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A male midwife examining a pregnant woman at a health center in Ethiopia

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Global Health & Medicine

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Global Health & Medicine (Print ISSN 2434-9186, Online ISSN 2434-9194) is an international, open-access, peer-reviewed journal, published by the National Center for Global Health and Medicine (NCGM), which is a national research and development agency in Japan that covers advanced general medicine, basic science, clinical science, and international medical collaboration.

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Global Health & Medicine is dedicated to publishing high-quality original research that contributes to advancing global health and medicine, with the goal of creating a global information network for global health, basic science as well as clinical science oriented for clinical application.

The articles cover the fields of global health, public health, and health care delivery as well as the seminal and latest research on the intersection of biomedical science and clinical practice in order to encourage cooperation and exchange among scientists and healthcare professionals in the world.

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We aspire to identify, attract, and publish original research that supports advances of knowledge in critical areas of global health and medicine.

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A male midwife examining a pregnant woman at a health center in Ethiopia

Ethiopia is one of the few countries that were able to successfully reduce maternal mortality rate between 1990 and 2015 by expanding the healthcare service network through establishing a good primary health care system. Midwives working at the health centers are core healthcare service providers for mothers and children in the communities.

(*Photo by Shinichiro Noda*)

Global Health & Medicine. 2019; 1(1):1-2.

EDITORIAL

DOI: 10.35772/ghm.2019.01005

Focusing on global health and medicine

Hiroaki Mitsuya, Norihiro Kokudo

National Center for Global Health and Medicine, Tokyo, Japan.

Abstract: Focusing on the theme of global health and medicine, at the beginning of the new era (Reiwa) in Japan, we are pleased and honored to launch *Global Health & Medicine*, an international, open-access, peer-reviewed journal, published by the National Center for Global Health and Medicine (NCGM), which is a national research and development agency in Japan that covers advanced general medicine, basic science, clinical science, and international medical collaboration. The journal is dedicated to publishing high-quality original research that contributes to advancing global health and medicine, with the goal of creating a global information network for global health, basic science as well as clinical science oriented for clinical application. We aspire to identify, attract, and publish original research that supports advances of knowledge in critical areas of global health and medicine.

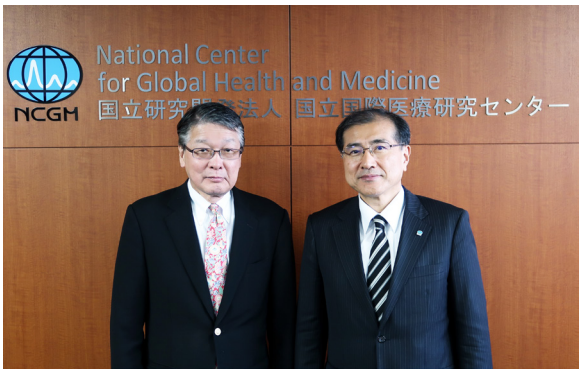
***Keywords*:** GHM, global health, medicine, health care, basic science, clinical science

Significant global health gains have been achieved in recent years, and life expectancy has been greatly extended in various areas and regions of the world thanks to socioeconomic development and public health interventions on vaccination, nutrition, and sanitation. However, economic, political, cultural, and environmental forces continue to drive changes in the burden of diseases, and people everywhere continue to face a complex mix of interconnected threats to their health and well-being, including communicable and non-communicable diseases, high-impact public health emergencies, and the emergence of antimicrobial resistance.

Thirteenth General Programme of Work 2019-2023 (GPW13) issued by the World Health Organization (WHO) emphasizes three interconnected strategic priorities to ensure healthy lives and well-being for all people at all ages: achieving universal health coverage, addressing health emergencies, and promoting healthier populations. Ensuring healthy aging is also central to universal health coverage, just as it is to the other priorities of GPW 13. The number of people over the age of 60 is expected to double by 2050, and such unprecedented demographic development will require a radical societal and global response.

Japan, as the "super-aged" society, with the highest proportion of the elderly worldwide, has long been a leader and advocate in highlighting the importance of a resilient and sustainable health care system to promote healthy aging. Continuous efforts have been made in Japan, such as aligning the health care system to the needs of elderly populations, enhancing the functioning of the elderly and the management of chronic diseases, developing systems of long-term care, creating age-friendly environments, and improving the measurement, monitoring, and understanding of healthy aging.

Focusing on the theme of global health and medicine including healthy aging, at the beginning of the new era (Reiwa) in Japan, we are pleased and honored to launch



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Global Health & Medicine (Print ISSN 2434-9186, Online ISSN 2434-9194), an international, open-access, peer- reviewed journal, published by the National Center for Global Health and Medicine (NCGM), which is a national research and development agency in Japan that covers advanced general medicine, basic science, clinical science, and international medical collaboration.

Global Health & Medicine is dedicated to publishing high-quality original research that contributes to advancing global health and medicine, with the goal of creating a global information network for global health, basic science as well as clinical science oriented for clinical application. We aspire to identify, attract, and publish original research that supports advances of knowledge in critical areas of global health and medicine. The first issue of *Global Health & Medicine* contains a delightfully diverse selection of research articles. The topics include global health, population aging, HIV/AIDS, hepatitis, hepatocellular carcinoma, and diabetes based on the national and global data.

As an international journal and an English language publication, *Global Health & Medicine* will accept submissions from around the world. We encourage submission of original research findings in the fields of global health, public health, and health care delivery as well as the seminal and latest research on the intersection of biomedical science and clinical practice in order to encourage cooperation and exchange among scientists and healthcare professionals in the world. We will also consider papers discussing cutting-edge findings driving the evolution of global health care, such as the rapid adoption of digital technologies in medical research and practice, including genomic sequencing and other -omics approaches as well as use of artificial intelligence to analyze "big data" from millions of individuals and to assist health practitioners and scientists for the more rapid and precise diagnosis and better treatment of illnesses. We look forward to your contributions.

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Population aging in Japan: policy transformation, sustainable development goals, universal health coverage, and social determinates of health

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Abstract: Japan is aging rapidly, and its society is changing. Population aging and social change are mutually linked and appear to form a vicious cycle. Post-war Japan started to invest intensively in infectious disease control by expanding health services and achieving universal medical insurance coverage in 1961. The high economic growth in the 1960s contributed to generate a thick middle class layer, but the lingering economic slump after the economic bubble crisis after 1991 and globalization weakened this segment of society. Health disparity has been acknowledged and social determinates of health have been focused. In this article, the author reviewed the response course to health challenges posed by population aging in Japan, and aims to offer lessons to learn for Asian nations that are also rapidly aging. The core viewpoints include: *i*) review health policy transformations until the super-aged society, *ii*) discuss how domestic issues in aging can be a global issue, *iii*) analyze its relationship with Japanese global health engagement, *iv*) debate the context of social determinates of health, and *v*) synthesize these issues and translate to future directions.

***Keywords*:** Population aging, Japan, policy transformation, SDGs, UHC, social determinates of health

Introduction

Japan is aging rapidly, those over 65 already constituted 27.7% of the total population in 2017. This figure is the highest in the world and is projected to grow continuously up to 38.4% in 2065 (*1*). However, population aging is a result of remarkable success in health improvement and economic development in a country or region, and a similar trend is becoming visible globally, particularly in Asia. Hence, Japan is only a front runner of a future aging world, and her experience will be beneficial for countries that are to follow. However, the demographic impact of aging is more complicated than just a growing number of senior citizens. Another side of the coin is that decline in birthrate to below the death rate results in population decrease, and especially reduction of the young workforce. The population dynamics in Japan are very dramatic, as shown in Figure 1. The Japanese population climbed to a peak within the twentieth century, and is projected to return to the level of the previous century within the next 100 years.

This demographic change poses challenges to all aspects of life for individuals and the society as a whole. How can we extend healthy lifespan, and not merely physical longevity? How is the extended lifespan supported financially? With an increasing in-need population and declining contributors, how can we sustain the social infrastructure including social security (medical insurance and pension) and other essential services such as transportation and response capacity to natural disasters? How can we perpetuate innovations and vitality in a predominantly aged society? All these are perceived as "clear and present dangers" and shared by Japanese leaders and the population as a whole.

Japanese were proud to achieve universal medical insurance coverage and pension in 1961 (*2*) and believe that this achievement has contributed to generate a thick healthy middle class layer, who has brought prosperity and stability in the 60', 70' and 80'. However, a success story itself could turn out to be a hurdle to introduce necessary changes, as Jared Diamond wrote in his book Collapse: How Societies Choose to Fail or Survive. The courage to make painful decisions about values, "Which of the values that formerly served society well can continue to be maintained under newly changed circumstances? Which of these treasured values must instead be jettisoned and replaced with different approaches?" is critical for sustaining a society (*3*). These words are particularly relevant to Japan, which is already a super-aged society with low birthrate and

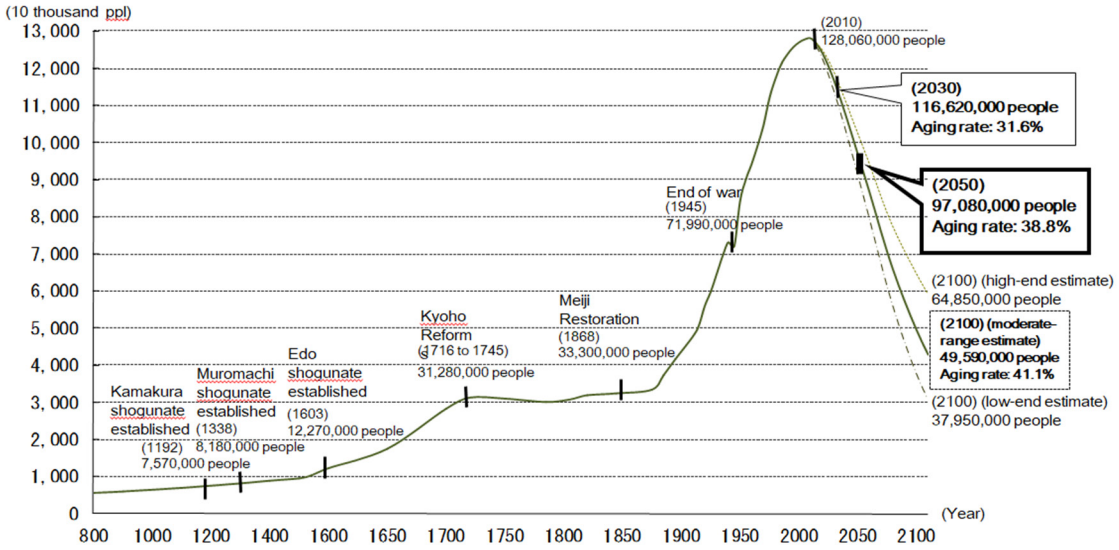

Figure 1. Long-term changes in total population and estimated future population. Data source: Population to 2010: materials prepared by National Spatial Planning and Regional Policy Bureau, Ministry of Land, Infrastructure Transport and Tourism (MLIT) based on the national census results by Ministry of Internal Affairs and Communications (MIC) and the analysis of long-term chronological population distribution data in the Japanese islands (1974) by National Land Agency. The population thereafter: materials prepared by National Spatial Planning and Regional Policy Bureau, MLIT based on *Population Projection for Japan* by National Institute of Population and Social Security Research (estimated in January 2012).

population decline.

With the above background, in this article, the author wishes to: *i*) review health policy transformations until the super-aged society, *ii*) discuss how domestic issues in aging can be a global issue, *iii*) analyze its relationship with Japanese global health engagement, *iv*) debate the context of social health determinates, and *v*) synthesize these issues and translate to future directions.

Transformation of health policy until the super-aged society

Demographic change is not readily visible in daily life and is only appreciated when it becomes too apparent suddenly. However, while experts in demography can illustrate future population size and composition relatively easily, such "inconvenient truth" is difficult to communicate to the public as well as policymakers. Early warning was voiced. For example, the late Dr. Taro Takemi, Past President of Japan Medical Association published an article on Monthly Chuo-Koron, entitled "How can we cope with the growing number of senior citizens ?" in 1955. He foresaw that population aging required changes in health care delivery and demanded a critical review of future design in social security. All his concerns have proven to be real nearly half a century later when the "inconvenient truth" becomes too visible. Figure 2 illustrates the historical development or transformation of health policy in Japan until Japan becomes a super-aged society. Aging in other countries is also plotted to show how they may plan to introduce significant policy changes when their populations become aged progressively in the future as in Japan.

In this regard, the post-World War II health policies in Japan can be categorized into four phases: *i*) towards UHC (until 1961); *ii*) expansion of social security (until 1980); *iii*) preparation for aging society (until 2000), and *iv*) enhancing sustainability (until 2025).

Towards UHC (until 1961)

The central policy issues in the '50s were expansion of medical insurance and pension coverage for every citizen in Japan. Coverage started from large companies, public sectors and local communities, but small industry workers and their families were left behind. Universal coverage was finally achieved in 1961 when GDP per capita was US$563 (current equivalent) (*4*). At that time, the average life expectancy was 70.2 years for women and 66.0 for men, and the average age of the Japanese population was 28 years. The respective figures for 2017 were US$38,428, 87 years, 81 years for men, and 47 years.

Expansion of social security (until 1980)

The '60s were remembered for the amazing rate of economic growth, and Japan became the number 2 global economic power in 1968, replacing West Germany. Industrialization and urbanization progressed at a rapid pace. Old family-based welfare, while offering public support for impoverished people, was

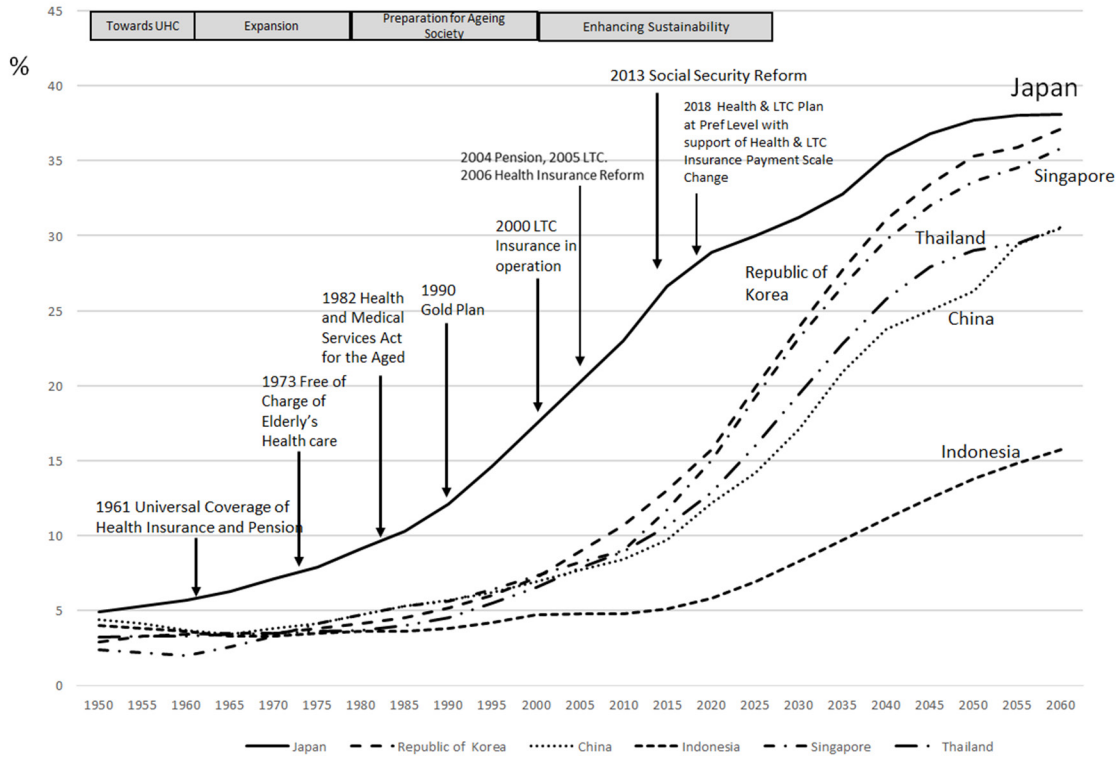

Figure 2. Aging rates of Asian countries and evolution of Japan's elderly care system. Data source: *http://www8.cao.go.jp/kourei/whitepaper/w-2018/html/zenbun/s1_1_2.html*

being challenged. The on Welfare for the Elderly Act was enacted in 1963, which expanded social support for senior citizens in need. According to the copayment system of medical insurance, service receivers should pay 30% of the cost each time they receive service (remaining 70% is directly claimed by the service providers to insurance bodies). Increasing political pressure that the 30% co-payment was discouraging senior citizens from accessing services drove the government to waive the copayment for seniors aged over 70 years in 1973. This scheme was judged feasible at that time, when the economy was strong and the proportion of senior citizens was less than 10%. The act was welcomed initially, but later proved too costly. Eventually, politicians paid a high political price when re-introducing the copayment, losing elections due to such an unpopular move. The evolution and remarkable outcomes as well as increasing challenges of the Japanese medical insurance system are well documented by Ikegami and Campbell (*5*).

Preparation for aging society (until 2000)

As the proportion of senior citizen increased to almost 10%, bureaucrats started to raise concerns over the trend of increasing medical expenditures. Mr. Hitoshi Yoshimura, Director-General of Insurance Bureau of Ministry of Health (1982 to 1984) and later Vice-Minister of Health (1984 to 1986) was a strong advocate of the urgent need for medical cost containment due to expensive health technologies and aging, among many other factors. Mr.Yoshimura was not shy to present his pessimistic view on the sustainability of social security, particularly medical insurance. He advocated various measures to "rationalize" medical cost. A significant outcome was the enactment of the Health and Medical Services Act for the Aged in 1982. The Act has two components: health promotion after age 40 and financial balancing mechanism to support medical insurance bodies, particularly community-based insurance bodies to pay medical bills of senior citizens.

Along with continued population aging and reduction of family size, Japan needed to consider socializing nursing care for the aged by expanding capacity of relevant institutions. Also, a consensus was reached that hospital admission due to "social" needs and not medical reasons should be a target of rationalization. This so-called "social hospitalization" phenomenon and the long waiting list to enter nursing homes became a political agenda in the national parliament as well as prefectural assemblies. This resulted in systematic investment from the public sector. The Ministry of Health, Ministry of Finance and Ministry of Home Affairs launched the "Gold Plan" in 1989 (over 65 population: 11.6%), investing 6 trillion yen to build more long-term care institutions. The plan

was modified in 1994 (over 65 population: 14.1) to expand home care programs (*6*). These developments provided the infrastructure to introduce the long-term care insurance (LTCI) (*7*), which came into operation in 2000 (over 65 population: 17.4%). The LTCI covers both home and institutional care according to the assessed level of disability.

Enhancing sustainability (until 2025)

By implementation of the LTCI, the social security architecture for the aged was completed, with medical care insurance for sickness, pension for livelihood support and LTCI for long-term disability. However, upon entering the 21st century, the continuation of rapid aging and sharp birthrate decline questioned the sustainability of medical care insurance and pension. Attempts were made to increase the premium and copayment of service receivers and to enhance the efficiency of service providers as well as to expand public support for medical insurance bodies. Significant reforms were legislated in 2006 and 2015. However, it has been noted that the reform of medical insurance should be discussed in the broader context of social security. For example, the increase in demand for LTCI is greater than that for medical insurance. From 2000 to 2018, the number of users of the LTCI increased three-fold from 1.49 to 4.74 million. In response to such an increase in demand, the service cost rose from 3.6 to 10 trillion yen from 2000 to 2016, and the per capita insurance premium of senior citizens themselves also increased from 2911 to 5514 yen (*8*).

On the other hand, the medical cost grew but at a much moderate pace from 30.1 trillion yen in 2000 to 42.2 trillion in 2017. This was a result of tighter control of the health insurance reimbursement scheme, but this capping strategy posed challenges for both health care professionals and health care industries. A book entitled Collapse of Medical System (*9*) that addressed this issue became a bestseller in 2006.

In addition, due to the continued Japan economic slump after the economic bubble collapse in 1991, many young people failed to find full-fledged employment and accepted irregular jobs with less wages and lower insurance and pension contributions. Thus, social insurance premiums from workers were not raised. Altogether, balance sheets of both medical and long-term care insurance have deteriorated from reduced contributions and increased demand. The financial gaps are filled by transferring medical costs from young people to seniors within insurance bodies and infusion from tax revenues. Consequently, out of the 97.7 trillion yen government budget in FY 2018 (*10*), 33.8 trillion yen was spent on social security. Hence, health is the biggest budget item, six times larger than that for education, and science and technology, which is generally regarded as investment for future human capital.

All these generated needs to look at all aspects of social security by avoiding silo approaches to medical insurance and LTCI, as well as disproportionate consumption of the general budget. The government solution was to increase the consumption tax from 5% to 8% and eventually to 10% by 2019. A bipartisan agreement was reached in 2012 to use a considerable portion of the increased revenue to enhance the sustainability of social security, ahead of 2025 when the post-war baby boomers (1947-49) would reach the age of over 75 and demand greater medical and long-term care services. Hence, the National Council of Social Security Reform was called by the Cabinet Office, and a report (*11*) including a road map of "total reform" was submitted to the Office on 6 August 2013.

The report was perceived as unique in addressing challenges in a cross-cutting manner and recommending well coordinated policy change, taking into account changes of social, family and individual values. The report presented the grand vision and proposed reform on social measures to address the declining number of children, medical and long-term care insurance, and pension.

Convergence of global and domestic health agendas

If one turns attention to global health, it is surprising to see a convergence of the Japanese domestic agenda with the global health agenda. The life expectancy of the world has reached 71 years and many "developing countries" have graduated from being recipient countries to mid-income countries with limited access to development aid from more affluent countries. This was made possible by massive investments in control of diseases and infections (HIV/AIDS, tuberculosis, malaria, and neglected tropical disease) followed by maternal and child health. The former included access to medicine and preventive measures such as anti-retroviral medicines for HIV/AIDS and long lasting insecticide-treated bed-nets for malaria. These brought a drastic decrease in deaths and an increase in a healthy workforce that drove socio-economic development in once communicable disease-affected low-income countries. In the case of tropical diseases, a typical example is onchocerciasis or river blindness. This parasitic disease was a common cause of blindness among populations along the river side in tropical regions, particularly in West Africa. River side fertile farming land was abandoned due to the disease. However, mass preventive use of ivermectin (Mectizan) almost eliminated river blindness and led to economic recovery. The vicious circle of ill health and poverty was broken. The same was observed in Japan during the early post-war period. In 1954, the leading cause of death was tuberculosis, which consumed 28% of the medical care budget (*12*). The most affected

population was young students and workers. Japan aggressively controlled tuberculosis by mass screening and case management at public health centers together with public financial support for care under official diagnosis and treatment regimens. That resulted in a sharp decline in mortality and morbidity. Healthier workers contributed to a remarkable economic growth, which was unprecedented in the world. Furthermore, the health infrastructure built for tuberculosis served as the basis for meeting changing health needs, such as control of non-communicable diseases (NCD). Mass screening originally designed for tuberculosis was used for early detection of hypertension, which was the main risk factor for brain hemorrhage and replaced tuberculosis as the leading cause of death in 1951.

