.436$, $r = -0.779$ and $P = 0.928$, $r = -0.091$, respectively). The median amount of IFN-γ produced by PBMCs of newly diagnosed TB stimulated *in vitro* with H37Ra was higher than that for relapsed and chronic TB ($P = 0.202$, $r = -1.275$ and $P = 0.982$, $r = -0.023$, respectively) (Fig. 4).

DISCUSSION

In this study, the correlations of plasma granulysin and IFN-γ concentrations with clinical disease in patients with newly diagnosed pulmonary, relapsed and chronic TB in northern Thailand, where TB is endemic, were evaluated. The effects of *in vitro* stimulation with PPD and H37Ra of PBMCs from these patients were also investigated. The finding of decreased circulating granulysin and increased IFN-γ in patients with newly diagnosed, relapsed and chronic TB before anti-TB therapy indicated involvement of granulysin and IFN-γ in host defense against TB infections.

In patients with newly diagnosed and relapsed pulmonary TB who had not yet received anti-TB therapy, plasma granulysin concentrations were significantly decreased compared to those of healthy individuals. This may be because granulysin is rapidly consumed during active disease, because of an ongoing effector immune response, or because plasma granulysin is reduced during active disease because of a reduction in the T cell subset dedicated to its production (15). However, granulysin