This success story was convincing enough for Japanese leaders and politicians to engage themselves in global health cooperation. During the period of the economic bubble in Japan from 1986 to 1991, Japanese ODA was greatly expanded, which reaffirmed its "soft power" in foreign policy. Meanwhile, reflecting the end of the cold war, a new paradigm was sought globally. As a nation with a constitutional commitment to renounce war, Japan welcomed and promoted the new paradigm of international cooperation, from "security against war" to "human security", which addresses both "freedom from fear of war and other insecurity issues" and "freedom from want of better health and other human conditions" for all people. Human security became the principal value of Japanese diplomacy, and naturally Japan started to voice proposals for global health. At the 1998 Birmingham Summit, then Prime Minister Hashimoto proposed several steps to improve the effectiveness of international cooperation against parasitic diseases. In 2000, the Kyushu-Okinawa G8 Summit adopted the Okinawa Infectious Diseases Initiative, which led global fights against three major infections; HIV/AIDS, tuberculosis and malaria. In every subsequent G7/8 summit hosted by Japan, health was on the agenda in head of state meetings. For example, the G8 Hokkaido Toyako Summit in 2008 addressed the importance of health systems to support disease control activities. With this background, Japan welcomed sustainable development goals (SDGs), and the Prime Minister personally expressed his commitment repeatedly at the United Nations (UN) and in his contribution to the Lancet (*13*). The G7 Iseshima Summit in 2016 was the first summit meeting after the adoption of SDGs at the UN. The leaders addressed universal health coverage (UHC) as an approach for global health strategies for both communicable and non-communicable diseases under the SDGs framework. At the same time, they emphasized that health systems and UHC are the needed infrastructure to tackle health emergencies such as epidemics, which have been viewed as an increased risk in the interconnected world. The health topics at the summits had expanded from communicable diseases control to a more inclusive approach such as health systems and UHC, which is the course that Japan had taken with not only many successes but also bitter lessons. The Japanese experience can be useful for other nations, particularly Asian countries, which are experiencing a similar course of development and foreseeing rapid aging of their populations.

At the same time, Japanese politicians and business leaders have become more sensitive about the shrinking domestic market due to population aging and decline, and recognize the need to cultivate new industries beyond the production of consumer goods. Advisers to the Prime Minister identified the time gap of population aging among nations as business opportunities for health- and aging-related industries. Being a forerunner in population aging, Japan has been developing systems and technologies for the "silver" market, and hence may have a relative advantage. The linkage of domestic health and technology with global needs and issues as well as business is being formulated. To facilitate such transformation, The Health and Medical Strategy Promotion Act was promulgated in May 2014, which led to the establishment of Headquarters for Healthcare and Medical Strategy (hereinafter referred to as "Headquarters") in June 2014. The Headquarters served as an engine of coordinated policy, and the Cabinet approved the Healthcare Policy in July 2014, including a sentence "Healthcare Policy shall promote overseas activities of the healthcare sector by building mutually beneficial relationships with foreign countries, especially in the fields of medicine and long-term care". In addition, in preparation for the new paradigm of international cooperation beyond the millennium development goals (MDGs), the Headquarters approved the Basic Design for Peace and Health (Global Health Cooperation) in September 2015. The Basic Design emphasizes the importance of UHC and our commitment to SDGs. With this background, the Asia Health and Wellbeing Initiative (AHWIN) (*14*) was launched in 2016 with wide participation by public and private entities in Japan and international collaborators.

Japan's engagement in global health

The forum for Japanese health diplomacy was G7/G8 summits and multi-lateral UN agencies such as WHO, mainly on the basic framework of human security and SDGs. However, the global environment changed with the emergence of other groups such as BRICs, and G7 leadership also changed the global picture of engagement of G20 countries. The USA and the UK, which had been generous donors in the past, are paying more attention to their domestic issues, while the relative position of Japan and Germany in global health has gained more weight. Also, UHC involves non-health sectors, particularly the Ministry of Finance, private

sectors and communities. From this perspective, Japan has started to expand collaboration with the World Bank group in establishing global financing facilities for health and nutrition for mothers and children, as well as pandemic emergency financing facilities. Such collaboration is being expanded to the Regional Banks such as the Asia Development Bank. Also, Japan's stewardship in organizing the G20 Meetings has several characteristics, including head of state meetings including both finance and health ministers, and a separate health minister meeting focusing on UHC, aging and emerging infections including antimicrobial resistance. The series of meetings and communiques left a legacy of serious involvement of G20 in global health as well as its own domestic issues.

In the World Health Organization (WHO), the new Director-General, Dr. Tedros Adhanom Gehbreyesus was appointed in July 2017 by direct voting of all member states. He started to transform WHO into the "engine" to accelerate the achievement of SDG3: ensure healthy lives and promote well-being for all at all ages, through the 13th General Programme of Work (*15*) (GPW13). The GPW13 sets the targets of triple billion by 2023: one billion more people benefit from UHC; one billion more people have better protection from health emergencies, and one billion more people enjoy better health and well-being. The main pillar of the triple billion is UHC which is defined in one of the 13 targets under SDG3. Each goal of SDGs has a set of targets and indicators for monitoring. Target 3.8 under SDG3 states "Achieve universal health coverage, including financial risk protection, access to quality essential health-care services and access to safe, effective, quality and affordable essential medicines and vaccines for all". The legitimacy of the UN comes from the approval of the heads of states gathered in the UN by the General Assembly's formal adoption of the SDGs in September 2015. Hence, in the next few years, all global health initiatives will link with the SDGs to justify their legitimacy, and Japan is committed to engage in both domestic and international activities.

Recognition of social determinants of health challenges and mitigation through SDGs/UHC

Commitment to SDGs urged Japan to review critically domestic challenges; for example, health gaps that were difficult to recognize in Japan. Under the post-war regime, the bipartisan political agenda was to generate and maintain a thick middle class layer, and the idea of social gaps and their linkage with health conditions tended to be rejected. However, the long economic slump after the economic bubble crisis together with globalization diminished traditional life-long full-time employment, and blue color jobs were increasingly taken up by Asian neighbors. Also, there were significant changes in the family and its value. Under such a social environment, the Japanese political climate became fluid, except the Koizumi Cabinet (2001-2006) which enjoyed populist support. However, Koizumi's market-oriented approach fueled the demand for fundamental social changes, leading to social movement that brought landslide victory for the opposition party, the Democratic Party of Japan (DPJ), in 2009. The DPJ raised many untouched issues such as poverty among young parents. The Party promised drastic overhaul of the county and attracted initial support. However, due to the rapid ascension of the Party, the leadership lacked skill and experience in running the government. Handling of the 11 March 2011 Tōhoku earthquake/tsunami and nuclear accident highlighted their weakness in governance. Eventually, they lost the general election in 2012. During the period of DPJ rule, evidence such as health disparity and social determinates of health was accumulated. Thereafter, the Liberal Democratic Party regained power and gave priority to economic revitalization, but did not forget the need to address social issues caused by aging and its consequences.

If we look at healthy longevity, Japan has a national health promotion strategy endorsed by the Cabinet since 1978 (revised in 1988). The earlier strategy emphasized the life-course approach, early detection of major NCDs and health promotive activities. The third version was renamed Health Japan 21 and was launched in 2000 with a clear aim of extending a healthy life expectancy. However, the progress report released in 2011 showed that out of 59 targets, only 16.9% were accomplished, 42.4% showed some progress, 23.7% were unchanged and 15.3% deteriorated. The unchanged or worsened targets include decreases in prevalence of metabolic syndrome, hyperlipidemia, and diabetic complications, and the number of steps walked per day. These results raised alarm. The second version of Health Japan 21 (*16*) issued in 2012 clearly states that the overarching objectives of the second version were to improve healthy life expectancy and narrow health gaps among prefectures (difference in lifespan of two years for men and 2.7 years for women). Then it urged national policies in the following areas: prevention of onset and progression of NCDs, maintaining functions for social wellbeing, cultivating an environment to support health maintenance, and improving lifestyle and social environment for nutrition, exercise, rest, alcohol consumption, smoking, and oral health.

After the turn of the century, the bipartisan political agenda has been the sustainability of social security; first pension, followed by medical insurance, and finally the long-term care insurance scheme. After several reform attempts of individual components, it was recognized that the individual approach had limitations and all components should be reviewed comprehensively. Also, there is broad consensus that our UHC would not be sustainable in the face of increasing

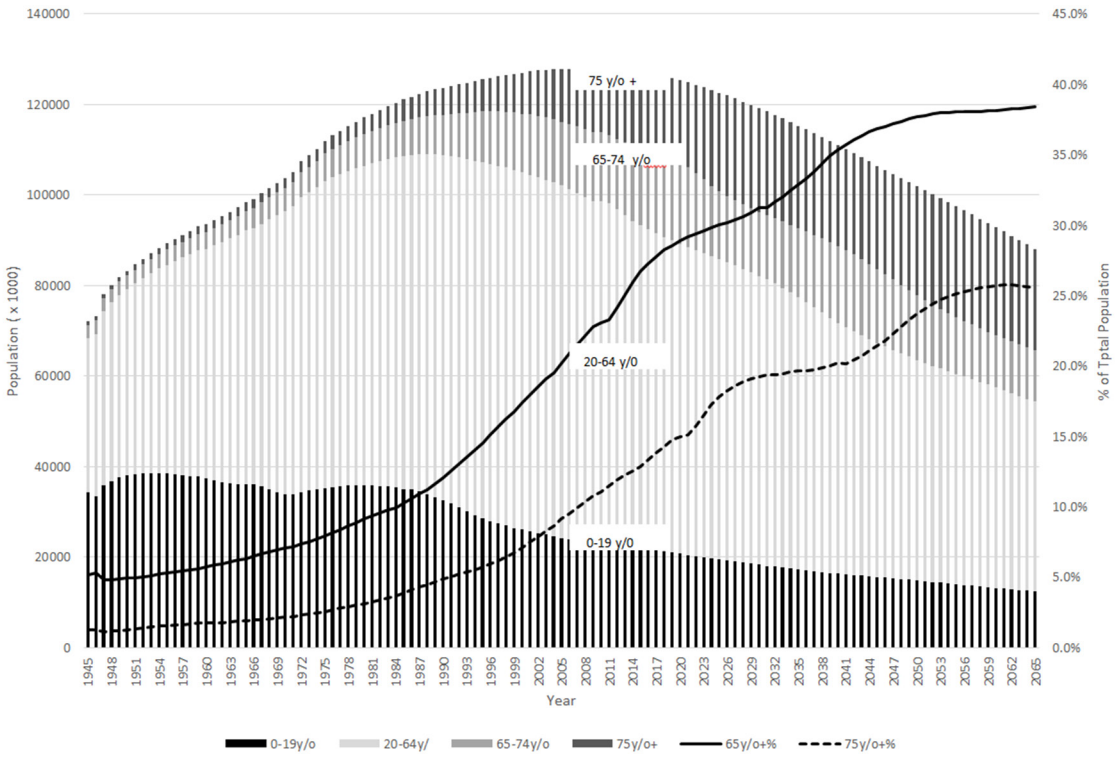

Figure 3. Changes of age groups in Japan. Data source: *http://www.ipss.go.jp/pp-zenkoku/j/zenkoku2017/pp_zenkoku2017.asp http://www.ipss.go.jp/ppzenkoku/j/zenkoku2017/db_zenkoku2017/db_s_suikeikekka_1.html*

senior citizens, declining workforce and increasingly intensive and costly care, as illustrated by the National Council of Reform of Social Security Report (*11*). The report proposed systematic and comprehensive reform across pension, medical insurance, long-term care insurance and support for child care. According to the Report, the government is moving to ensure universal coverage of client-centered comprehensive health, medical and nursing care support at the community level. This requires significant transformation of service provisions. For example, for a community with a high proportion of senior citizens, acute care service is likely over-supplied while services for chronic illness and rehabilitation may remain under-supplied. All prefectures are mandated by the revised Medical Care Act to plan and transform service provisions ahead of 2025 when the baby boomers become over 75.

However, aging challenges will continue. As illustrated in Figure 3, the absolute number of senior citizens over 65 will reach a peak around 2042 when the sons or daughters of baby boomers become over 65. The ratio of senior citizens will continue to increase, but the absolute number will decline. However, the cohort of new seniors will be entirely different from previous generations. The likelihood of them being single and part-time workers before retirement will be much higher than the previous generation. The social determinants of health will matter very much because this generation may be disadvantaged in social capital.

The ways forward: review and comments on recent policy

Prime Minister Abe was in office for 2,616 days at the end of February 2019 and became the second longest serving prime minister in post-war Japan, after Mr. Eisaku Sato who served 2,798 days. His priority for domestic policy is economic revitalization and active measures against aging and low birthrate. Moreover, he views these also from an international perspective, particularly Asia where rapid aging is progressing. In this context, the Basic Principles of the Asia Health and Wellbeing Initiative launched in 2016 was revised in 2018 (*14*). The underlying philosophy is to enhance cooperation to meet the common challenges of aging by *i*) sharing Japanese experience (both positive and negative), *ii*) expanding services with the concept of UHC, *iii*) accepting care workers to train in Japan who will return home to serve their own aging populations, and *iv*) R&D taking advantage of Japanese health services and products. This initiative is coordinated by the Cabinet Office, and inter-ministerial works have begun. For example, to accept more care workers, the Immigration Act was amended in 2018 to expand the target from care for the elderly to broader aspects of services and products that support long life, including

housing and food.

As describe above, the Cabinet Office launched the Headquarters in May 2016 aiming to lead implementing the SDGs both domestically and internationally. Furthermore, the initiatives include establishment of the SDGs Promotion Roundtable Meeting, where a wide range of stakeholders (including government, NGO/NPOs, experts, private sectors, international organizations and domestic organizations) engage in constructive dialogue. Business communities also participate because they see tremendous opportunities. Echoing such government initiatives, the Japan Business Federation launched Society 5.0 for SDGs (*17*) with broad participation by business communities.

Synergetic efforts participated in by both public and private sectors have been started to address both domestic and international health challenges. Such spirit of public-private partnership to achieve win-win relations is a rare phenomenon in Japan, and is anticipated to create new value out of collaboration beyond social/corporate responsibilities. SDGs serve as a catalyst for collective efforts toward sustainable development and surely will occupy a central position in future health agendas in Japan and beyond.

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The role of the G20 economies in global health

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Abstract: The Meetings of Health Ministers of the Group of Twenty (G20) that started at the G20 Summit in Berlin, Germany in 2017 have provided a platform for the discussion of global health matters such as antimicrobial resistance (AMR), public health emergencies, and universal health coverage. Similar issues are also discussed at meetings of the G7 and the World Health Assembly (WHA). This article will examine recent data to explore the characteristics of the G20 and its potential for improving health outcomes. G20 countries have a leading role to play in helping other countries improve global health outcomes because member countries have already faced many issues associated with aging society and increased prevalence of non-communicable diseases (NCDs). Indeed, 71% of the world's elderly population lives in the G20 countries and most of these countries have a high proportional mortality from NCDs of more than 70%. G20 countries are also responsible for a disproportionate share of global impacts. For instance, 72% of CO_2 emissions are produced by G20 countries. Migration dynamics and its consequences also need to be considered from the perspective of optimizing health outcomes. Moreover, 78% of the world's top 50 pharmaceutical companies are located in the G20 countries. There is ample room for G20 countries to pursue collaborative and cooperative approaches that can complement the roles of the G7 and WHA in similar health issues. The G20 could, for example, share experiences on dealing with aging and NCDs, reduce their CO_2 emissions, prohibit the production of low-quality medicines, and use standardized health check-up formats for migrants and refugees to transfer their own health information. As a group, the G20 countries have the potential to solve global health problems and other issues. The convening of high-level health meetings at G20 summits has the potential to facilitate such endeavors.

***Keywords*:** Aging, G20, health, migrants, non-communicable diseases (NCDs), pharmaceuticals, refugees

Introduction

Historically, the Group of Twenty (G20) summits have been characterized by meetings between finance ministers and representatives of central banks and have had mandates to promote cooperation towards sustainable economic growth (*1*). The G20 has significant influence globally. For instance, the G20 countries collectively occupy around 54% of the land mass worldwide (*2*), and they account for 64% of the world's total population and 86% of global GDP (*3*). In recent years, the G20 has addressed issues beyond finance and trade. For example, the first Meeting of Health Ministers of G20 held in Berlin, Germany in 2017 discussed global health matters, including antimicrobial resistance (AMR), public health emergencies, and universal health coverage (UHC) (*4,5*). However, G7 Summits such as the Ise-Shima Summit (*6*) and the World Health Assembly (WHA) have also hosted similar discussions on global health issues (*7*), which raises the questions of what role the G20 countries have in health and how necessary and useful the G20 Meetings of Health Minsters are.

This article will examine recent data to identify and explore some characteristics of the G20 and its potential role in the health domain.

Present state

Aging

In recent years, population aging has become an important issue around the world (*8*). Japan has the highest proportion of elderly people (33% of its population), followed by Italy and Germany. The world average is around 12.7% (*8*). However, in terms of the number rather than proportion of elderly people, China has more elderly people than any other country, followed by India and the USA (*8*). Overall, 71% of the world's elderly

population lives in G20 countries (Figure 1).

Disease dynamics in G20 countries

Globally, in the 1990s the leading causes of Disability-Adjusted Life Year (DALY) lost were due to maternal, childhood, and neonatal diseases, respiratory infections, tuberculosis, and diarrhea. Since then, Non-Communicable Diseases (NCDs) such as ischemic health diseases, stroke, chronic obstructive pulmonary diseases, cancers, and psychiatric disorders have become predominant (*9*). According to the World Health Organization (WHO), 70% of the mortality associated with NCDs occurs in 17 of the 19 G20 countries excluding the EU (Figure 2) (*10*). This is not just a trend observed in developed countries; many developing countries are also seeing an increase in NCDs (*10*).

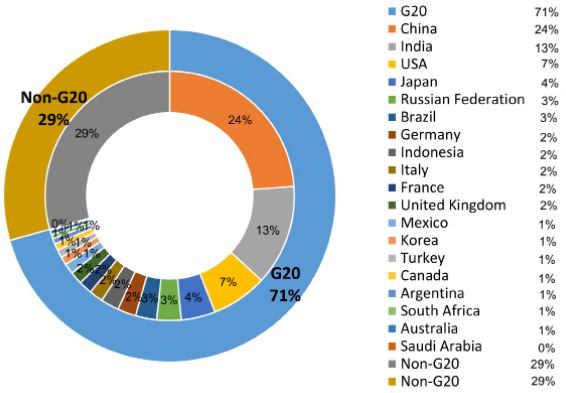

Figure 1. Proportion of the population aged 60 years or over (2017). Data source: *http://www.un.org/en/development/desa/population/publications/pdf/ageing/WPA2017_Highlights.pdf* (*8*).

Climate change

According to the U.N. Paris Declaration in October 2018, the threats posed by anthropogenic climate change are substantial (*11*). Therefore, the loss of life due to climate change is increasing year on year through, for example, heatwaves and droughts, changes in precipitation dynamics, and extreme weather events (*12*). The key greenhouse gas responsible for these impacts is carbon dioxide (CO_2) (*13*) and, collectively, the G20 countries are responsible for around 72% of global CO_2 emissions (*14*) (Figure 3).

Mobility and Migration

The popularity of air travel continues to increase worldwide (*15*), and with it, international population mobility is also on the rise. Both the displacement (*16*) and international migration (*17*) of people are also increasing globally, with 57% of international migrants coming from G20 countries such as India, Mexico, Russia, and China. However, 78% of international

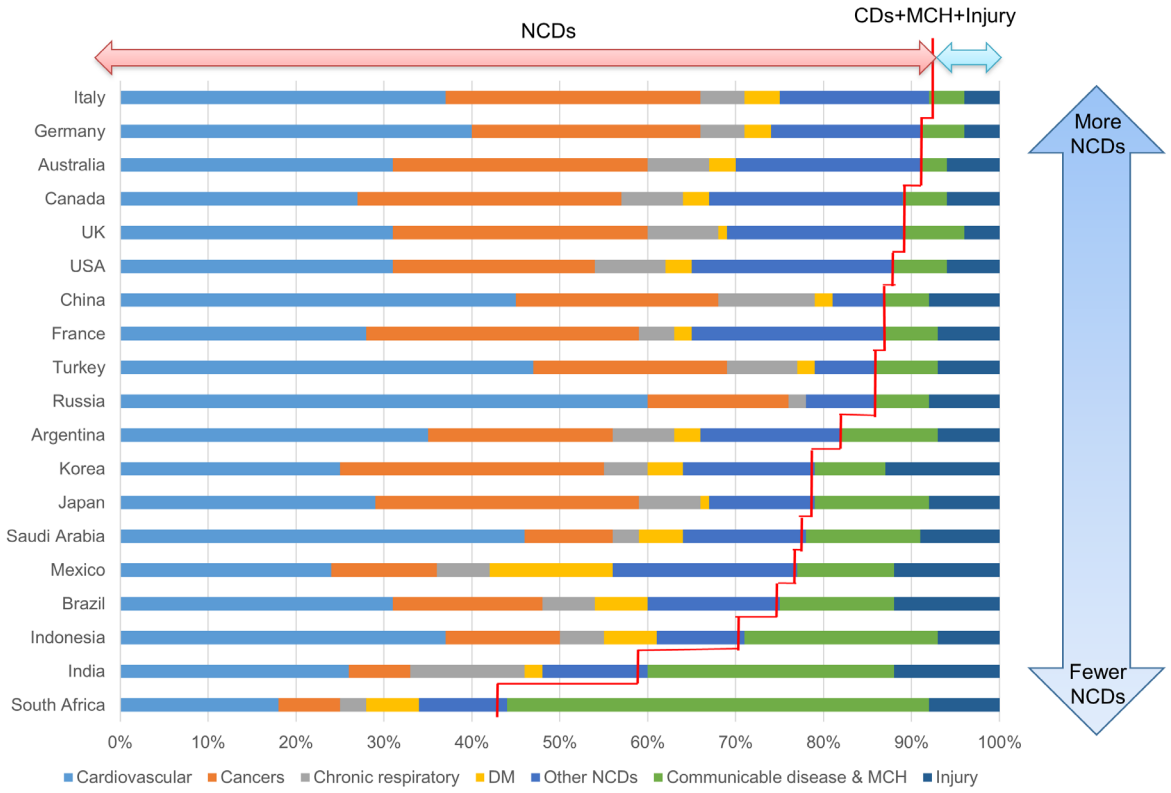

Figure 2. Proportional mortality (G20, except EU) (% of all deaths, all ages, both sexes). Data source: *https://apps.who.int/iris/bitstream/handle/10665/128038/9789241507509_eng.pdf;jsessionid=BEEB2253AC4BD65717F996270A1B200F?sequence=1* (*10*).

migrants relocate to G20 countries such as the USA, Germany, Russia, and Saudi Arabia (*18*) (Figure 4).

Pharmaceutical companies

Of the world's top 50 pharmaceutical companies, 78% are located in G20 countries such as the USA, Japan, the UK, and Germany. However, the second most important country in this respect is a non-G20 nation, Switzerland, where 13% of the top 50 pharmaceutical companies are located (*19*) (Figure 5).

Discussion

The G20 is heavily represented in many global topics and fields of interest. This means that the G20 countries can have an important role to play in global health because of their experience dealing with particular issues. For instance, many G20 countries have an aging society and are therefore developing novel approaches, frameworks, and policies to deal with the issue. One such approach is long-term care insurance in Japan.

The majority of G20 countries are also faced with threats in terms of mortality and morbidity associated with NCDs, including cardiovascular diseases, neoplasms, and diabetes mellitus (*10*). Consequently, they should prepare strategies that minimize the occurrence of, and optimize the response to, NCDs. Each G20 nation must develop its own approaches. Therefore, the lessons G20 nations have learned in terms of cost, benefits, impacts, and uncertainties could be useful for other countries that are likely to face increasing prevalence of NCDs in the years to come as their own aging society (*8*).

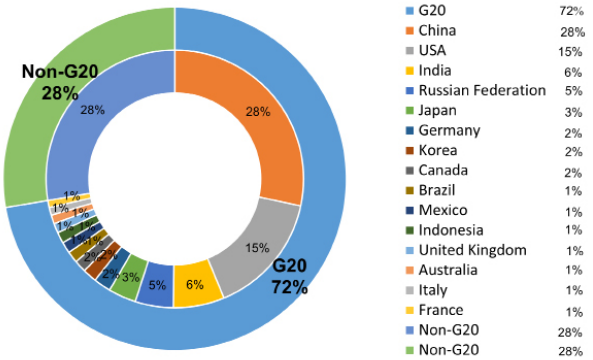

Figure 3. Global carbon dioxide emissions (2015). Data source: *http://www.jccca.org/chart/chart03_01.html* (*14*).

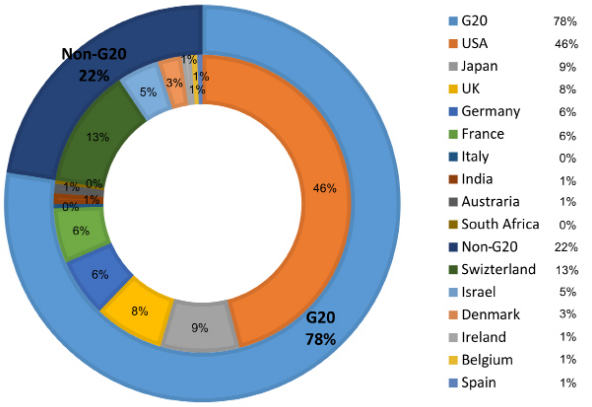

Figure 5. Locations of the world's top 50 pharmaceutical companies. Data source: *http://www.pharmexec.com/pharm-execs-top-50-companies-2017* (*19*).

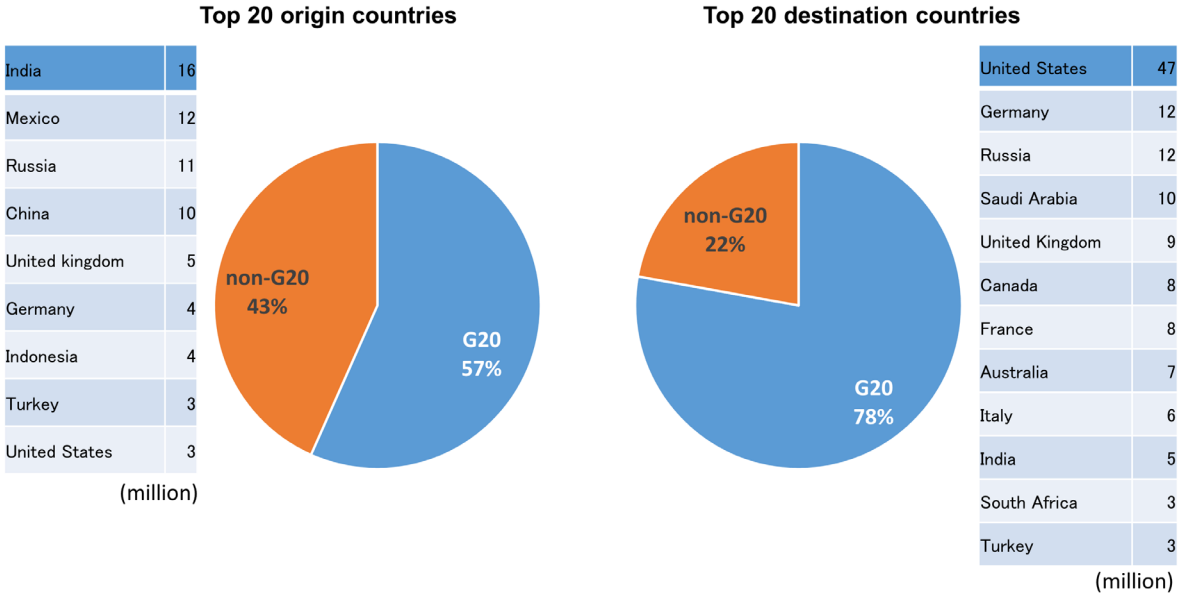

Figure 4. Top 20 origin and destination countries of international migrants (2015). Data resource: *http://www.un.org/en/development/desa/population/migration/publications/migrationreport/docs/MigrationReport2015_Highlights.pdf* (*18*).

G20 countries are responsible for some issues of concern such as CO_2 emissions, but they may have difficulty agreeing on commitments to reduce CO_2 emissions. The USA, for instance, announced its withdrawal from the Paris Declaration (*20*) even though it is responsible for a disproportionate amount of global CO_2 emissions (*14*). However, that does not mean that effective cooperation is not possible in other domains. For instance, the issue of counterfeit and substandard medicines could be managed if the pharmaceutical companies in G20 countries, which account for 78% of the global market, agreed not to produce low-quality medicines. If Switzerland were to join such a campaign, the share would become 91% (*19*). In addition, the G20 has considerable leverage with respect to influencing the pharmaceutical sector to reduce and perhaps control the cost of expensive diagnostics and treatments for NCDs.

Population growth, easy access to other countries, and armed conflicts lead to mass migration in the form of refugees, workers, and tourists (*15*). This can increase the spread of emerging and re-emerging infectious diseases such as Ebola-type diseases that necessitate a Public Health Emergency International Concern (PHEIC) as well as AMR diseases such as multi-drug resistant tuberculosis. NCDs may affect migrants (*i.e.*, refugees, workers, and tourists), so their health care costs can become a burden on host (recipient) countries. If the G20 would agree to use standardized health check-up formats and frameworks for migrants to transfer their own health information such as one's immunization history to other countries or other organizations, it could help many migrants more easily receive appropriate health services, and the recipient countries could protect their own populations from infectious diseases.

Thus, G20 countries may be able to collaborate on developing and implementing frameworks rather than merely setting global policy goals for AMR, UHC, and PHEIC.

Conclusions

Because of their influence on the global economy, G20 countries have the collective potential to solve many global health issues. The G20 can take on roles and tasks unlike those of the G7 and WHA even while addressing similar issues. In an era of Sustainable Development Goals, collaboration among actors in different fields is clearly necessary if global health outcomes are to be improved. Thus, the issues that G20 countries must tackle go beyond health and include social and environmental matters, too. The world expects the G20 countries to play an important role in achieving those outcomes.

Acknowledgements

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Challenges to eliminating the AIDS pandemic in China

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Abstract: Based on data from the Chinese Center for Disease Control and Prevention (CDC), there were a total of 861,042 people with human immunodeficiency virus (HIV)/ immune deficiency syndrome (AIDS) as of December 31, 2018 in China, a total of 148,589 new HIV infections, and 38,134 AIDS-related deaths in the year 2018. As of 2017, only 74% of people living with HIV knew their status, 80% of people living with HIV were receiving treatment, and 91% were virally suppressed in China. Although mankind has made great progress in the fight against AIDS in recent years, the vision of ending the AIDS epidemic still faces many challenges in China. Due to the huge population and the imbalance in the prevalence of HIV/AIDS in China, expanding HIV screening and early detection remains the key to China's response to HIV. Limitations of antiviral therapy (ART), rejection or discontinuation of an immediate ART strategy by people infected with HIV, and the difficult search for a cure for AIDS all limit the coverage and quality of treatment. The high price of drugs and lack of vaccines present enormous challenges; social discrimination still exists, and participation by non-governmental organizations in prevention, treatment, and care is limited. As part of the future response to HIV, HIV eradication programs should continue to be explored, and attention should be paid to long-term care for people living with HIV.

***Keywords*:** HIV/AIDS, diagnosis, treatment, prevention, social support

Introduction

Due to complexity and lethality of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), there were globally a total of 36.9 million people living with HIV and 1.7 million people newly infected with HIV in 2018 (*1*). On February 5, 2019, the United States announced the goal to end the HIV epidemic in the United States within 10 years. The US Department of Health and Human Services (HHS) then proposed a new initiative to address this ongoing public health crisis with the goal of first reducing the number of incident infections in the United States by 75% within 5 years and then by 90% within 10 years (*2*). The initiative features strategies in 5 areas (Table 1). In recent years, mankind has made great progress in the fight against AIDS, but the vision of ending the AIDS epidemic still faces many challenges in terms of HIV screening, treatment, prevention, and social support.

Outbreaks and characteristics in China

Based on data from Chinese Center for Disease Control and Prevention (CDC), there were a total of 861,042 people with HIV/AIDS as of December 31, 2018 in China, a total of 148,589 new HIV infections, and 38,134 AIDS-related deaths in the year of 2018 (*3-4*).

The epidemiological characteristics of HIV/AIDS in China are as follows: *i*) AIDS remains prevalent at a low level nationwide, with higher prevalence in some areas and groups; *ii*) The number of people living with HIV/AIDS continues to increase (Figure 1) (*3-10*), with the prevalence of AIDS differing widely in different groups; *iii*) Sexual transmission is the most prevalent method of transmission, and sexual transmission between men has increased markedly.

Given these circumstances, a comprehensive strategy for prevention, drug treatment, and even a functional cure will be crucial for curbing the HIV epidemic in China in the coming days.

Challenges of eliminating the AIDS epidemic

Key issue of HIV Screening

Based on data from the 5th National Conference on HIV/AIDS, as of 2017, only 74% of people living with HIV knew their status, 80% of people living with HIV were receiving treatment, and 91% were virally

Table 1. The plan for ending the HIV epidemic in the US*

Key strategies	Content
Diagnose	Implement routine testing during key healthcare encounters and increase access to and options for HIV testing.
Treat	Implement programs to increase adherence to HIV medication, help people get back into HIV medical care, and research innovative products that will make it easier for patients to access HIV medication.
Protect	Implement extensive provider training, patient awareness, and efforts to expand access to PrEP.
Respond	Ensure that states and communities have the technological and personnel resources to investigate all related HIV cases to stop chains of transmission.
HIV HealthForce	A boots-on-the-ground workforce of culturally competent and committed public health professionals that will carry out HIV elimination efforts in HIV hot spots.

*Generalized from the article "Ending the HIV epidemic: A plan for the United States" by Fauci *et al*. (*2*).

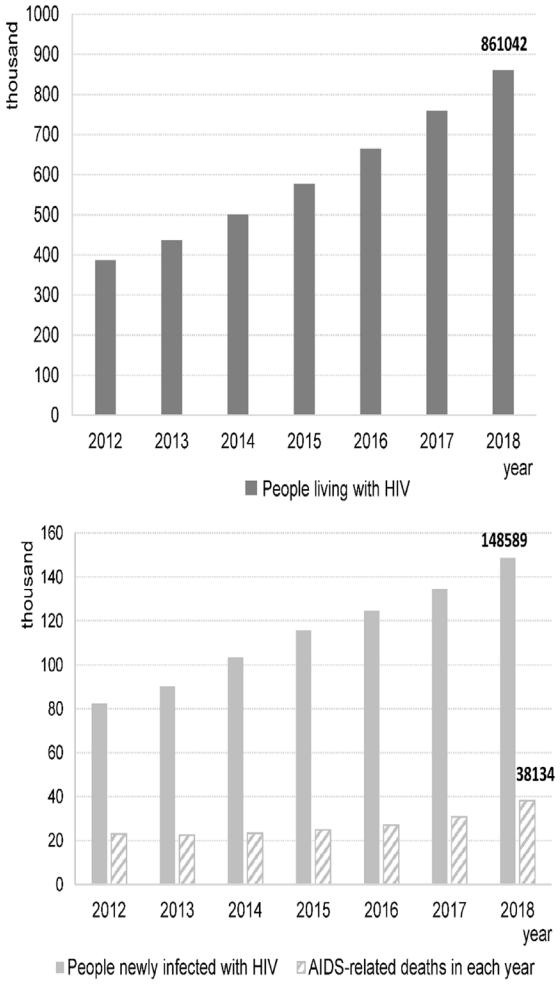

Figure 1. HIV/AIDS epidemics in China annually from 2012 to 2018. There were a total of 861,042 people living with HIV as of December 31, 2018, 148,589 people newly infected with HIV, and 38,134 AIDS-related deaths in 2018 in China. New HIV infections have steadily increased in China over the past few years. Data sources: Chinese Center for Disease Control and Prevention (*3-10*).

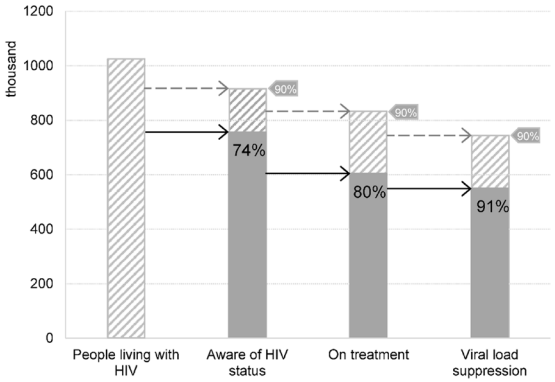

Figure 2. The status of "90-90-90" targets in 2017 in China. As of 2017, only 74% of people living with HIV knew their status, 80% of people living with HIV were receiving treatment, and 91% were virally suppressed in China. HIV screening still has difficulty preventing and controlling HIV/AIDS. Data source: The 5th National Conference on HIV/AIDS (*11*).

suppressed in China (*11*) (Figure 2). HIV screening still has difficulty preventing and controlling HIV/AIDS.

A fact worth noting is the sudden increase in new HIV infections in 2018, and this was closely related to the expansion of HIV testing in China (*12-13*). China has implemented an expanded HIV testing strategy, with the number of people tested increasing from 100 million in 2012 to 200 million in 2017 (*14*). However, expanding HIV screening and early detection remain the key to China's response to HIV because of the country's huge population and the imbalance in the prevalence of HIV/AIDS in China (*15*). Most of the people infected with HIV were found passively, and many of them had entered the middle and late stages of the disease, precluding the possibility of early treatment. Moreover, some people who are infected still do not know their infection status and have not taken the initiative or are unwilling to go to a medical facility for testing. Therefore, the current methods of detection at medical facilities cannot fully meet the goal of finding people infected with HIV as soon as possible (*16*).

Treatment quality needs to be improved

The number of people receiving ART in China

increased from 171,000 in 2012 to 610,000 in 2017, treatment coverage was 80.4% in 2017, and the success rate of treatment remained above 90% (*14*). Nonetheless, the treatment of HIV/AIDS still faces the following challenges.

Limitations of antiviral therapy (*ART*). At present, integrase inhibition has become the first-line recommendation of major guidelines worldwide, but the types of free medicines offered in China are still relatively limited. Free drugs do not include integrase inhibitors, protease inhibitors offered are only effective against Klebsiella spp., few varieties of nucleoside inhibitors are offered, and few combination therapies are offered (*17*). Coverage of medicines by health insurance will help to further improve compliance with and the willingness to receive antivirals, so such a policy might prompt development in the future.

An immediate ART strategy is rejected or discontinued by people infected with HIV. Patients are worried about the physical impact of adverse drug reactions and drug-drug interactions (DDI) (*18-22*).

Searching for a cure for AIDS. A patient in Germany, the "Berlin Patient" appeared to be free of HIV a decade ago, and two new patients Britain and Germany, the "London patient" and the "Dusseldorf patient," now also appear to be free of the virus. The two patients both received a bone marrow transplant from a donor with a CCR5 gene mutation, and these may be the second and third cases in which AIDS was "cured." These findings provide new evidence for use of hematopoietic stem cell transplantation to cure AIDS, and modification of the CCR5 gene may be a breakthrough for a functional cure for AIDS. However, points worth noting are that the CCR5Δ32 mutation is very rare and that the rate of donor and recipient matching in stem cell transplantation is already very low, so this approach has little practical value at present (*23*).

HIV Prevention is not widely used

Pre-exposure prophylaxis (*PrEP*). Although many research institutes in China have conducted pilot studies on the efficacy and safety of PrEP in high-risk HIV populations, Truvada has not been formally approved by the State Food and Drug Administration for HIV pre-exposure prevention. In addition, Truvada is currently sold at a higher price in China and it is unlikely to be self-financing because the medication would need to be affordable for long-term use. Moreover, the best way to prevent HIV infection with Truvada would be to combine it with regular checkups and drug-related counseling and education. However, HIV infection is still highly stigmatized in China, and people infected with HIV are still seriously discriminated against in all aspects of life and work, which may lead to the failure of effective screening and counseling related to PrEP. Therefore, PrEP in China is still in its infancy and pilot stage, and it still faces enormous challenges in terms of its use and subsidized cost.

Development of AIDS vaccines. Since the discovery of HIV, researchers from around the world have applied the previous concepts of viral vaccine development to the development of an HIV vaccine, but few vaccines are able to progress to clinical trials. Even if they progress to clinical trials, they fail to exhibit clinical efficacy. Vaccines are crucial to HIV prevention and curing AIDS, but the research and development of suitable vaccines for HIV/AIDS still has a long way to go.

Barriers of stigma and discrimination

Stigma and discrimination are still major barriers to expansion of the scale of prevention and treatment of and care for people living with HIV in China. This is particularly true for some key populations, such as men who have sex with men (MSM), injection drug users, and sex workers. Discrimination may occur in health care settings, barring people from accessing health services, enjoying quality health care, and receiving poor treatment in educational and work settings, all of which limit access to HIV testing, treatment, and other HIV services.

Moreover, non-governmental organizations (NGOs) are still not sufficiently participating in prevention, treatment, and care. Most NGOs involved in HIV/AIDS prevention and control in China lack legal protection and have limited ability to raise funds. Their activities are mainly concentrated in the field of campaigns and education, and they mainly operate in small and medium-sized cities and rural areas.

Conclusion

As treatment programs continue to advance, HIV/AIDS has gradually evolved from an incurable disease into a controllable and treatable chronic disease. That said, a point worth remembering is that eliminating AIDS does not mean the end of HIV. If AIDS is ended by 2030, 35 million people will still be living with HIV for 30 years or longer afterwards. Sustained care and support will be required unless a cure is found. As part of the future response to HIV, HIV eradication programs should continue to be explored, and attention should be paid to long-term care for people living with HIV.

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Efficient and practical dissemination of information on viral hepatitis in Japan: an effort by the Hepatitis Information Center, National Center for Global Health and Medicine

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Abstract: One of the important missions of the Hepatitis Information Center is to disseminate information regarding liver disease. The Hepatitis Information Center, National Center for Global Health and Medicine (NCGM) has been endeavoring to ensure that reliable and up-to-date information on liver disease is accessible to all people, regardless of age, disability, and background. Described here are several initiatives with regard to the dissemination of information about liver disease including: *i*) Education tool for youth, *ii*) Conversion of materials on liver diseases into audio format for the visually impaired, and *iii*) Hepatic Disease Medical Navigation System (Hepatic Navi). Hepatic Navi is a web-based search tool that informs users of the location and other information concerning medical centers where people can be tested for the hepatitis virus for free or at reduced cost. Hepatic Navi consolidates data from 47 prefectures into one database. The system depicts data *via* an interface that can be accessed anywhere with a PC, tablet, smart phone, or mobile phone. As a result, it has become possible for anyone from anywhere to access information on hepatitis virus testing. By using Hepatic Navi, it is anticipated that general people in need feel free to access to the testing and further treatment for virus hepatitis.

***Keywords*:** Education for youth, visual impairment, Hepatic Disease Medical Navigation System (Hepatic Navi)

Introduction

If viral hepatitis is left untreated, it may progress to serious conditions such as cirrhosis and liver cancer. There are an estimated 3.0-3.7 million patients infected with the hepatitis B and/or C virus in 2000, making it one of the most common infectious diseases in Japan (*1*). The Hepatitis Information Center, National Center for Global Health and Medicine (NCGM) was established in 2008, and it has implemented various initiatives involving comprehensive measures to combat hepatitis (*2*). In specific terms, the Center's mission includes: *i*) provision of current information *via* the Internet and other media, including medical guidelines for liver disease and domestic and foreign information about hepatitis management; *ii*) provision of support for information-sharing among regional core centers, whereby the Center plays an administrative role in a committee composed of regional core centers; and *iii*) training and education for medical personnel.

Described here are the recent achievements of the Hepatitis Information Center regarding dissemination of information on hepatitis including: *i*) Education tool for youth, *ii*) Conversion of materials on liver diseases into audio format for the visually impaired, and *iii*) Hepatic Disease Medical Navigation System (Hepatic Navi).

Education tool for youth

This tool named "Basic knowledge of hepatitis for young people" is information dissemination mainly for young people using an educational approach (*3*). The purposes of the program are to inform young people (mainly junior high school students) about hepatitis, to prevent hepatitis virus infection, and to eliminate prejudice and discrimination against the infection and patients. The program involves learning by considering seven problems. These tasks can be done both in an educational setting and also at home with a parent. This web tool was created based on the outcomes of the Policy Research for Hepatitis Measures in fiscal year 2012 to 2013 (Principal Investigator: Dr. Kato, Keio University) and was posted on the website of the Hepatitis Information Center in February 2018. The tool is expected to help raise awareness, increase knowledge, and promote respect for human rights.

Conversion of materials on liver diseases into audio format for the visually impaired

This initiative is part of the efforts of the Hepatitis Information Center to provide information to people with disabilities, such as visual impairment (*4*). The "Act for Eliminating Discrimination against Persons with Disabilities" seeks to eliminate discrimination on the grounds of disability in order to create a society where all citizens respect each other. Enacted in June 2013, the law stipulates that public institutions (such as government agencies) and companies are obligated to provide "reasonable accommodations" to people with disabilities. At the Hepatitis Information Center, efforts were made in collaboration with other organizations to ensure information on liver disease is accessible to the visually impaired. Specific materials are provided on the Hepatitis Information Center website for the visually impaired. This website was originally created by a research group funded by a Grant-in-Aid for Scientific Research (B; Principal Investigator: Dr. Yamaki, National Cancer Center Japan).

The following materials are provided in audio format for the visually impaired:

(*i*) Disease information (acute hepatitis, hepatitis B, hepatitis C, nonalcoholic fatty liver disease, alcohol-related liver disease, cirrhosis, liver cancer, and other liver diseases);

(*ii*) Illness and treatment counseling (information on counseling centers for liver disease at regional core centers for treatment of liver disease);

(*iii*) Information on reducing the cost of medical treatment (hepatitis treatment medical cost subsidy system);

(*iv*) Benefits for people infected through specific causes (benefits for patients with hepatitis B or hepatitis C);

(*v*) Guidelines to preventing the spread of viral hepatitis in daily life (*5*);

(*vi*) Information about mother-to-infant transmission of the hepatitis B virus (*6*);

(*vii*) Education tool for youth (as described above).

Hepatic Disease Medical Navigation System (Hepatic Navi)

In Japan, the cost of testing for the hepatitis virus has been fully or partially subsidized by national and local governments since 2008. From July 9, 2018, the Hepatitis Information Center provides a new web-based search tool which aids people to find approximately 30,000 medical institutions (regional core centers, specialized institutions, public health centers and contracted medical institutions) where they take government-supported virus hepatitis testing (*7*). For the establishment of Hepatic Navi, in cooperation with the Ministry of Health, Labor and Welfare, local government provided information of such institutions. Since November 29, 2018, it also became possible to search by Hepatic Navi for medical institutions that can support liver cancer and severe cirrhosis patients enforced by the Ministry of Health, Labor and Welfare. Hepatic Navi prioritizes security measures, such as performing a penetration test before publication, adopting SSL encryption for all communications, and a propriety analysis of visitor access. Cesium is used as the map engine (*8*). The map engine was developed by the laboratory of Dr. Hidenori Watanabe at the University of Tokyo.

Some important aspects of this system are the following:

(*i*) Format creation: Contains data related to medical institutions for hepatitis testing in each prefecture. All such data have been compiled into one database in Japan for the first time;

(*ii*) Visualization of data: In the form of map data, information related to medical institutions for hepatitis testing and general information on liver disease is accessible to all people at all times *via* a personal computer, tablet, smart phone, or mobile phone of any model;

(*iii*) Data linkage: Linkage of data are executed using map information from the Geospatial Information Authority of Japan. This includes measures that take into consideration the effective use of domestic resources;

(*iv*) Raising awareness: Information can be transmitted to citizens directly, and at the same time, useful information can be provided to doctors, nurses, social workers, local governments, etc. throughout the country.

As of December 2018, the number of visitors to Hepatic Navi has been increasing and reached to 630,948/month (Figure 1).

Discussion

Since 2016, the Hepatitis Information Center has been conducting awareness-raising activities including

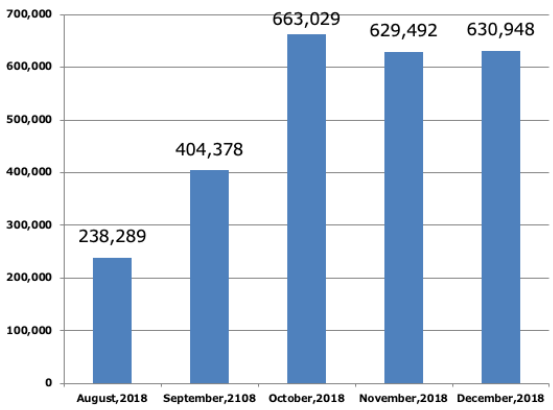

Figure 1. The number of visitors per month to the webpage of Hepatic Disease Medical Navigation System (Hepatic Navi).

for young people and people with disabilities. This approach is based on the policy that all citizens should be able to access information regarding liver disease. In this article, we reported the recently developed resources include education tool for the youth, conversion of materials on liver diseases into audio format for the visually impaired, and Hepatic Navi.

In Japan, about 7.4% of the total population has physical disabilities (4,360,000 people), intellectual disabilities (1,082,000), or mental disorders (3,924,000) (*9*). Thus, accessibility of information needs to be ensured so that the elderly and people with disabilities are able to obtain necessary information, especially in countries such as Japan where the elderly population is rapidly growing.

Hepatic Navi has consolidated data from 47 prefectures into one database for the first time.

The system depicts data *via* an interface that can be accessed with a PC, tablet, smart phone, or mobile phone, allowing anyone to access information on facilities testing for the hepatitis virus from anywhere. This tool is expected to bring the progress in spreading the measures of hepatic disease throughout the country. As for the system management, because the information of all medical institutions listed was based on public information, the data handling is relatively less strict compared with that of personal information. In the future, it may become conceptually possible to input other resource data onto Hepatic Navi, which could potentially serve as a user-friendly information platform.

The Hepatitis Information Center has been making efforts to ensure that all citizens can obtain correct information on liver diseases. In addition to Hepatic Navi, a system needs to be developed and conditions need to be improved so that people can easily access information on liver disease and information on related medical and welfare services, regardless of their disability, age, or region.

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Hepatocellular carcinoma with non-B and non-C hepatitis origin: epidemiology in Japan and surgical outcome

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Abstract: During the last two decades, there has been a dramatic increase in so-called non-B non-C hepatocellular carcinoma (NBNC HCC) in Japan. Majority of NBNC HCC are considered as so-called metabolic HCC and some could be related to occult HBV infection. Although there have been some reports on histological features predominant in metabolic HCC, very few specific driver genes for NBNC HCC have been reported. Most of the NBNC HCC are found incidentally and are relatively large in size. Since liver function is generally normal or subnormal, such patients have a higher chance for undergoing curative surgery. Although there has been slightly conflicting long-term outcomes reported for NBNC HCC, slightly better outcomes may be expected compared to other etiologies after curative surgery. However, risk of recurrence depends on the background liver. NBNC HCC in cirrhotic patients have a persistently higher risk of tumor recurrence requiring a long-term postoperative surveillance. It would be safe to conclude at this moment that NBNC HCCs should be treated using the same surgical strategy as HCCs with viral origin, same operative indications and same follow-up protocol.

***Keywords*:** non-B non-C HCC, hepatitis, epidemiology, surgical strategy, recurrence

Introduction

More than 70% of cases with hepatocellular carcinoma (HCC) in Japan have long been from hepatitis C origin (C-HCC) followed by hepatitis B (B-HCC) and other etiology. These etiological features in Japan have been very unique in East Asia where most of the HCCs are derived from hepatitis B. Since the century changed, however, the proportion of so-called non-B and non-C HCC (NBNC HCC) has dramatically increased whereas C-HCC decreased significantly and B-HCC is unchanged (Figure 1) (*1*). Acting antivirals (DAA) treatment for hepatitis C virus (HCV) was introduced with the coverage by social insurance and the special subsidy program for anti-viral treatment around 2014, and most of the infected patients in Japan have enjoyed the benefit of DAA treatment. Incidence of de novo HCC in HCV infected patients is therefore expected to decrease in the next decade.

Another significant change in HCC patient background is aging. According to nation-wide biannual HCC survey by the Liver Cancer Study Group of Japan (LCSGJ), average age of new HCC patients has been steadily increasing up to 69 years-old in the latest survey (Figure 2). Since average age of HCV derived HCC patients is one of the oldest among all etiology subgroups (*2,3*) and incidence of carcinogenesis increases by age (*4*), HCV-HCC population who benefits by curative surgery may shrink rapidly in the near future. In contrast, proportion of NBNC HCC in surgical candidates has been steadily increasing and have outnumbered HCV-HCC patients in the recent few years (Figure 3).

NASH/NAFLD as a cause of NBNC HCC

According to the INUYAMA NOBLESSE Study group report covering 5,326 NBNC HCC patients in Japan, major etiologies for NBNC HCC were alcohol (27%), nonalcoholic fatty liver disease (NAFLD) (11%) followed by primary biliary cholangitis (PBC) (6%) and autoimmune hepatitis (AIH) (6%) (*5*). However, more than half (54%) of the cases has an unclassified etiology and many of them could be so-called metabolic HCC related to metabolic syndrome including diabetes mellitus, central obesity, hypertension, and dyslipidemia. Globally speaking, prevalence of obesity has not been relatively high in Japan with median

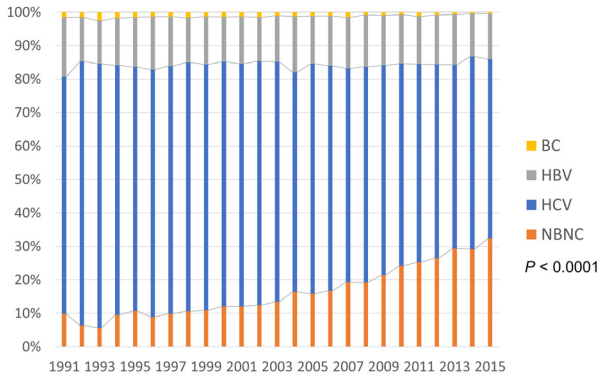

Figure 1. Trend in background liver disease for HCC in Japan (*1*).

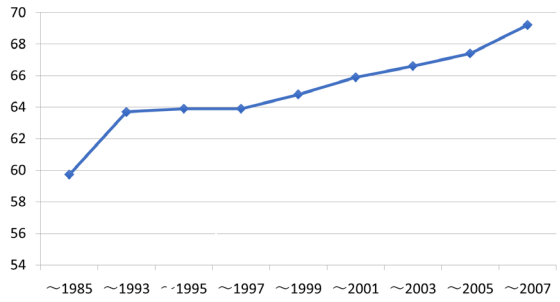

Figure 2. Average age of HCC patients diagnosed in Japan (Nation-wide survey by Japanese Study Group of Liver Cancer 1984-2007).

body mass index (BMI) under 25 in both sexes (*6*). However, because of recent Westernization of food and other habits, increasing obesity and metabolic related diseases (metabolic syndrome) have been recent issues in Japan especially in males. Actually, prevalence of NAFLD jumps up when BMI exceeds 26 (*6*). Thirty to 40 % of Japanese males are estimated to have NAFLD and fibrosis may progress very slowly in approximately 30% of cases (*7*). Once cirrhotic liver is established, annual HCC development is estimated to be around 2%.

Occult HBV infection as a cause of NBNC HCC

Another important etiology for NBNC HCC is so-called occult HBV. Occult HBV or history of previous HBV infection is defined by positive anti-hepatitis B core antibody (HBcAb) with negative hepatitis B surface antigen (HBsAg). Although HBcAb is not routinely tested in daily practice in most of the specialized institutions for HCC in Japan, occult HBV infection may consist of 30-40% of NBNC HCC in Japan (*5*). It is well known that even a history of HBV infection, *i.e.* positive HBcAb and negative HBsAg, can be a risk factor for HCC among patients with negative serology results for active HBV infection (*8-10*). Since 2002, we have started to routinely test HBc antibody for all patients undergoing liver resection at the University

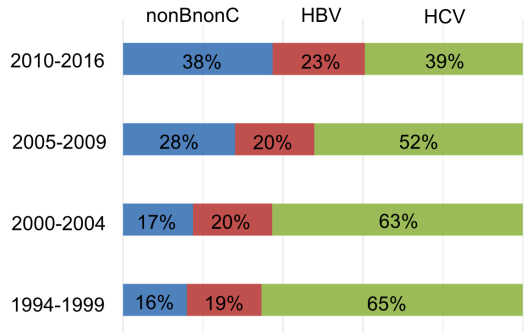

Figure 3. Etiological trend in HCC patients undergoing liver resection at the University of Tokyo (1994-2016).

of Tokyo Hospital. Incidence of occult HBV infection among surgical candidates was 29.0% (45/155) (*2*).

Genomic study

Although various etiologies and the number of gene signatures that have been identified recently (*11*), the global landscape of the genetic changes in HCC genomes underpinning different epidemiological backgrounds still remains uncharted (*12*). Recent HCC genome sequencing research covering 503 liver cancer genomes uncovered 30 candidate driver genes and 11 core pathway modules. They found that telomerase reverse transcriptase (TERT) is a central driver gene and promising molecular target. Furthermore, they identified frequently mutated genes including TP53 and CTNNB1 (b-catenin) (*13*). The Cancer Genome Atlas (TCGA) projects have revealed that HCC contains intra- and inter-tumor heterogeneity with numerous passenger mutations (*14*). It is thus suggested that different combinations of mutations contribute to the development of HCC. There are very few altered genes specific to NBNC HCC. They only reported that ARID1A mutation was more frequent while AXIN1 mutation was infrequent in NBNC HCC genomes. At present, there are no molecular targeted agents specifically effective to NBNC HCC. By using genome-wide random mutagenesis with Sleeping Beauty transposons, Kodama *et al*. reported that Sav1 is one of the candidate driver genes in steatohepatitis-based HCC (*15*). Integrated analysis, covering exome, transcriptome, methylome and high-throughput screens shed light on inhomogeneous profiles of individual HCC nodules in the whole liver, resulting in the establishment of next-stage etiology-specific molecular targeted therapy against HCC.

Steatohepatitic HCC

Steatohepatitic HCC (SH-HCC) is a recently established histological subcategory of HCC associated with the patient's metabolic condition and the presence of steatosis or steatohepatitis in the background

liver. Histological diagnostic for SH-HCC is made when the tumor fulfills four of the following five criteria: steatosis (> 5% tumor cells), ballooning or Mallory–Denk body formation, interstitial fibrosis and inflammatory infiltrates (*16,17*). SH-HCC is not equal to either NBNC-HCC, metabolic HCC, or NAFLD derived HCC. However, patients with SH-HCC were characterized by a higher frequency of diabetes mellitus and hypertension, along with higher serum levels of cholesterol and triglycerides, than those with conventional HCC (*18*). The background liver of SH-HCC patients showed steatosis and steatohepatitis more frequently. Although there has not been sufficient evidence to conclude prognostic features of SH-HCC, so far, there seems to be no significant differences compared to other histological types of HCC.

Surgical strategy for NBNC HCC

Surgical indications for NBNC HCC have been essentially the same as that for HCC with other etiologies. R0 resection with negative surgical margin is a basic strategy and anatomic resection is recommended whenever possible because of potential eradication of micrometastases in the tumor-bearing portal territory (*19*). Substantial surgical margin is not always achievable and because of the non-invasive and well-encapsulated nature of HCC, minimal or zero surgical margin without tumor exposure is considered acceptable with a non-inferior surgical outcome (*20*). Safety limit of liver resection is determined by the degree of liver functional reserve represented by the presence of ascites and jaundice, and the indocyanine green (ICG) retention test. These criteria are known as so called "Makuuchi criteria" and they assure a very low surgical mortality (Figure 4) (*21,22*).

Typical recent cases

Case 1: A 67-year-old male on a routine medical checkup and chest X-ray examination revealed an elevation of right diaphragm. Subsequent CT showed a tumor, 10 cm in diameter, in the right liver (Figure 5A). He was diabetic (HbA1c: 9.7%) and hypertensive. Alfa fetoprotein (AFP) was normal, but DCP (PIVKA-II) was 3,517 mAu/mL. HBcAb was negative. Child-Pugh score 5 and ICG retention rate at 15 min (ICG-R15) was 6.8%. He underwent right hepatectomy. Pathological findings were: Moderately to poorly differentiated hepatocellular carcinoma, 11 × 8 × 7 cm, eg, fc(-), sf(+), vp0, vv0, b0. Background liver was normal. He is fine with no evidence of disease for 21 months.

Case 2: A 72-year-old female was diagnosed to have a renal cyst and follow-up US detected a liver tumor. AFP was 9,303 ng/mL and DCP (PIVKA-II) was 672 mAu/mL. HBcAb was negative. A contrast enhanced CT revealed a 6 cm mass in the caudate lobe (Figure 5B). She had a history of hypertension and hyperlipidemia. She underwent left hepatectomy. Pathological findings were: Well- to moderately differentiated hepatocellular carcinoma, 6.7 × 5.5 × 4.9 cm, eg, fc(+), sf(+), vp0, vv0, b0. Background liver was mild fibrosis (F1). She is fine with no evidence of disease for 11 months.

Case 3: A 68-year-old male had a history of hypertension and hyperlipidemia. He suddenly developed severe abdominal pain and he was transferred by ambulance to ER. An emergent contrast enhanced CT revealed a large enhancing mass, 17 cm in diameter, in

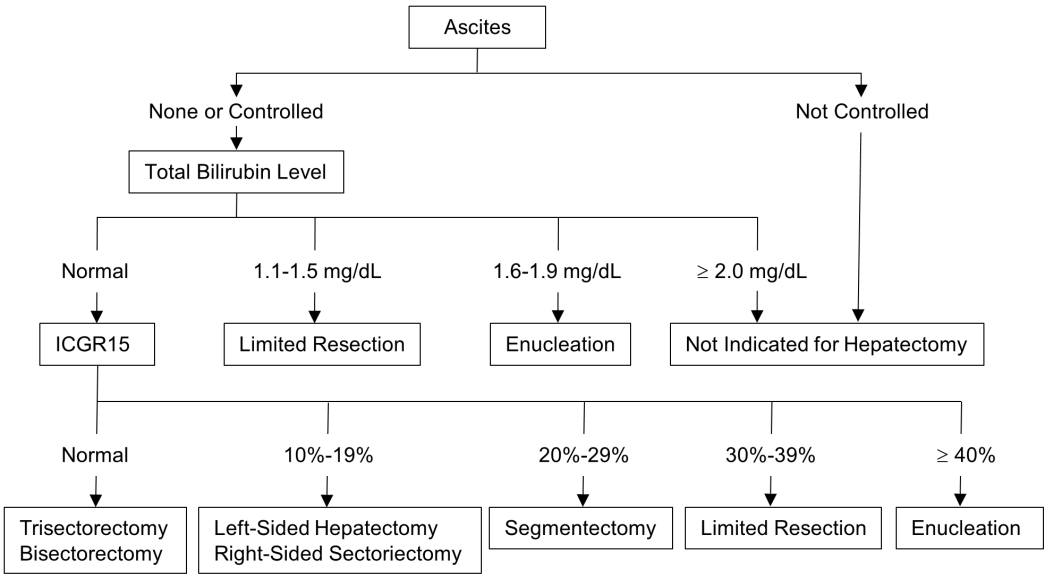

Figure 4. Makuuchi Criteria: Decision criterion for selection of operative procedures in patients with impaired liver function reserve. To convert total bilirubin from milligrams per deciliter to micromoles per liter, multer, multiply by 17.1 ICG15 indicates indocyanine green retention rate at 15 minutes (*21,22*).

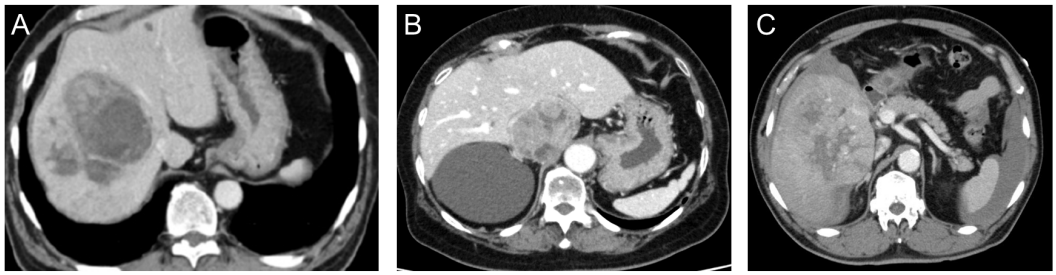

Figure 5. Contrast enhanced CT. (A), Case 1 showing a large HCC with mosaic pattern; **(B)**, Case 2 showing a 6 cm sized tumor in the caudate lobe; and **(C)**, Case 3 showing a large HCC in the right liver and intra-abdominal fluid collection.

the right liver and intraabdominal fluid collection (Figure 5C). AFP was 4,237 ng/mL and DCP (PIVKA-II) was 111,669 mAu/mL. Rupture of an HCC was suspected and he underwent TACE followed by right hepatectomy with a 3-day interval. Pathological findings were: Moderately differentiated hepatocellular carcinoma, 11.8 × 10.5 × 8.0 cm, eg, fc(+), sf(+), vp0, vv1, b0. Background liver was chronic hepatitis with mild fibrosis (F1, A2). He is fine with no evidence of disease for 8 months.

Short-term outcome after liver resection

Operative morbidity or mortality depends on liver function and comorbidities. Since NBNC HCC patients are frequently associated with obesity and so-called metabolic diseases including diabetes mellitus, central obesity, hypertension, and dyslipidemia, presence of such comorbidities may affect short-term outcomes. Yoshida *et al*. reported significantly higher incidence of postoperative complications in patients with metabolic HCC compared with those with cryptogenic HCC (40.0 *vs*. 22.7 %, $p = 0.049$) (Yoshida (*23*)). Several authors in the US have reported a near two-fold increased risk of complications among patients with obesity and metabolic HCC (Bhayani (*24*), Mathur (*25*), Pawlik (*26*)). As a basic perioperative management, all comorbidities in NBNC HCC patients should be precisely evaluated and controlled in a multi-discipline approach.

Long-term outcome after liver resection

Although there have been a number of reports on the long-term outcome of NBNC HCC or metabolic HCC, whether the long-term outcome of such HCCs is better than or comparable to that of HCC with other etiologies has not been conclusive (Wakai (*27*), Reddy (*28*), Kaneda (*29*), Yoshida (*23*), Vigano (*30*)). These discrepancies in the literature may be because of differences in background liver and in the etiologies of the control groups.

According to a nation-wide survey in Japan covering 57,450 HCC patients (Jan. 2000 to Dec. 2005), 9,307 patients were identified as NBNC HCC. Occult HBV infection was not excluded because HBc Ab was not routinely tested in most of the centers. After excluding those with extrahepatic disease or Child-Pugh C status and those undergoing other treatments, 4,741 NBNC HCC patients underwent the 3 major treatments, liver resection (HR: 2,827 cases) Radiofrequency ablation (RFA: 432 cases), and trans-arterial chemoembolization (TACE: 1,437 cases). As expected, the degree of liver damage in the HR group was significantly lower than that in the RFA and TACE groups. On the other hand, the HR and TACE groups had significantly more advanced tumors than the RFA group. The 5-year survival rates after HR, RFA, and TACE were 66%, 49%, and 32%, respectively. Stratifying the survival rates, according to the TNM stage and the Japan Integrated Staging (JIS) score (Kudo (*31*)), showed the HR group to have a significantly better long-term outcome than the RFA group in the stage II and in the JIS scores "1" and "2." Figures 6 A and B shows comparisons of overall survival according to treatment modalities in stage II with multiple tumors (A) or in stage II with > 2 cm tumors (B). HR offered significant prognostic advantages over TACE and RFA (Utsunomiya 2014 (*3*)).

Using the same national registry data, patient outcomes after HR was analyzed according to the etiology of HCC (Utsunomiya 2015 (*32*)). Of the 11,950 HCC patients undergoing HR, 2,194 were HBV derived HCC, 7,018 were HCV derived HCC, and 2,738 were NBNC HCC. Patients with both HBV and HCV infection ($n = 309$) were excluded. Liver function in the HCV positive group was significantly worse than that in the HBV positive and NBNC HCC groups. The NBNC HCC group had significantly more advanced disease than other groups probably because they are not on the surveillance program. The 5-year overall survival rates after HR in the HBV-HCC, HCV-HCC, and NBNC HCC groups were 65%, 59%, and 68%, respectively. The 5-year recurrence-free survival (RFS) rates in these 3 groups were 41%, 31%, and 47%, respectively (Figures 6 C, D). After stratification according to the TNM stage, the NBNC HCC group had a significantly better RFS than the HBV HCC group in stages II, III, and IVA, and significantly better than the HCV-HCC group in stages I and II. Multivariate analysis revealed a significantly

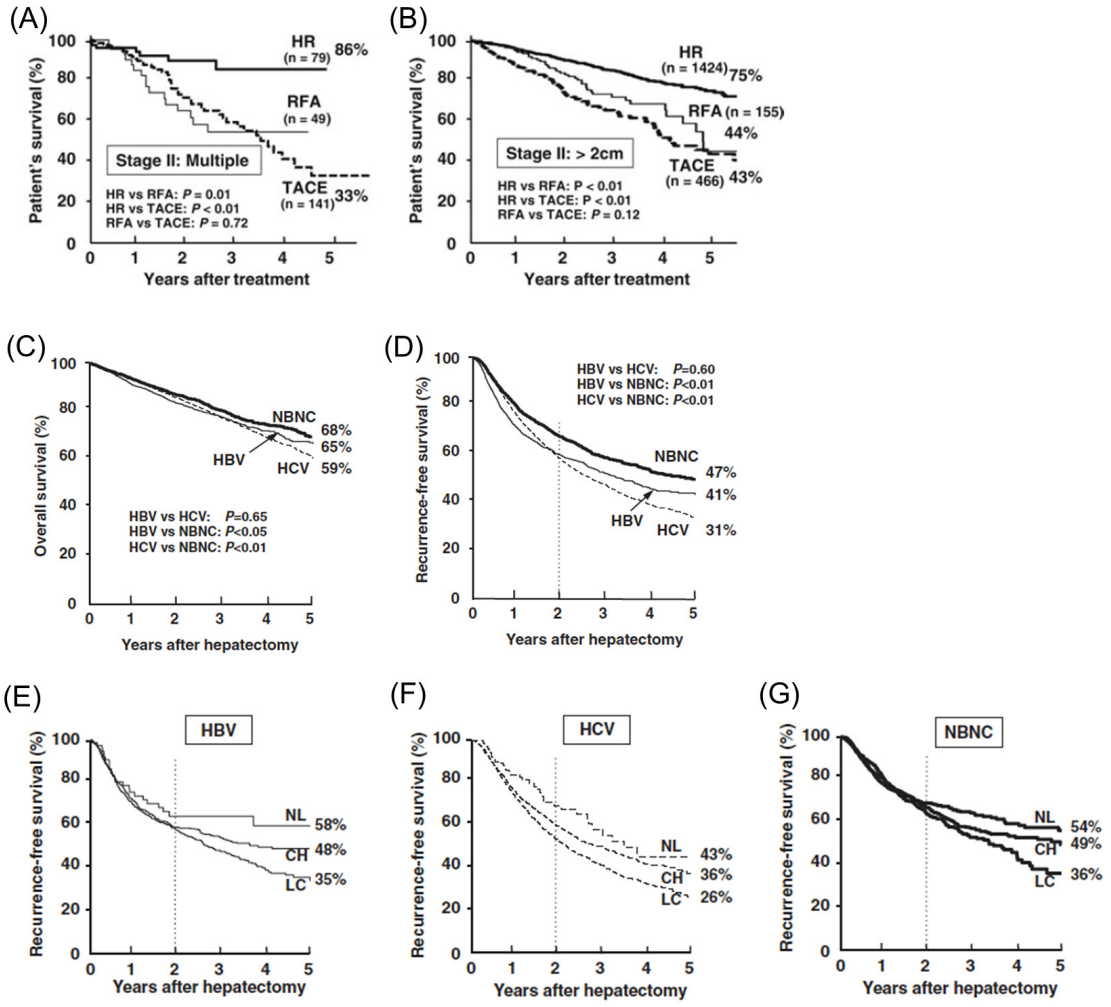

Figure 6. (A,B), OS for NBNC HCC (multiple or > 2cm) according to treatment options in Japan; (C,D), OS and RFS after liver resection for HCC according to viral status in Japan; (E,F, and G), Impact of background liver on RFS after liver resection for HCC according to viral status in Japan (Nationwide survey by Japanese Study Group of Liver Cancer 2000-2005) (*3,32*).

better RFS in the NBNC HCC group. They concluded that patients with NBNC HCC had a significantly lower risk of tumor recurrence than those with HBV and HCV derived HCC. However, tumor recurrence significantly depended on the histology of background liver regardless of the etiology. RFS curves of NBNC HCC in cirrhotic liver persistently went down to 36% at 5 years suggesting the need for long-term surveillance after resection (Figure 6 E, F, G).

As mentioned above, the national registry data do not include information on HBc Ab status to differentiate occult HBV infection. Omich reported a single center series of resectable NBNC HCC including this important information (Omichi (*2*)). They have compared outcomes of curatively resected HCC according to viral status, HCV (n = 260), HBV (n = 111), occult HBV (Positive HBc-Ab, n = 45), or pure NBNC HCC (n = 110). Long-term outcomes (OS and RFS) of pooled NBNC HCC (pure NBNC plus occult HBV) groups was not significantly different from those for HCV or HBV groups. However, OS and RFS for positive Hbc-Ab NBNC HCC (occult HBV) was significantly better than that of HCC with other etiologies including pure NBNC (Figure 7A, B). The positive Hbc-Ab NBNC HCC group had slightly favorable prognostic factors, *i.e.* higher proportion of well-differentiated HCC, a lower level of AFP, and a globally better functional reserve. Another reason for better outcomes for the positive Hbc-Ab NBNC HCC (occult HBV) would be very low (trace level) viral load compared to that for the HBV HCC group, because it is well-known that HBV viral load itself affects long-term outcome of HBV derived HCC (Yeo (*33*), Zhou (*34*)). Since serum HBV DNA data were not available, reasons for the better outcome for positive Hbc-Ab NBNC HCC (occult HBV) warrants further investigation. However, it would be safe to conclude that NBNC HCC patients (especially pure NBNC) are persistently at higher risk of recurrence for at least 10 years even after curative resection, thus requiring life-long surveillance.

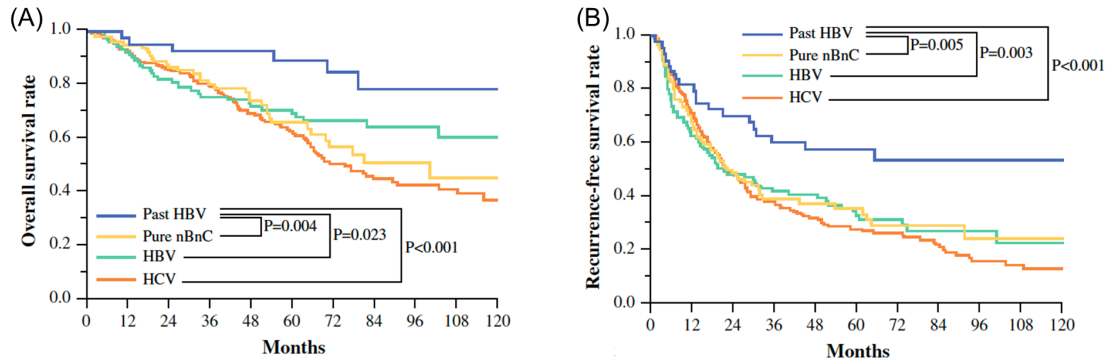

Figure 7. OS (A) and RFS (B) after liver resection for HCC according to viral status at the University of Tokyo (2002-2010) (*2*).

Conclusion

In conclusion, NBNC HCC is increasing in Japan particularly in surgical candidates. The majority of NBNC HCC in Japan are considered as so-called metabolic HCC and some could be related to occult HBV infection. Most of the NBNC HCC are found incidentally and are relatively large in size. Liver function is generally normal or subnormal thus providing a higher chance for curative surgery. Slightly better long-term outcomes may be expected compared to other etiologies, however, risk of recurrence depends on background liver. So far, NBNC HCCs should be treated with the same surgical strategy as HCCs of viral origin, same operative indications and same follow-up protocol.

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Diabetic dyslipidemia: evaluation and mechanism

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Abstract: Diabetes is one of the well-established independent risk factors for cardiovascular diseases. Diabetes induces dyslipidemia which is characterized by elevated fasting triglyceride (TG) and reduced high-density lipoprotein-cholesterol (HDL-C), and such diabetic dyslipidemia is a crucial determinant for atherogenesis and atherosclerotic progression in patients with diabetes. Previous measurement methods of lipoproteins have problems including time-consuming (ultracentrifugation) and inaccurate and impossible measurements of TG-rich lipoproteins such as chylomicron, intermediate-density lipoprotein (IDL) and very low-density lipoprotein (VLDL). Our developed anion-exchange high-performance liquid chromatography (AEX-HPLC) can measure all fractions of lipoproteins accurately. Our studies using AEX-HPLC showed that IDL and VLDL in type 2 diabetes were higher than non-diabetic subjects, and IDL and VLDL were higher in the order of type 2 diabetic patients with obesity, type 2 diabetic patients without obesity, and non-diabetic subjects. Here, we also describe the underlying mechanisms for development of diabetic dyslipidemia.

***Keywords*:** Diabetes, high-performance liquid chromatography, insulin resistance, lipoproteins, triglyceride

Introduction

Diabetes is one of the well-established independent risk factors for cardiovascular diseases (*1*). Diabetes induces atherosclerosis and resulting atherosclerotic diseases such as cerebral vascular diseases, ischemic heart diseases, and peripheral arterial diseases, which are major causes of death in patients with diabetes and significantly reduce their quality of life (*2-4*). Although various factors such as hyperglycemia are involved in progression of atherosclerosis in diabetes, diabetic dyslipidemia may be a crucial determinant for atherogenesis and atherosclerotic progression in patients with diabetes.

Diabetic dyslipidemia is characterized by elevated fasting triglyceride (TG), reduced high-density lipoprotein-cholesterol (HDL-C), and elevated low-density lipoprotein-cholesterol (LDL-C) and small dense LDL (*5*). Here, we show the characteristics of diabetic dyslipidemia, which have been demonstrated by our developed anion-exchange high-performance liquid chromatography (AEX-HPLC) (*6*). We also discuss the underlying mechanisms for development of diabetic dyslipidemia.

The measurement of lipoproteins in patients with diabetess

Profiles of serum lipoproteins

Profiles of serum lipoproteins are shown in Figure 1 (*7*). Lipoproteins are classified by density, which is determined mainly by content of TG. The particle sizes of chylomicron (CM) and very low-density lipoprotein (VLDL) are relatively large, however, densities of such lipoproteins are low due to high content of TG. CM, VLDL and intermediate-density lipoprotein (IDL) are called TG-rich lipoproteins, which are increased in type 2 diabetes, insulin resistance and obesity. Studies of lipoprotein/arterial wall interactions have demonstrated that the larger the lipoprotein particle, the lower the influx into intima. Therefore, CM are unlikely to induce atherosclerosis because CM do not seem to enter intima (*8*). Among TG-rich lipoproteins, IDL and VLDL are important for atherogenesis. LDL is a cholesterol-rich lipoprotein, which is a well-established atherogenic lipoprotein (*9*). HDL contains small amounts of TG and cholesterol, and plays an important role in anti-atherogenesis by reverse cholesterol transport. The incidence rates of coronary heart disease and definite myocardial infarction were three to four times higher in the lowest HDL-C quartile (< 1.24 mmol/L) than the highest quartile (≥ 1.66 mmol/L), and there was a significant dose response for definite myocardial infarction (*10*).

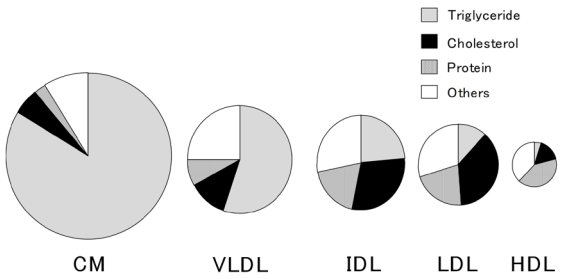

Figure 1. Profiles of serum lipoproteins. We made this figure by modification of figure, which we previously made in reference 7.

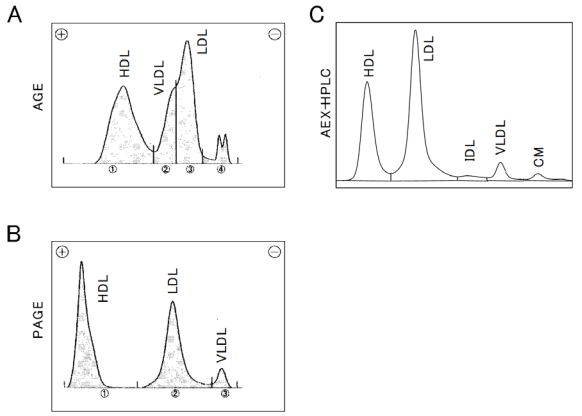

Figure 2. Results of measurement of the same sample by agarose gel electrophoresis (AGE), polyacrylamide-gel electrophoresis (PAGE) and anion-exchange high-performance liquid chromatography (AEX-HPLC). CM, chylomicron; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

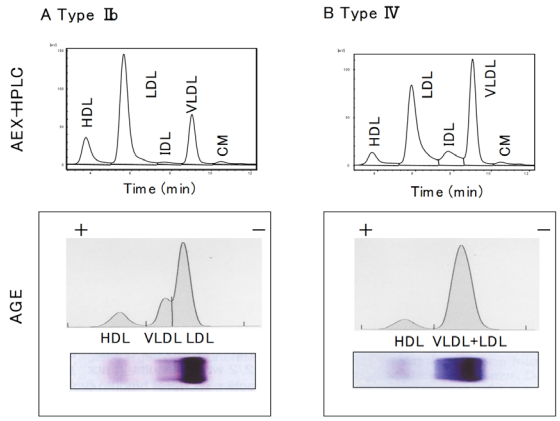

Figure 3. Results of measurement of serum of patients with hyperlipoproteinemia type IIb and type IV by agarose gel electrophoresis (AGE) and anion-exchange high-performance liquid chromatography (AEX-HPLC). CM, chylomicron; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

Excellence of AEX-HPLC as compared with previous methods to measure lipoproteins

Various methods for analysis of lipoproteins by ultracentrifugation (*11-13*), electrophoresis (*14-16*), gel-permeation chromatography (*17,18*), and anion-exchange chromatography (*19*) have been reported. The cholesterol levels of all major classes of lipoproteins in serum can be measured by ultracentrifugation, but it takes a long time to complete the analysis (*11-13*). The other methods have poor ability to measure IDL -C levels (*14-19*).

Very recently, in measurements of lipoprotein fraction, AEX-HPLC became commercially available, in addition to agarose gel electrophoresis (AGE) and polyacrylamide-gel electrophoresis (PAGE), in Japan. Results of measurements of the same sample by AGE, PAGE and AEX-HPLC are shown in Figure 2. AGE and PAGE could not measure CM and IDL, and AGE could not distinguish between LDL and VLDL, however, AEX-HPLC could accurately measure all fractions of lipoproteins.

Diabetic dyslipidemia is characterized by elevated TG and LDL-C, and reduced HDL-C (*5*). Therefore, hyperlipoproteinemia type IIb and type IV (WHO classification) are commonly observed in patients with diabetes (*20*). Results of measurements of serum of patients with hyperlipoproteinemia type IIb and type IV by AGE and AEX-HPLC are shown in Figure 3. AGE could not measure CM and IDL, and could not distinguish between LDL and VLDL. However, AEX-HPLC could distinguish and measure all fractions of lipoproteins in patients with hyperlipoproteinemia type IIb and type IV.

The characteristics of lipoproteins in patients with diabetes

We compared lipoprotein profiles obtained by our previous studies using AEX-HPLC (*21*), which included studies using young lean men (*22*), subjects with low Framingham risk score (FRS) (*23,24*), type 2 diabetic patients without and with obesity (*25,26*) (Figure 4). HDL-C in type 2 diabetes, especially in type 2 diabetic patients with obesity was lower than young lean men and low FRS subjects. IDL-C in type 2 diabetes was higher than the other two groups, and IDL-C was higher in the order of type 2 diabetic patients with obesity, type 2 diabetic patients without obesity, low FRS subjects, young lean men. VLDL-C clearly showed higher values in the order of type 2 diabetic patients with obesity, type 2 diabetic patients without obesity, low FRS subjects, and young lean men. LDL-C and CM-C did not show a difference between patients with and without diabetes. According to the accumulation of our previous AEX-HPLC data (*22,24-26*), characteristics of diabetic dyslipidemia are reduced HDL-C, and increased IDL-C and VLDL-C, which further deteriorated due to complication with obesity.

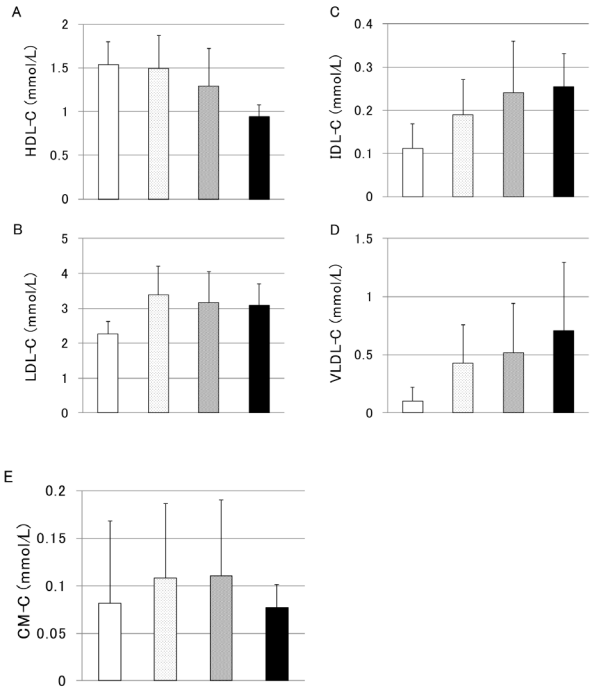

Figure 4. Serum concentration of HDL-C, LDL-C, IDL-C, VLDL-C and CM-C in young lean men (white boxes), subjects with low Framingham risk score (FRS) (dotted boxes), type 2 diabetic patients without (shaded boxes) and with obesity (black boxes).

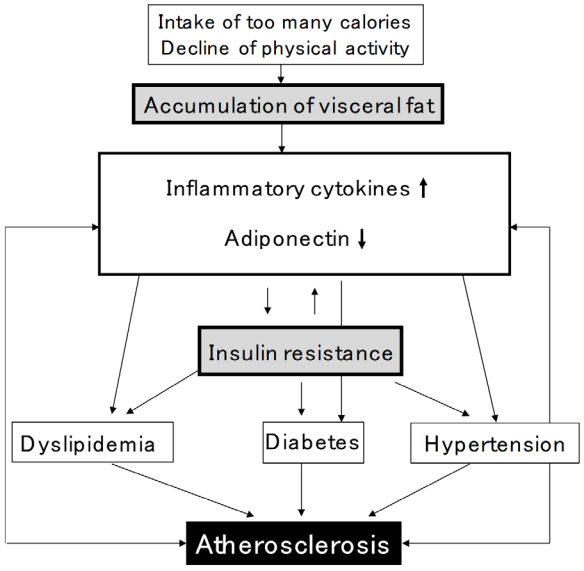

Figure 5. Pathophysiology of the metabolic syndrome. We made this figure by modification of figure in reference 27.

The mechanisms of development of diabetic dyslipidemia

A significant association of visceral obesity and insulin resistance with diabetic dyslipidemia

Pathophysiology of the metabolic syndrome is shown in Figure 5 (*27*). Accumulation of visceral fat and resulting insulin resistance may play a crucial role in inducing diabetic dyslipidemia.

A significant influence of visceral obesity on diabetic dyslipidemia

Our previous study showed a significant and positive association between serum TG levels and the ratio of visceral fat area (VFA) to subcutaneous fat area (SFA) in obese individuals (*28*). Further, our other previous studies demonstrated a significant and negative correlation between VFA and HDL-C levels and a significant and positive correlation between VFA and TG levels in patients with type 2 diabetes (*29,30*), supporting a significant influence of visceral fat on diabetic dyslipidemia.

Significance and mechanism for elevation of VLDL-C in patients with type 2 diabetes

We previously observed a significant and positive correlation between VFA and VLDL-C levels, suggesting a significant influence of visceral obesity on VLDL (*29,30*). In the accumulated analysis of AEX-HPLC studies, VLDL-C clearly showed higher values in the order of type 2 diabetic patients with obesity, type 2 diabetic patients without obesity, and non-diabetic individuals (*21*), indicating an importance of VLDL in diabetic dyslipidemia.

Our previous study showed that supervised aerobic exercise training (60 min/day, 2 to 3 times/week) markedly reduced VLDL-C at week 8 (-45%) and week 16 (-50%) with an improvement of insulin resistance, in individuals with dyslipidemia (*31*). Another study showed that the glucagon-like peptide-1 analogue (GLP-1A) used as treatment for type 2 diabetes reduced serum TG and VLDL-C with an improvement of glycemic control, in type 2 diabetic patients with obesity (*26*). In this study, reduction of TG by GLP-1A was significantly correlated with a decrease in VLDL-C (Figure 6A). Further, reduction of TG was significantly correlated with small dense LDL, which is a strong atherogenic lipoprotein (Figure 6B). Overproduction of VLDL is metabolically associated with preponderance of small dense LDL (*32*). In Japanese patients with type 2 diabetes, serum TG level was a leading predictor of coronary heart disease, comparable to LDL-C (*33*), supporting the importance of VLDL in diabetic dyslipidemia.

VLDL is the leading actor in diabetic dyslipidemia (*34*). The main mechanism for elevation of VLDL-C in patients with type 2 diabetes is shown in Figure 7. Insulin resistance increases activity and expression of hormone-sensitive lipase (HSL) in adipose tissue, which catalyzes the breakdown of TG, releasing free fatty acids (FFA) (*35*). Increased FFA entry into liver elevates hepatic production of VLDL. Insulin resistance also decreases the activity of lipoprotein lipase (LPL), the rate-limiting

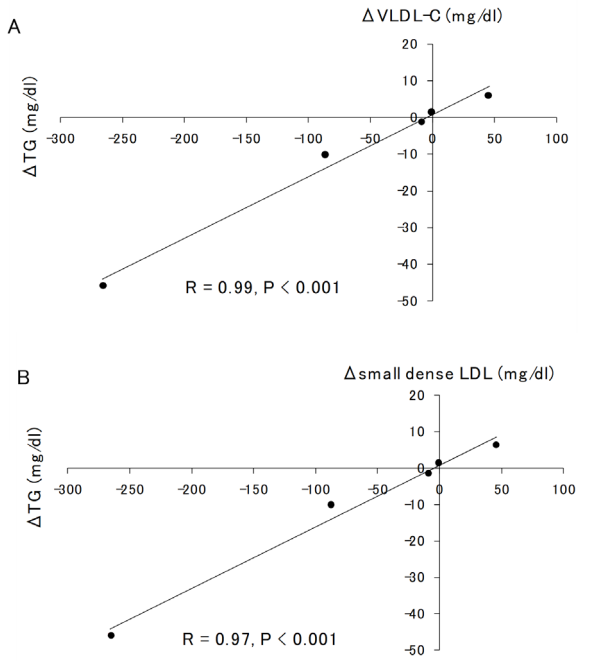

Figure 6. Correlation of changes in serum triglyceride with changes in VLDL-C and small dense LDL by glucagon-like peptide-1 analogue in patients with type 2 diabetes. Correlation coefficient was analyzed by Spearman's rank correlation.

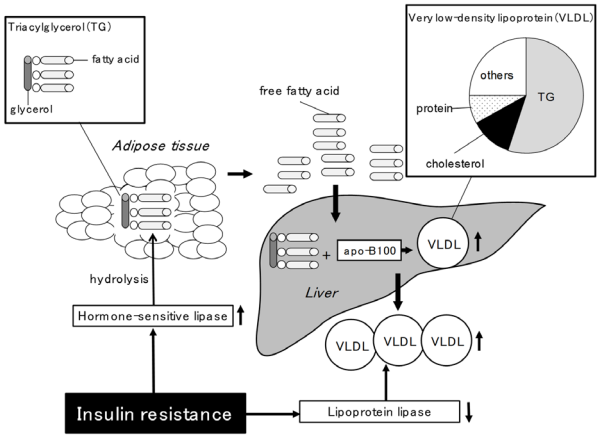

Figure 7. The main mechanism for elevation of VLDL in patients with type 2 diabetes. We made this figure by modification of figure in reference 34.

enzyme of catabolism of VLDL (*36*). Therefore, reduced LPL activity increases VLDL in diabetes.

Molecular mechanisms for development of diabetic dyslipidemia

Molecular mechanisms for development of diabetic dyslipidemia are shown in Figure 8 (*21*). Insulin resistance increases activity and expression of HSL in adipose tissue, which catalyzes the breakdown of TG, releasing FFA (*35*). Insulin promotes apoB100 degradation, and hepatic insulin resistance reduces apoB100 degradation (*37*). Insulin resistance also

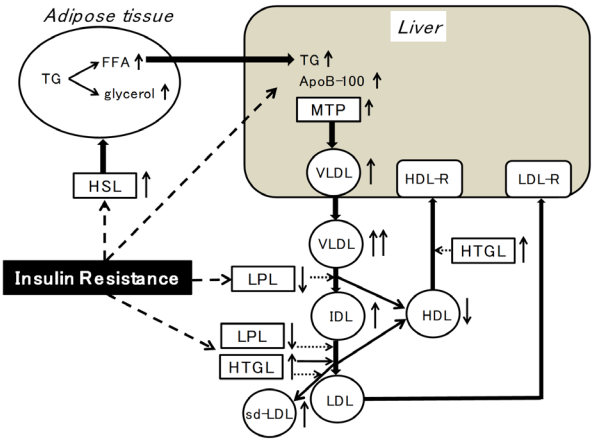

Figure 8. Molecular mechanisms for development of diabetic dyslipidemia. We made this figure by modification of figure in reference 21. FFA, free fatty acids; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; HTGL, hepatic triglyceride lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; sd-LDL, small dense LDL; TG, triglyceride; VLDL, very low-density lipoprotein.

enhances expression of microsomal TG transfer protein (MTP), a key enzyme involved in VLDL assembly (*38*). In type 2 diabetes, increased FFA entry to liver, reduced degradation of apoB100, and enhanced expression of MTP may elevate hepatic production of VLDL. Insulin resistance also decreases the activity of LPL, the rate-limiting enzyme of the catabolism of TG-rich lipoproteins such as CM, VLDL and IDL (*36*). The formation of HDL is related to the catabolism of TG-rich lipoproteins by LPL (*39*). Therefore, reduced LPL activity increases IDL and VLDL, and reduces HDL. The activity of hepatic TG lipase (HTGL), the enzyme that facilitates the catabolism of HDL, is correlated with insulin resistance (*40*). In type 2 diabetes, low serum HDL-C may be partially due to an increased rate of clearance by HTGL (*40*). LDL size and buoyancy are inversely proportional to HTGL activity (*41*), and patients with high HTGL have smaller, denser LDL particles, as compared to subjects with low HTGL activity (*42*). Increased HTGL activity due to insulin resistance/relative insulin deficiency may increase super-atherogenic lipoprotein, small dense LDL, in type 2 diabetes. Further, overproduction of hepatic VLDL is metabolically associated with a preponderance of small dense LDL and reduced large cholesterol-rich HDL (*32*).

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A novel HIV-1 protease inhibitor, GRL-044, has potent activity against various HIV-1s with an extremely high genetic barrier to the emergence of HIV-1 drug resistance

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Abstract: We designed, synthesized, and identified two novel nonpeptidic HIV-1 protease inhibitors (PIs), GRL-037 and GRL-044, containing P2-tetrahydropyrano-tetrahydrofuran (Tp-THF), P1-benzene and P1-methoxybenzene, respectively, and P2'-isopropyl-aminobenzothiazole (Ip-Abt), based on the structure of the prototypic PI, darunavir (DRV). The 50% inhibitory concentrations (IC_{50}s) of GRL-037 and GRL-044 against wild-type HIV-1_{NL4-3} were 0.042 and 0.0028-0.0033 nM with minimal cytotoxicity profiles compared to the IC_{50} values of four most potent FDA-approved PIs, ranging from 2.6 to 70 nM. GRL-044 was also potent against HIV-2_{EHO} (IC_{50}=0.0004 nM) and various PI-resistant HIV-1 variants (IC_{50} ranging from 0.065 to 19 nM). In the selection assays we conducted, the emergence of HIV-1 variants resistant to GRL-044 was significantly delayed compared to that against DRV. Thermal stability test using differential scanning fluorimetry employing purified HIV-1 protease (PR) and SYPRO® Orange showed that both GRL-037 and GRL-044 tightly bound to PR. A28S substitution emerged in the homologous recombination-based selection assays with GRL-044. Structural analyses showed that the larger size of GRL-044 over DRV, enabling GRL-044 to fit better to the hydrophobic cavity of protease, contributed to the greater potency of GRL-044 against HIV-1. Structural analyses also suggested that the van der Waals surface contact of GRL-044 with A28' appears to be better compared to that of DRV because of the larger surface of Ip-Abt of GRL-044, which may be partially responsible for the emergence of A28S. The present antiviral data and structural features of GRL-044 should provide molecular insights for further design and development of potent and "resistance-repellant" novel PIs.

***Keywords*:** Protease inhibitor, HIV-1, AIDS, drug resistance

Introduction

Combined antiretroviral therapy or cART for HIV-1 infection and AIDS has dramatically changed the clinical profiles and prognosis of HIV-1 infection/AIDS and significantly extended the life expectancy of people with HIV-1 infection/AIDS. In fact, cART has been definitely shown to reduce HIV-1 transmission at very high rates (*1*). The most recently completed 7-year PARTNER2 study has revealed no cases of HIV transmission among ~800 sero-discordant gay male couples, who engaged in condomless anal sex a total of 76,088 times while the HIV-1-infected partners were virally suppressed on cART. The study supports the message of the U=U (undetectable=untransmittable) campaign and the benefits of early testing and cART (*2*). Nevertheless, individuals who initially attain complete suppression of HIV-1 to undetectable levels may suffer treatment failure because of the flaws of cART such as inherent adverse effects and the propensity of HIV-1 to develop drug resistance. Thus, development of therapeutics for HIV-1 infection/AIDS that are more potent, less or least toxic, and do not permit or delay the emergence of drug resistance with more favorable dosing regimen capabilities is critically important (*3-6*).

In the present work, we designed, synthesized, and identified two novel nonpeptidic HIV-1 protease inhibitors (PIs), GRL-037 and GRL-044, containing (*i*) P2-tetrahydropyrano-tetrahydrofuran (Tp-THF), (*ii*) P1-benzene and P1-methoxybenzene, respectively, and (*iii*) P2'-isopropyl-aminobenzothiazole (Ip-Abt) on the structure of darunavir (DRV) (*7,8*), which is the last approved PI for the treatment of HIV-1 infection/AIDS and has been most used worldwide (*8-10*). Both GRL-

037 and GRL-044 showed potent antiviral activity against wild-type HIV-1, but GRL-044, in particular, exerted potent antiviral activity against wild-type HIV-1, HIV-2, and various drug-resistant HIV-1 variants. Structural analyses showed that the larger and "right" size of GRL-044 over DRV contributed to the greater potency of GRL-044 against HIV-1. The present data suggest that the combination of the three moieties in GRL-044 resulted in highly potent activity against wild-type and multi-PI-resistant HIV-1 strains and contributed toward its extremely high genetic barrier to the emergence of HIV-1 variants to GRL-044 compared to its prototypic PI, DRV. The antiviral properties and structural features of GRL-044 should shed lights in the design and development of more potent and "resistance-repellant" PIs with novel structural features.

Materials and Methods

Antiviral agents

Two nonpeptidic PIs, GRL-0476 and GRL-1398, and their properties were previously published (*11,12*), while GRL-037 and GRL-044 were newly designed and synthesized. The method of synthesis of GRL-037 and GRL-044 will be published elsewhere by A. K. Ghosh *et al*. DRV was synthesized as previously described (*7*). Amprenavir (APV), lopinavir (LPV), atazanavir (ATV), saquinavir (SQV), and zidovudine (AZT) were purchased from Sigma-Aldrich (St. Louis, MO).

Cells and viruses

MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal bovine serum (BenchMark™ Fetal Bovine Serum, Gemini Bio-Products) plus 50 U of penicillin and 50 µg of kanamycin per mL. The following HIV-1 strains were used for the drug susceptibility assay and *in vitro* selection experiments: eleven HIV-1 clinical strains (HIV_A, HIV_B, HIV_C, HIV_G, HIV_{TM}, HIV_{MM}, HIV_{JSL}, HIV_{SS}, HIV_{ES}, HIV_{EV}, and HIV_{13-52}), which were originally isolated from patients with AIDS who were enrolled in an open-labeled clinical study of APV and abacavir at the Clinical Center, National Institutes of Health, were randomly chosen from the enrollees, who had failed APV-plus-ABC therapy in a phase I/II study of tenofovir disoproxil fumarate (*13,14*). Such patients had failed existing anti-HIV-1 regimens after receiving 7 to 11 anti-HIV-1 drugs over the previous 24 to 83 months in late 1990s. Such clinical strains contained 8 to 16 amino acid substitutions in the protease-encoding region (Figure 4), which are associated with HIV-1 resistance to various PIs and have been genotypically and phenotypically characterized to be multi-protease inhibitor (PI)-resistant HIV-1 (*15*). Five HIV-1 variants resistant to PI that had been selected *in vitro* with each of four FDA-approved PIs (DRV, APV, ATV, and LPV) were also employed (*16,17*). All the variants used in the present study were confirmed to have acquired multiple amino acid substitutions in protease (see Table 1 footnote), which have reportedly been associated with viral resistance to PIs. Each of the PI-selected HIV-1 variants (HIV_{DRV^RP10}, HIV_{DRV^RP30}, $HIV_{APV\text{-}5\mu M}$, $HIV_{ATV\text{-}5\mu M}$, and $HIV_{LPV\text{-}5\mu M}$) was highly resistant to the corresponding PI, with which the variant was selected, and the differences in the IC_{50}s relative to the IC_{50} of each drug against wild-type HIV-1 ($HIV\text{-}1_{NL4\text{-}3}$) ranged from >14 to >303-fold (Table 1). All the HIV-1 strains used in this study were stored at -80°C until use.

Antiviral and cytotoxicity assays

Antiviral assays were conducted as previously described (*14*). Briefly, the designated concentrations of each compound tested were prepared by ten-fold serial

Table 1. Antiviral activity of GRL-037 and GRL-044 against *in vitro* PI-selected HIV-1 variants and multi-drug resistant HIV-1 clinical isolates

Virus	Mean IC_{50} (nM) ± SD							
	APV	LPV	ATV	DRV	GRL-0476	GRL-1398	GRL-037	GRL-044
$HIV\text{-}1_{NL4\text{-}3}$	70 ± 41	57 ± 3	3.3 ± 0.5	3.5 ± 0.7	4.8 ± 1.0	0.29 ± 0.05	0.042 ± 0.012	0.0033 ± 0.0024
$HIV_{APV\text{-}5\mu M}$[a]	>1,000 (>14)	390 ± 62 (6.8)	4.5 ± 0.3 (1.4)	336 ± 65 (96)	ND[c]	ND[c]	47 ± 5 (1,119)	4.5 ± 0.2 (1,364)
$HIV_{ATV\text{-}5\mu M}$[a]	>1,000 (>14)	378 ± 70 (6.6)	>1,000 (>303)	13 ± 4 (3.7)	ND[c]	ND[c]	0.44 ± 0.12 (10)	0.065 ± 0.024 (20)
$HIV_{LPV\text{-}5\mu M}$[a]	>1,000 (>14)	>1,000 (>18)	503 ± 31 (152)	409 ± 23 (117)	ND[c]	ND[c]	40 ± 3 (952)	3.8 ± 0.3 (1,152)
HIV_{DRV^RP10}[a]	>1,000 (>14)	>1,000 (>18)	>1,000 (>303)	140 ± 41 (40)	382 ± 55 (80)	45 ± 7 (154)	23 ± 4 (548)	3.3 ± 0.2 (1,000)
HIV_{DRV^RP30}[a]	>1,000 (>14)	>1,000 (>18)	>1,000 (>303)	465 ± 107 (133)	393 ± 61 (82)	173 ± 29 (598)	83 ± 18 (1,976)	19 ± 2 (5,758)
HIV_B[b]	>1,000 (>14)	>1,000 (>18)	>1,000 (>303)	40 ± 8 (11)	ND[c]	ND[c]	46 ± 5 (1,095)	4.6 ± 0.4 (1,394)
HIV_C[b]	>1,000 (>14)	>1,000 (>18)	418 ± 49 (127)	38 ± 2 (11)	ND[c]	ND[c]	5.5 ± 0.3 (131)	2.6 ± 0.8 (788)

The amino acid substitutions identified in protease of $HIV_{APV\text{-}5\mu M}$, $HIV_{ATV\text{-}5\mu M}$, $HIV_{LPV\text{-}5\mu M}$, HIV_{DRV^RP10}, HIV_{DRV^RP30},HIV_B, and HIV_C, compared to the wild-type $HIV\text{-}1_{NL4\text{-}3}$ include L10F/V32I/L33F/M46L/I54M/A71V, L23I/E34Q/K43I/M46I/I50L/G51A/L63P/A71V/V82A/T91A, L10F/V32I/M46I/I47A/A71V/I84V, L10I/ I15V/K20R/L24I/V32I/M36I/M46L/L63P/K70Q/V82A/L89M, L10I/I15V/K20R/L24I/V32I/M36I/M46L/L63P/K70Q/V82A/I84V/L89M,L10I/L33I/M36I/M46I/ F53L/K55R/I62V/L63P/A71V/G73S/V82A/L90M/I93L, and L10I/I15V/K20R/L24I/M36I/M46L/I54V/I62V/L63P/K70Q/V82A/L89M, respectively. Numbers in parentheses represent fold changes in IC_{50}s for each isolate compared to the IC_{50}s for wild-type $HIV\text{-}1_{NL4\text{-}3}$. All assays were conducted in triplicate, and the data shown represent mean values (± 1 standard deviation) derived from the results of three independent experiments. [a]Laboratory-selected drug-resistant HIV-1 variants; [b]multidrug-resistant HIV-1 clinical isolates; [c]ND: not determined

dilution using the working solution of the compound (2 μM) directly in 96-well microtiter culture plates. MT-4 cells (105/mL) were exposed to fifty 50% tissue culture infective dose ($TCID_{50}$) of each HIV-1 strain in the presence or absence of various concentrations of the compound and cultured at 37°C. On the day 7 of culture, the supernatant was harvested and the amount of p24 Gag protein was determined using the fully automated chemiluminescent enzyme immunoassay system (Lumipulse G1200; Fujirebio Inc., Tokyo, Japan). The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% inhibitory concentrations; IC_{50}) were determined by comparison with the level of p24 production in drug-free control cell cultures. All assays were performed in triplicate. To determine the drug susceptibility of HIV-2_{EHO}, MT-4 cells (10^5/mL) were exposed to 100 $TCID_{50}$ of the strain in the presence or absence of various concentrations of each compound, followed by cultivation at 37°C for 7 days. The number of viable cells was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and the magnitude of viral inhibition by each compound was determined based on their inhibitory effects of the virally-induced cytopathicity in MT-4 cells. For cytotoxicity, cells were plated in 96-well microtiter culture plates at a density of 10^5/mL and continuously exposed to various concentrations of each compound throughout the entire period of the culture. The number of viable cells in each well was determined using Cell Counting Kit-8. The 50% cytotoxic concentrations (CC_{50}) were determined as the concentration required to reduce the number of the cells by 50% compared to that of drug-unexposed control cultures.

In vitro generation of highly GRL-044-resistant HIV-1 variants

We attempted to select HIV-1 variants resistant against GRL-037 and GRL-044 as previously described (*15*). Briefly, thirty TCID50 of each of eleven HIV-1 clinical strains was mixed and propagated in a mixture of an equal number of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs)(5×10^5) and MT-4 cells (5×10^5), in an attempt to adapt the mixed viral population for their replication in MT-4 cells. The cell-free supernatant harvested on day 7 of the co-culture was referred to as HIV_{11MIX}. In the first passage, MT-4 cells (5×10^5) were exposed to the HIV_{11MIX}-containing supernatants and cultured in the presence of each compound at an initial concentration of an IC_{50} dose. On the last day of each passage (weeks 1 to 3), 1.5 mL of the cell-free supernatant was harvested and transferred to a culture of fresh uninfected MT-4 cells in the presence of increased concentrations of the compound for the following round of culture. In this following round of culture, three drug concentrations (increased by one-, two-, and three-fold compared to the previous concentration) were employed. When the culture supernatant contained > 200 ng/mL of p24 Gag protein, the HIV-1 isolate was assumed to have substantially replicated and the highest drug concentration among the three concentrations was used to continue the selection (for the next round of culture). This protocol was repetitively used until the drug concentration reached the targeted concentration (regularly 5 μM). Proviral DNA preparations obtained from the lysates of infected cells at indicated passages were subjected to nucleotide sequencing.

Determination of nucleotide sequences

Molecular cloning and determination of the nucleotide sequences of HIV-1 strains passaged in the presence of each compound were performed as previously described (*16*). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-4 cells by using the InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by nucleotide sequence determination. The PCR primers used for the protease-encoding region were KAPA-1 (5'-GCA GGG CCC CTA GGA AAA AGG GCT GTT GG-3') and Ksma2.1 (5'-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3'). The PCR mixture consisted of 1 μL proviral DNA solution, 10 μL Premix *Taq* (Ex *Taq* Version; Takara Bio Inc., Otsu, Japan), and 10 pmol of each PCR primer in a total volume of 20 μL. The PCR conditions used were an initial 1 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 20 sec at 55°C, and 1 min at 72°C, with a final 10 min of extension at 72°C. The PCR products were purified using spin columns (illustra MicroSpin S-400 HR columns; GE Healthcare Life Science., Pittsburgh, PA), cloned directly, and subjected to sequencing using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). The Sanger sequencing was conducted at the CCR Genomics Core of the National Cancer Institute.

Thermal stability analysis using differential scanning fluorimetry (DSF)

Wild-type HIV-1 protease (PR^{WT}) was purchased from ProSpec-Tany TechnoGene (Ness-Ziona, Israel). The final concentrations of each protein and compounds were 5 μM. Differential scanning fluorimetry (DSF) assays were conducted as previously described (*18,19*), using a Protein Thermal Shift dye kit (Thermo Fisher Scientific) according to the manufacturer's instructions. All of the preparation procedures were conducted on ice and samples were kept on ice until the instrument run. Twenty microliters of PR^{WT} was successively heated from 25 to 95°C, and the changes in the fluorescence intensity were measured by using a StepOne real-time PCR system (Thermo Fisher Scientific). The data

were analyzed using StepOne software version 2.3 and Protein Thermal Shift software version 1.0 (Thermo Fisher Scientific) and melting temperature (T_m) values were obtained. All of the assays were conducted in duplicate on two different occasions.

Structural Analyses of GRL-044 Interactions with Wild-type HIV-1 Protease.

We started from the X-ray crystal structure of GRL-142 complexed with wild-type PR (PR^{WT}) (PDB ID: 5TYS). An initial conformation of the Tp-THF moiety was built by taking the coordinates from the conformation of the Tp-THF moiety of GRL-015 (PDB ID: 5CON (*11*)). The conformation of GRL-142 was used to build the other moieties of GRL-044. The assembled structure was suitably modified to build an initial conformation of GRL-044. GRL-044 built in the active site of wild type protease was fully minimized using OPLS3 force field and used for subsequent analyses. Hydrogens were added to the crystal structure of DRV in complex with PR^{WT} (PDB ID 4HLA (*20*)), protonation states of aspartates were assigned. A full minimization using OPLS3 force field was carried out. A cut-off distance of 3.1 Å between a polar hydrogen and an oxygen or nitrogen atom, a minimum donor angle of 60° between D-H-A, and a minimum acceptor angle of 90° between H-A-B were used to define the presence of hydrogen bonds (D, A, and B are donor, acceptor, and atom connected to acceptor, respectively). Connolly molecular surfaces for the inhibitors and selected PR residues from the active site were generated using a water sphere with a radius of 1.4 Å as a probe. Software tools from Schrödinger, LLC, New York, NY, as implemented in the Maestro interface (version 10.7.015, Release 2016-3), were used for model building, visualization, and analysis.

Results

GRL-044 potently inhibits the replication of wild-type HIV-1

Based on the structure of DRV (*8*), we designed, synthesized, and identified two novel nonpeptidic PIs, GRL-037 and GRL-044, which contain P2-tetrahydropyrano-tetrahydrofuran (Tp-THF), P1-benzene and P1-methoxybenzene, respectively, and P2'-isopropyl-aminobenzothiazole (Ip-Abt) (Figure 1). Two previously reported PIs, GRL-0476 (*11*) and GRL-1398 (*12*), both of which served as controls, and GRL-037 and GRL-044 were all active against the wild-type HIV-1 strain (HIV-1_{NL4-3}) with 50% inhibitory concentration (IC_{50}) values of 4.8, 0.29, 0.042, and 0.0033 nM, respectively in the drug susceptibility assays. Amprenavir (APV) and lopinavir (LPV) were much less active (IC_{50} = 70 and 57 nM, respectively) and atazanavir (ATV) and darunavir (DRV) were

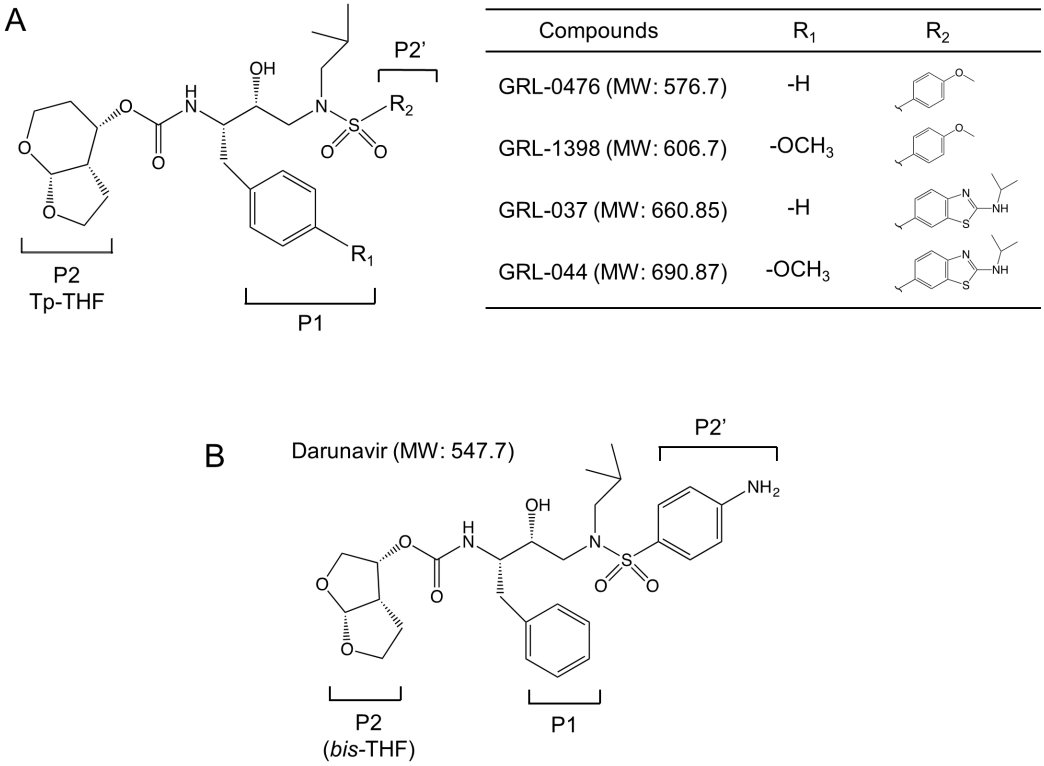


Figure 1. Structures of GRL-0476, GRL-1398, GRL-037, and GRL-044. Structures of GRL-0476, GRL-1398, GRL-037, and GRL-044 (Panel A). The structure of darunavir is shown as a reference (Panel B).

Table 2. Antiviral activity of GRL-044 against HIV-1_{NL4-3} and HIV-2_{EHO} and its cytotoxicity *in vitro*

Drug	Mean IC_{50} (nM) ± SD		CC_{50} (μM)	Selectivity index[a]
	HIV-1_{NL4-3}	HIV-2_{EHO}		
APV	39 ± 12	375 ± 63	152 ± 6	3,900
ATV	2.6 ± 0.3	60 ± 23	40 ± 1	15,400
DRV	3.3 ± 0.4	3.8 ± 1.2	145 ± 12	43,900
GRL-044	0.0028 ± 0.0008	0.0004 ± 0.0002	52 ± 3	18,571,000

The data shown represent mean values (±1 standard deviation) derived from the results of three independent experiments. [a]Each selectivity index denotes a ratio of CC_{50} in MT-4 cells to IC_{50} against HIV-1_{NL4-3}.

comparably potent (IC_{50} = 3.3 and 3.5 nM, respectively) even as compared to the least active experimental PI, GRL-0476, tested in the present study (Table 1).

GRL-044 exerts potent activity against PI-resistant and multi-drug-resistant HIV-1 variants

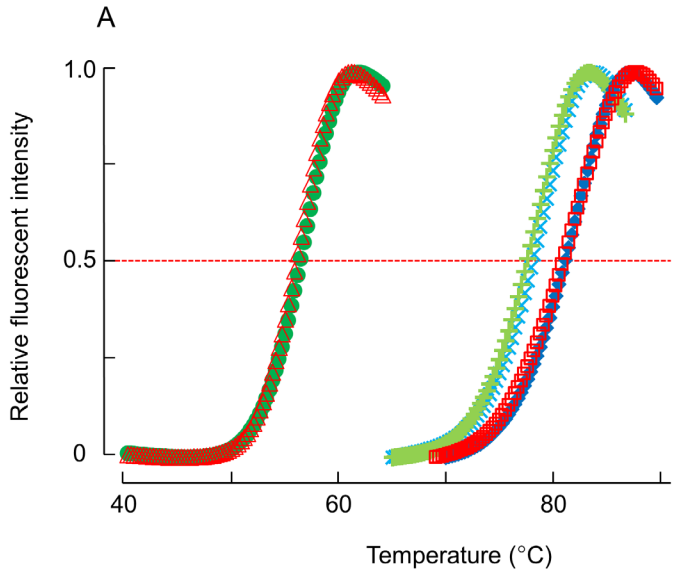
We next examined whether GRL-044 was active against a variety of HIV-1 variants that had been selected *in vitro* with each of three FDA-approved PIs: APV, ATV, and LPV (Table 1). Those HIV-1 variants had been selected *in vitro* in the presence of increasing concentrations of each PI (*16,17*). All the three variants were confirmed to have multiple amino acid substitutions in the virally-encoded protease, which have reportedly been associated with viral resistance to PIs (see the footnotes of Table 1). Two *in vitro* DRV-selected HIV-1 variants, HIV_{DRV^RP10} and HIV_{DRV^RP30}, were obtained by propagating a mixture of eight multi-drug resistant HIV-1 variants (HIV_{8MIX}) in the presence of increasing concentrations of DRV (*17*). Each of the PI-selected variants ($HIV_{APV\text{-}5\mu M}$, $HIV_{ATV\text{-}5\mu M}$, and $HIV_{LPV\text{-}5\mu M}$, HIV_{DRV^RP10}, HIV_{DRV^RP30}) was highly resistant to the corresponding PI, which the variant was selected against, and the fold differences in the IC_{50}s relative to the IC_{50} of each drug against HIV-1_{NL4-3} ranged from >14 to >303, although ATV exerted potent activity against $HIV_{APV\text{-}5\mu M}$ (Table 1). Two multi-drug-resistant HIV-1 strains, HIV_B and HIV_C, that were isolated from patients experiencing treatment failure following long-term ART (*14*), were also employed. The three FDA-approved PIs were all least active against HIV_B and HIV_C. DRV was moderately active against $HIV_{ATV\text{-}5\mu M}$, HIV_B, and HIV_C, while it had lost its activity against $HIV_{APV\text{-}5\mu M}$, $HIV_{LPV\text{-}5\mu M}$, HIV_{DRV^RP10}, and HIV_{DRV^RP30}. GRL-0476 and GRL-1398 were substantially less active against HIV_{DRV^RP10} and HIV_{DRV^RP30} with the fold-changes ranging from 80 to 598. GRL-037 also lost its activity to most of the variants examined with IC_{50} values ranging from 5.5 to 83 nM, with fold-changes ranging from 131 to 1,976, while it retained its activity against $HIV_{APV\text{-}5\mu M}$ with an IC_{50} value of 0.44 nM 10-fold change. GRL-044, the most potent PI against HIV-1_{NL4-3} among the two novel PIs, also decreased in potency against all the HIV variants examined with fold-changes ranging from 20 to 5,758. However, its IC_{50} values remained substantially low, with a range from 0.065 to 19 nM (Table 1).

GRL-044 is potent against HIV-2_{EHO} with favorable toxicity profiles

In general, PIs that are active against PI-resistant HIV-1 variants are also active against HIV-2 strains (*19,21*). We thus examined GRL-044 together with APV, ATV, and DRV against HIV-2_{EHO} in the drug susceptibility assays. As expected, both APV and ATV, which significantly lose their activity against various PI-resistant HIV-1 variants (Table 1), had significantly greater IC_{50} values against HIV-2_{EHO} (Table 2). DRV, which exerts its potent activity against various HIV-1 variants resistant to other FDA-approved PIs [except for saquinavir (SQV)(*8*), had a comparable potency against HIV-2_{EHO} compared to that against HIV-1_{NL4-3}. Since GRL-044 was highly potent against various highly drug-resistant HIV-1 variants (except against HIV_{DRV^RP30}) with IC_{50} values ranging from 0.065 to 4.6 nM (Table 1), we also examined whether GRL-044 was active against HIV-2_{EHO} as well. Interestingly, GRL-044 proved to be significantly more potent (by 7-fold) against HIV-2_{EHO} (IC_{50} = 0.0004 nM) than against HIV-1_{NL4-3} (IC_{50} = 0.0028 nM) (Table 2). Such a feature was seen in GRL-142, which is more potent against most of PI-resistant HIV-1 variants examined than against wild-type HIV-1 strain (*19*). It is of note that GRL-044 showed a highly favorable selectivity index of 18,571,000 compared to those of the three FDA-approved PIs, APV, ATV, and DRV (Table 2).

Greater thermal stability of PR^{WT} in the presence of GRL-044 compared to that of SQV or DRV

In order to examine the specificity of possible binding of GRL-037 and GRL-044 to protease, thermal stability of PR^{WT} in the absence or presence of AZT, SQV, DRV, GRL-037 or GRL-044 was determined using the differential scanning fluorimetry employing SYPRO® orange as previously described (*18,19*). The T_m (50% melting temperature) values were determined as the temperature, at which the relative fluorescent



B

Compound	Mean Tm (°C) ±SD (Δ Tm)
No drug	56.3 ± 0.2
AZT	56.0 ± 0.2 (-0.3)
SQV	77.5 ± 0.1 (21.2)
DRV	78.0 ± 0.2 (21.7)
GRL-037	81.0 ± 0.1 (24.7)
GRL-044	81.0 ± 0.2 (24.7)

Figure 2. Thermal stability of PR^{WT} with AZT, DRV, SQV, GRL-037, or GRL-044 as determined using differential scanning fluorimetry (DSF). Relative fluorescence intensities determined by DSF using SYPRO orange (Panel A). T_m values shown represent the temperatures at which the relative fluorescence intensity was 0.5, and ΔT_m values indicate the Tm difference between protease complexed with each compound and that with no compound (Panel B).

intensity became 50%. As shown in Figure 2, the mean T_m values without agent and with a nucleoside reverse transcriptase inhibitor (NRTI), zidovudine (AZT), were 56.3°C and 56.0°C, respectively, while those with DRV and SQV were comparably greater with 77.5°C and 78.0°C, respectively. The mean T_m values of GRL-037 and the most potent GRL-044 were also comparably high with 81.0°C. It is of note that the antiviral potency of GRL-044 against HIV-1_{NL4-3} was greater than that of GRL-037 by approximately 12-folds, although the T_m values of both compounds were comparable.

In vitro selection of HIV-1 variants resistant to GRL-044.

In an attempt to relatively quickly and effectively select HIV-1 variants *in vitro*, we previously used a mixture of 8 and 11 multi-PI-resistant clinical HIV-1 isolates (HIV_{MDRs}), HIV_{8MIX} and HIV_{11MIX}, respectively, as a starting HIV-1 population, resulting in successful rapid emergence of highly DRV- or TPV-resistant HIV-1 variants (*15,17*). We, therefore, employed HIV_{11MIX} to possibly successfully obtain resistant HIV-1 variants against GRL-044 in the present study (Figure 3). Figure 4 illustrates the amino acid sequences of protease of each of the 11 HIV_{MDRs}. When selected in the presence of increasing concentrations of APV, the virus quickly became resistant to the drug and was capable of replicating even in the presence of a high concentration, 5 µM, by 13 and 16 weeks of selection in the two independently conducted selection experiments (Figure 3). The HIV_{11MIX} also acquired resistance against DRV as well and became capable of replicating in the presence of 5 µM DRV, by 29 weeks of selection (Figure 3, Experiment 1). However, as depicted in the left panel of Figure 3, HIV_{11MIX} exposed to GRL-037 and GRL-044 did not replicate in the presence of 2 µM even at week 40 of the passage. In an attempt to reproduce the selection feature of Experiment 1, we conducted the second selection assay under similar conditions. As shown in the right panel of Figure 3, the selection feature was virtually the same and HIV_{11MIX} failed to replicate in the presence of relatively high concentrations of GRL-044. No further attempt to select GRL-044-resistant variants was made and the selection was terminated at week 27.

The homologous recombination-based selection expedites the emergence of resistance-associated amino acid substitutions

We determined the amino acid sequences by deducing from nucleic acid sequences of the protease of HIV_{11MIX} exposed to APV, DRV, GRL-037, and GRL-044 over 13, 29, 40, and 40 weeks in experiment 1 ($HIV_{11MIX}^{APV\text{-}1\text{-}WK13}$, $HIV_{11MIX}^{DRV\text{-}1\text{-}WK29}$, $HIV_{11MIX}^{037\text{-}1\text{-}WK40}$, and $HIV_{11MIX}^{044\text{-}1\text{-}WK40}$) respectively (Figure 5). The amino acid sequences showed distinctive differences between the former 2 strains and the latter 2 strains. The amino acid sequences of $HIV_{11MIX}^{APV\text{-}1\text{-}WK13}$ and $HIV_{11MIX}^{DRV\text{-}1\text{-}WK29}$ revealed that both selected variants had acquired V32I substitution as shown in green in Figure 5, which none of the eleven multi-PI-resistant clinical HIV-1 variants had contained before the selection as far as judged from the direct sequencing conducted for each of HIV_{11MIX} (Figure 4). One reason why these two variants have similarity in the amino acid substitution patterns

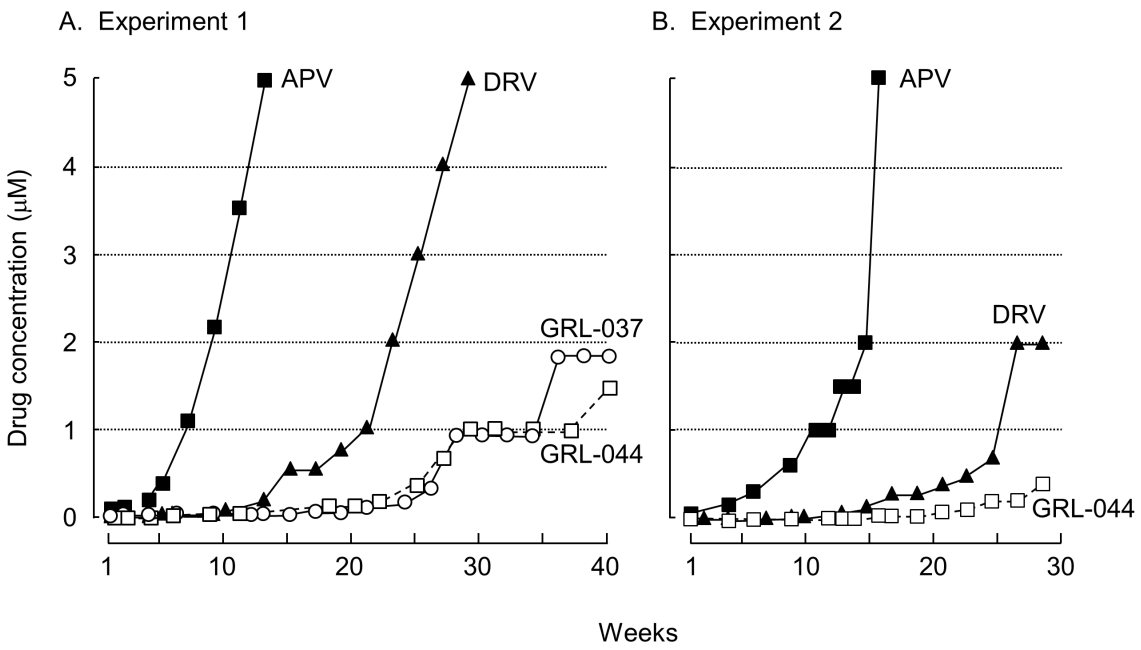


Figure 3. *In vitro* selection of HIV-1 variants against GRL-037, GRL-044, APV, and DRV. A mixture of 11 multi-PI-resistant HIV-1 isolates (HIV_{11MIX}) was propagated in MT-4 cells in the presence of increasing concentrations of GRL-037, GRL-044, APV, or DRV. The selection was conducted by passage of cell-free virus. Amino acid substitutions appeared in each HIV-1 during selection are illustrated in Figure 5.

```
                    10         20         30         40         50         60         70         80         90        99
HIV_NL4-3 PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF
HIV_A     .........I ....V..... .......... ....D.E... ....R..... ...V...... ..P....... V......... .T.......M ..L.F....
HIV_B     .........I  ......... .......... ..I..I.... .....I.... ..L.R..... .VP....... V.S....... .A.......M ..L......
HIV_C     .........I ....V....R ...I...... .....I.... .....L.... ...V...... .VP......Q .......... .A......M. .........
HIV_G     .........I IE..V...I. .......... .......... K....L.... .......... ..P....... T......... .A.......M .........
HIV_TM    .........I ...R...... .......... .......... K....L.... ...V...... ..P....... V......... .A.......M ..L......
HIV_MM    .........I .......R.. .......... .......... ..T..L.... ...V...... ..P....... V......... .A.......M ..K......
HIV_SS    .........R .......... .......... ......D... .....T.... .......... .VP....... V.S...I... .T.......M ..L......
HIV_JSL   .........I .......... ...I...... ..F.DIS... .....L..E. ...V..K... .VP....... V.S....... .A.......M .........
HIV_EV    .........V .E...A..IR .......... ..F.DI.... .....I...V ..LVR.K..E .VP....... V......... .A......M. .........
HIV_ES    .........I .......... .......... .......... .....L.... ....R..... .VP....... .LC...I... ....V....M .........
HIV_13-52 .......... .......... .......... ......D... .....I.... .......... .VP....... V.....I... .A.......M ..L......
```

Figure 4. Amino acid sequence of protease of 11 multi-PI-resistant HIV-1 isolates. The amino acid sequences of protease deduced from nucleotide sequences of the protease-encoding region of each of the eleven multi-PI-resistant HIV-1 isolates are shown. The consensus sequence of HIV_{NL4-3} is illustrated at the top as a reference. Identity with sequence at individual amino acid positions is indicated by dots.

in protease, i.e., the acquisition of V32I, is that APV and DRV share structural similarity (*8*). $HIV_{11MIX}^{DRV\text{-}1\text{-}WK29}$ had also acquired I84V substitution (highlighted in blue in Figure 5), which often emerges when HIV-1 becomes highly resistant to various PIs (*17,22*), often resulting in conferring significant resistance to PIs on HIV-1 (*22,23*). It is thought that the emergence of V32I and I84V took place relatively quickly because of the use of HIV_{11MIX} as a starting virus population, which led to homologous recombination occurring from one isolate to another in the presence of escalating doses of DRV, expediting the emergence of highly DRV-resistant

Figure 5. Amino acid sequences of protease region of HIV_{11MIX} selected by GRL-037, GRL-044, APV, and DRV

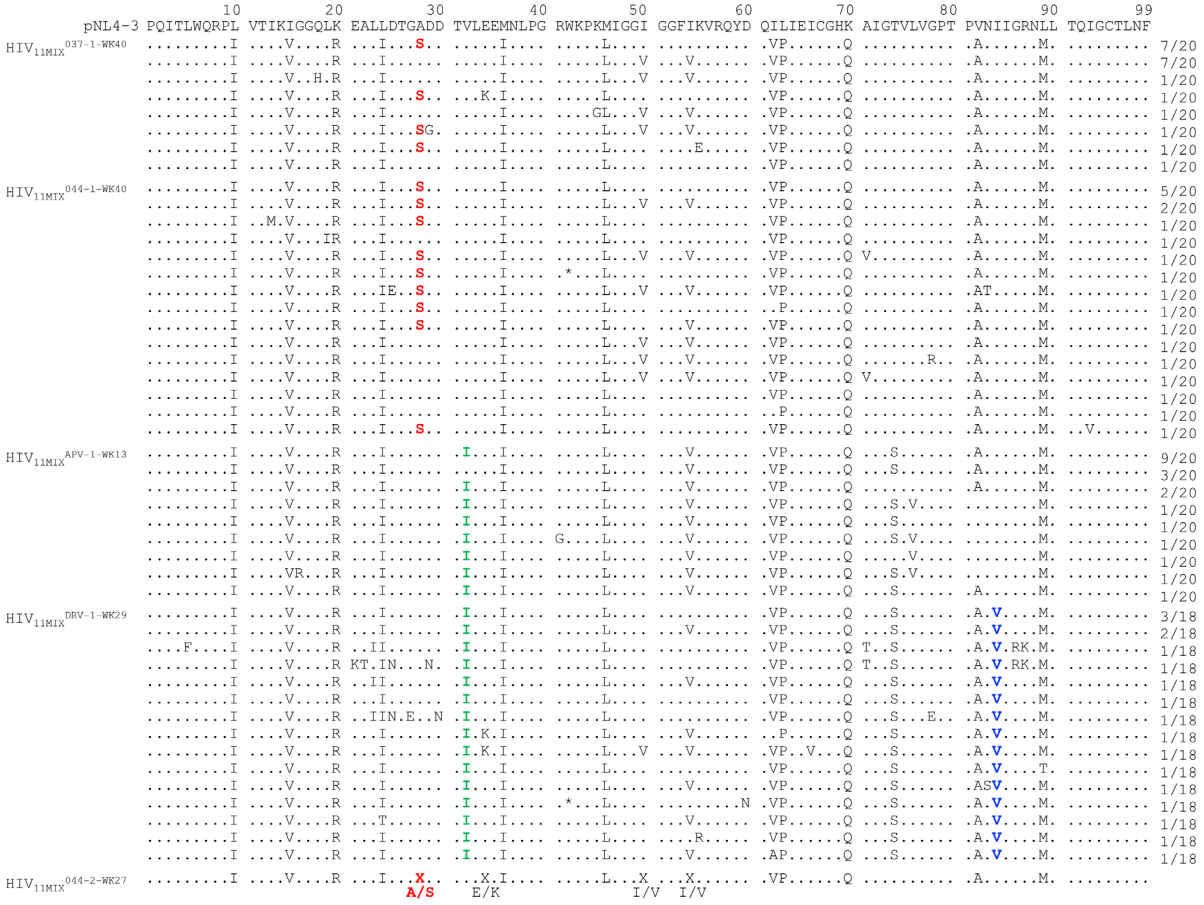


Figure 5. Amino acid sequences of the protease of HIV_{11MIX} selected with APV, DRV, GRL-037, and GRL-044 *in vitro*. Amino acid sequences deduced from the nucleotide sequences of the protease-encoding region of proviral DNA isolated from HIV_{11MIX} selected with APV at 13 weeks, DRV at 29 weeks, GRL-037 at 40 weeks, and GRL-044 at 40 weeks in experiment 1 and GRL-044 at 27 weeks in experiment 2 are shown. The consensus sequence of HIV_{NL4-3} is illustrated at the top as a reference. Identity with sequence at individual amino acid positions is indicated by dots. Fractions on the right are the number of viruses which each clone is presumed to have originated from over the total number of clones examined. The amino acid sequence of $HIV_{11MIX}^{044\text{-}2\text{-}WK27}$ was determined by the deduction of the direct nucleic acid sequence obtained for $HIV_{11MIX}^{044\text{-}2\text{-}WK27}$.

HIV-1 variants (*22*).

Interestingly, in both $HIV_{11MIX}^{037\text{-}1\text{-}WK40}$, and $HIV_{11MIX}^{044\text{-}1\text{-}WK40}$, the unique substitution, A28S, known to lead to a significant reduction in protease activity (*24*) and poor viral fitness (*25*), had been acquired by passage 40 (highlighted in red in Figure 5), while both resultant variants had not apparently acquired high levels of resistance to GRL-037 or GRL-044 and did not replicate well in the presence of either of the two compounds (Figure 3). Since the A28S substitution has been seen with a few PIs, such as TMC-126 (*26*), GRL-98065 (*27*), brecanavir (*25*), and GRL-1398 (*12*) and not with any of the currently available FDA-approved PIs except only in a few cases (*28,29*), we repeated the selection assay with APV, DRV, and GRL-044 (Figure 3 , Experiment 2). When we determined the amino acid sequence of the protease of HIV_{11MIX} with direct nucleic acid sequencing after HIV_{11MIX} was selected in the presence of GRL-044 in the second selection assay, the same A28S substitution was identified by week 27 as highlighted in red at the bottom of Figure 5.

Structural interactions of GRL-044 and DRV with wild-type protease.

We finally attempted to determine the structural interactions of GRL-044 with wild-type protease (PR^{WT}). In this attempt, we started from the crystal structure of GRL-142 complexed with PR^{WT} and modified the structure of GRL-142 (*19*) to generate GRL-044-PR^{WT}. A full minimization of GRL-044-PR^{WT} using OPLS3 force field was carried out to interrogate the structural interactions. We also wished to compare the interactions with those of DRV with PR^{WT}. Starting from a crystal structure of DRV-PR^{WT} (*20*), hydrogen atoms were added, followed by assignment of protonation states of aspartates and a full minimization using OPLS3 force field. The hydrogen bond and van der Waals surface interactions in GRL-044-PR^{WT} and DRV-PR^{WT} were subsequently analyzed and compared. The analyses helped understand the similarities and differences of polar and non-polar interactions of these two inhibitors complexed with PR^{WT}.

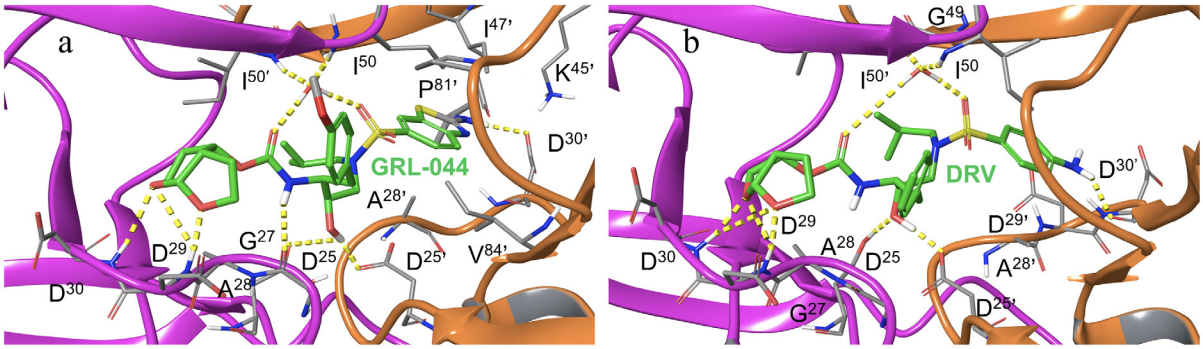


Figure 6. Polar interactions of GRL-044 and DRV with wild-type protease. The polar interactions of GRL-044 and DRV with wild-type protease are shown in Panels-a and -b, respectively. The protease dimer is shown in a ribbon representation, with the monomers shown in purple and orange. The residues in the immediate vicinity of the inhibitors (< 3 Å) are shown. The hydrogen bond interactions are shown by yellow dotted lines. GRL-044 and DRV are in green carbons, and thick sticks, the protease residues are in gray carbons.

The polar interactions of GRL-044 with PR^{WT} are shown in Figure 6a. The Tp-THF moiety of GRL-044 has hydrogen bond interactions with D29 and D30. The carbonyl and sulfonyl oxygens form polar interactions with I50 and I50' present in the protease flap through a bridging water molecule. This interaction with the protease flap is seen for various PIs (*8,14,19*). An amine group of the inhibitor makes a hydrogen bond interaction with G27 and the hydroxyl group of the inhibitor makes hydrogen bond interactions with the catalytic aspartates. The amine from the benzothiazole in the P2' group makes a hydrogen bond interaction with the side chain of D30'. DRV has many of these polar interactions with PR^{WT}. The residues identified within 3Å distance from the inhibitors are shown in panels a and b. Because of the slightly larger size of GRL-044 over DRV, there are a few additional residues of PR^{WT} that are closer to the former.

We then focused on the moieties that are different between GRL-044 and DRV and examined how the identified differences impact the van der Waals interactions with PR^{WT}. GRL-044 has an Ip-Abt as P2' ligand compared to the aminobenzene of DRV. The P2'-Ip-Abt has a better van der Waals interaction with D30'/K45' than the aminobenzene of DRV (Figure 7a, b). The P2' moiety of GRL-044 is also closer to R8, forming weak van der Waals interactions, whereas the R8 group is much further away from the aminobenzene of DRV (Figure 7c, d). While both P2-Tp-THF of GRL-044 and P2-*bis*-THF of DRV have van der Waals surface contact with I47, the P2'-Ip-Abt of GRL-044 has a better van der Waals contact with I47' than does the P2'-aminobenzene of DRV (Figure 7e, f). GRL-044 also makes better contact with G48 than does DRV (Figure 7g, h). The P1-paramethoxyphenyl substituent of GRL-044 makes better contact with G49 than the phenyl substituent alone of DRV (Figure 7i, j). Of note, residues 47-49 are in the flap region of the protease. Interactions of an inhibitor with the flap region probably help keep the region in a closed conformation resulting in stronger binding (*11,30*).

A28 is seen present in the active site cavity. The P2-*bis*-THF of DRV and P2-Tp-THF of GRL-044 have van der Waals contacts with A28. Interestingly, both P2′-Ip-Abt of GRL-044 and P2'-aminobenzene of DRV also have van der Waals contacts with A28'. The van der Waals surface contact of P2'-Ip-Abt of GRL-044 with A28' appears to be better compared to aminobenzene of DRV because of the larger surface of Ip-Abt (Figure 7k, l). Whether these interactions are wholly or partially responsible for the emergence of A28S substitution is not known at this time and needs to be explored.

Overall, the improved van der Waals interactions of GRL-044 with multiple PR^{WT} residues located in different protease domain should explain at least in part why GRL-044 has the significantly stronger binding to PR^{WT} and greater antiviral potency over DRV (Table 1).

Discussion

In the present work, we designed, synthesized, and identified two novel nonpeptidic PIs, GRL-037 and GRL-044, containing (*i*) P2-Tp-THF, (*ii*) P1-benzene and P1-methoxybenzene, respectively, and (*iii*) P2'-Ip-Abt (Figure 1). GRL-044 that contains (*i*) P2-Tp-THF, (*ii*) P1- methoxybenzene, and (*iii*) P2'-Ip-Abt proved to be most potent against HIV-1_{NL4-3} with IC_{50} values of 0.0028-0.0033 nM (Tables 1 and 2). The IC_{50} values against various PI-resistant HIV-1 variants also proved to be highly favorable ranging from 0.065 to 19 nM (Table 1). We have previously described that GRL-077 and GRL-058, both of which contain P2-C5-modified Tp-THF, P1-methoxybenzene, and P2'-Ip-Abt were potent against HIV-1_{NL4-3} and various PI-resistant HIV-1 variants (*30*). But the IC_{50} value of GRL-077 and GRL-058 were 21 and 3.5 nM, less potent by 6,364- and 1,061-fold, respectively than GRL-044. There is only one difference: both GRL-077 and GRL-058 have C5-modified Tp-THF, while GRL-044 has unmodified

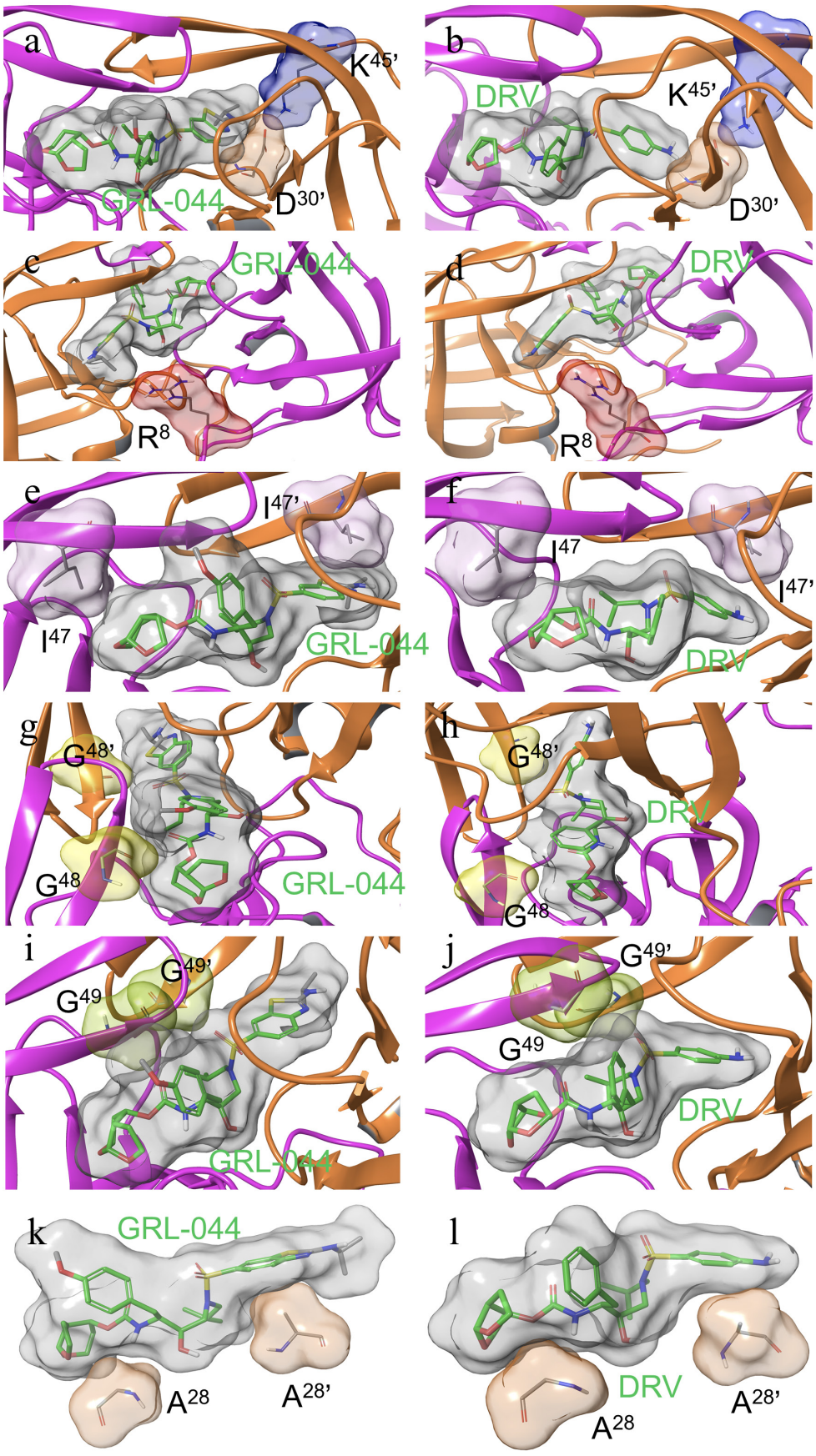


Figure 7. van der Waals surface interactions of GRL-044 and DRV with wild-type protease. The van der Waals surface interactions of GRL-044 and DRV with important residues are shown in panels a-l. The inhibitor surfaces are shown in gray, D30' is in orange, K45' in blue, R8 in red, I47 and I47' in plum, G48 and G48' in yellow, G49 and G49' in yellowish green, A28 and A28' in orange.

Tp-THF, strongly suggesting that the C5-modification significantly weakens the potency against HIV-1_{NL4-3}, although the potency of GRL-077 and GRL-058 against various PI-resistant variants including $HIV_{DRV^{R}P10}$ and $HIV_{DRV^{R}P30}$ is comparable with that of GRL-044.

Determination of thermal stability using the differential scanning fluorimetry employing SYPRO® orange helps understand the specificity and binding force of protein and its ligand (*19,29,31*). The T_m values of DRV and SQV, which were greater than those without agent and with AZT, were the lowest and those with GRL-037 and GRL-044 were further greater (Figure 3). However, in the present study, the mean T_m values of GRL-037 and the most potent GRL-044 were virtually identical, while GRL-044 was more potent by ~12-folds than GRL-037, showing that the T_m values in the DSF assay are not always proportionate to the biological activity of compounds such as antiretroviral potency.

It has been demonstrated that upon reverse transcription, reverse transcriptase (RT) frequently switches a template from one viral genomic strand to another, producing recombinant proviral DNA or a mosaic proviral DNA containing multiple parent genomic pieces. In fact, a single $CD4^{+}$ target cell can be infected with multiple HIV-1 virions (*32,33*). If newly produced proviral DNA has mutations in one spot conferring HIV-1 resistance to one drug and mutations in the other spot that are associated with resistance to the other drug, the daughter virions acquire resistance to both drugs, a process called homologous recombination (*34-36*). Thus, homologous recombination is likely to accelerate the development of multi-drug and multi-class drug resistance in the setting of HIV-1 infection. As we previously demonstrated, when the selection of HIV-1 variants with DRV was attempted using a single viral strain as the starting virus population, no DRV-resistant variants emerged; however, when a mixture of eight multi-drug-resistant (as well as multi-PI-resistant) clinical HIV-1 strains (HIV_{8MIX}) was employed as the starting virus population, highly DRV-resistant HIV-1 variants such as $HIV_{DRV^{R}P10}$ and $HIV_{DRV^{R}P30}$ were relatively quickly obtained, highly likely through the homologous recombination (*17,23*). In the current homologous recombination-based selection assay, HIV_{11MIX} exposed to APV and DRV over 13 and 29 weeks, resulting in $HIV_{11MIX}^{APV\text{-}1\text{-}WK13}$ and $HIV_{11MIX}^{DRV\text{-}1\text{-}WK29}$, respectively, had acquired V32I substitution (highlighted in green in Figure 5). The V32I substitution does not appear alone when wild-type HIV-1 (HIV^{WT}) as a starting virus population is selected with DRV, since V32I confers on HIV^{WT} greater susceptibility (~14-fold) to DRV as well as a highly compromised replication fitness (22). The V32I substitution is, therefore, thought to be a key substitution, which hardly emerges when selected in test tube and in the clinical setting; but once V32I substitution emerges with other amino acid substitutions, the V32I substitution predisposes HIV-1 to quickly develop high-level DRV-resistance. It is thought that the emergence of V32I took place relatively quickly because of the use of HIV_{11MIX} as a starting virus population, which led to the homologous recombination occurring from one isolate to another in the presence of escalating doses of DRV, expediting the emergence of highly DRV-resistant HIV-1 variants (*22*).

It is noteworthy that, when HIV_{11MIX} was selected with GRL-037 or GRL-044, the A28S substitution, known to lead to a ~1,500-fold reduction in *kcat/Km* in protease activity (*24*) and the poor viral fitness (*25*), had been acquired by passage 40 (Figure 5), although both resultant variants $HIV_{11MIX}^{037\text{-}1\text{-}WK40}$ and $HIV_{11MIX}^{044\text{-}1\text{-}WK40}$ were not much resistant to either of the compounds (Figure 3). The reason why both $HIV_{11MIX}^{037\text{-}1\text{-}WK40}$ and $HIV_{11MIX}^{044\text{-}1\text{-}WK40}$ were not sufficiently replicative in the homologous recombination-based selection assay could be due to the acquisition of A28S substitution. The A28S substitution has been seen in the selection assay of HIV-1 with a few PIs, such as TMC-126 (*26*), GRL-98065 (*27*), brecanavir (*25*), and GRL-1398 (*12*), and not with any of the currently available FDA-approved PIs except only in a few cases (*28, 29*). Of note, however, the population size of HIV-1 in the present homologous recombination-based selection assay is assumed to be significantly smaller than the size of HIV-1 variants such as polymorphic amino acid substitution-containing variants that reside in HIV-1-infected individuals, the appearance of mutations is affected by stochastic phenomena. Therefore, the rates of mutation appearance in culture may not be reliable. Moreover, in the initial selection, we observed the appearance of the rare A28S substitution in both $HIV_{11MIX}^{037\text{-}1\text{-}WK40}$ and $HIV_{11MIX}^{044\text{-}1\text{-}WK40}$. Thus, we repeated the selection assay with GRL-044. As shown in the bottom line of Figure 5, the A28S substitution was reproducibly identified through direct sequencing. Structurally, the P2-*bis*-THF of DRV and P2-Tp-THF of GRL-044 have van der Waals contacts with A28. Both P2′-Ip-Abt of GRL-044 and P2′-aminobenzene of DRV also have the contacts with A28′. However, apparently the van der Waals surface contact of P2′-Ip-Abt of GRL-044 with A28' appears to be better compared to aminobenzene of DRV because of the larger surface of Ip-Abt. Whether these interactions are wholly or partially responsible for the emergence of A28S substitution is not known at this time and needs to be explored.

Taken together, in our efforts to maximize the antiviral potency of the prototypic protease inhibitor, DRV (Figure 1), which has potent activity against various wild-type and drug-resistant HIV-1 species (*8*) and a reasonably high genetic barrier to the emergence of HIV-1 variants resistant to DRV (*17,18,37,38*), we successfully designed and identified GRL-044, which has much greater potency and shows greater genetic barrier compared to the prototypic DRV. The structural mechanisms by which GRL-044 acquired the much greater potency than the potency of DRV appear to stem

from the design of GRL-044 that the three moieties were substituted: (*i*) P2-Tp-THF, (*ii*) P1-methoxybenzene, and (*iii*) P2'-Ip-Abt, starting from the chemical structure of DRV. The present antiviral data and structural features of GRL-044 should shed lights in the further design and development of potent and "resistance-repellant" novel PIs.

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Non-AIDS-defining malignancies in Japanese hemophiliacs with HIV-1 infection

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Abstract: Along improvement of prognosis of HIV-1-infected patients due to successful anti-retroviral therapy, main causes of death in the patients have been changing from AIDS to non-AIDS defining malignancies (NADM) recently. However, little is known about the prevalence and incidence of NADM in patients, and especially in HIV-1-infected hemophiliacs. We prospectively conducted NADM screening with FDG-PET, chest CT, upper gastrointestinal endoscopy, tumor markers, and stool occult blood in hemophiliacs with a mean age of 48.9 years. Screening was done twice from December 2016 through March 2019; the first screening was used to calculate prevalence in 69 patients and the second was used to calculate incidence in 56 patients. The first screening revealed 4 cases of malignancies; three were cases of thyroid cancer and one was a case of a neuroendocrine tumor in the pancreas; prevalence was 5.8% (95% CI: 0.2-11.4%). During a mean follow-up of 1.2 years with 68.2 person-years (PYs), cancer was diagnosed in 2 cases (pancreatic and liver cancer) during the second screening. Incidence was 2.99/100 PY. It can be speculated that there might be around 40 cases of undiagnosed NADM currently and 20 cases of new NADM annually in this population, because 718 HIV-1-infected hemophiliacs are surviving in Japan according to the 2018 Nationwide Survey on Coagulation Disorders. Screening for NADM in HIV-1-infected hemophiliacs at other hospitals is strongly recommended.

***Keywords*:** Cancer, screening, prevalence, incidence

Introduction

The prognosis for HIV-1-infected patients improved drastically thanks to continuous advances in antiretroviral therapy (ART), the life expectancy of those patients was estimated to be almost the same as that of the general population, especially after 2000 (*1-4*). Along with that improvement, HIV-1-infected patients now face issues related to aging. Some patients need to be treated for lifestyle-related co-morbidities such as hypertension, chronic kidney diseases, cardiovascular diseases, and diabetes mellitus (*5*). In addition to these co-morbidities, HIV-associated neurocognitive disorders (HAND) have been recognized as a major issue for patients in this decade (*6*). We performed the Japanese nationwide study of HAND (the J-HAND study), which revealed 25% of HIV-1-infected patients were diagnosed as HAND, though hemophiliacs were not included in that study (*7*). Moreover, the risk of non-AIDS-defining malignancies (NADM) has been increasing (*8-10*), and NADM have become one of the major causes of death in HIV-infected patients (*11*).

In Japan, around 30% of all hemophiliacs were infected with HIV-1 through contaminated blood products produced in the US before 1986 (*12*), when use of non-heat-treated products was prohibited in Japan. The number of HIV-1-infected hemophiliacs was 1,439 at that time. ART was introduced in Japan at the end of 1996, and the prognosis for hemophiliacs improved to that of other HIV-1-infected patients (*13*). However, nearly 99% of Japanese hemophiliacs were also infected with hepatitis C virus (HCV). Disease progression of hepatitis C in HIV-1 co-infected patients was reported to be more advanced than that in HCV mono-infected patients (*14*). Indeed, hepatitis C status has already advanced to cirrhosis in almost half of hemophiliacs (*15*). Therefore, hepatitis C has been

extensively treated with interferon and/or direct-acting antiviral drugs (DAA) for two decades, and a sustained virological response (SVR) has been achieved in almost all HIV-1-infected hemophiliacs (*16*). Although the risk of hepatocellular carcinoma (HCC) can be reduced after SVR with DAA (*17*), there is still a substantial risk of HCC, and especially in patients with cirrhosis. In summary, HIV-1-infected Japanese hemophiliacs have unique characteristics: *i*) they were infected with HIV-1 more than 33 years ago, *ii*) they were also co-infected with HCV, although they obtained SVR, *iii*) they had a long history of treatment with nucleoside reverse transcriptase inhibitors (NRTI), indicating that they might have received mitochondrial toxicities, *iv*) their mean age was 48.9 years, which is close to the age of cancer onset in HIV-1-infected patients (*10*), *v*) they were not received examination for HAND.

Therefore, it could be postulated that HIV-1-infected Japanese hemophiliacs might have a substantial risk of NADM. However, little is known about that risk in this population. The aims of the current study were to explore the prevalence and incidence of NADM and to conduct co-screening for HAND using 18F-fluorodeoxyglucose-positron emission tomography (FDG-PET). This study focused only on NADM, and the HAND results in this population will be reported elsewhere.

Patients and Methods

Patients

A single center, prospective, longitudinal study of screening for NADM was conducted in HIV-1-infected Japanese hemophiliacs attending the AIDS Clinical Center (ACC), National Center for Global Health and Medicine (NCGM). All hemophiliacs were approached, and all eligible patients who agreed to participate in this study were included. Patients who were already diagnosed with an NADM were excluded in order to determine the undiagnosed prevalence of NADM.

Screening was conducted twice from December 2016 through March 2019. The first screening aimed to determine the prevalence of NADM and the second aimed to calculate its incidence in this population. In both screenings, patients underwent whole body FDG-PET, chest computed tomography (CT), gastric fibroscopy (GF), two tests for occult blood in stool, and measurement of tumor markers including carcinoembryonic antigen (CEA) for colon cancer, α-fetoprotein (AFP) for hepatocellular carcinoma, carbohydrate antigen (CA19-9) for biliary tract cancer or pancreatic cancer, pancreatic cancer-associated antigen (DUPAN-2) for pancreatic cancer, SPan-1 antigen (SPan-1) for pancreas and biliary tract cancer, prostate-specific antigen (PSA) for prostate cancer, cytokeratin 19 fragment (CYFRA) for non-small lung cancer, and pro-gastrin releasing peptide (ProGRP) for small cell lung cancer. If at least one of these examinations was positive, further specific examinations were added to identify or exclude NADM. This study was approved by the ethical committee at the NCGM (NCGM-G-2065-00), and all patients provided written informed consent in accordance with the Declaration of Helsinki. This study was registered with UMIN CTR (ID: UMIN000024741).

Statistical analysis

Mean age ± standard deviation (SD), AIDS status, cirrhosis status, CD4 count, and plasma HIV viral load (pVL) were calculated at the time of the first screening. The prevalence of NADM was expressed with 95% confidential intervals (95% CI), and the incidence was calculated per 100 person-years (100 PYs).

Results

Study participants

A summary of the study participants is listed in Table 1. HIV-1-infected Japanese hemophiliacs were included while patients with von-Willebrand disease were not. Thus, all study participants were male. Among the 85 hemophiliacs seen by the ACC, 69 patients gave consent for the first screening and 58 for the second screening. The flow of this study is shown in Figure 1.

The mean age (± SD) of the 69 patients during the first screening was 48.9 ± 8.01 years, with the youngest age being 37 years and the oldest being 71 years.

Table 1. Characteristics of 69 HIV-1-infeceted Japanese hemophiliacs during the first screening in 2016/2017

Characteristic	N (%)
Age, Years	
- 39	8 (11.6)
40-49	33 (47.8)
50-59	20 (29.0)
60 -	8 (11.6)
Mean ± SD[#1]	48.9 ± 8.01
History of AIDS-related illnesses	
Yes	11 (15.9)
No	58 (84.1)
Cirrhosis status	
Yes	13 (18.8)
No	56 (81.2)
CD4 count, cells/mm^3	
< 200	2 (2.9)
200-500	29 (42.0)
> 500	38 (55.1)
Mean ± SD	575 ± 285
Plasma viral load, copies/mL	
< 50	66 (95.7)
> 50	3 (55[#3]/57/44,400) [#2]

[#1], standard deviation; [#2], plasma viral load of each patient; [#3]; elite controller.

All patients received ART except one who had never received any ART because he was an elite controller. His CD4 count was 703 cells/mm^3, and pVL was 55 copies/mL without ART. Although eleven patients (15.9%) had a history of AIDS-related illnesses, no patient had an active AIDS-defining illness during screening. Therefore, the mean CD4 count was 575 cells/mm^3, and pVL was below 50 copies/mL in 95.7% of patients. Overall, HIV-1 infection in this population was quite well controlled. Although an SVR had been achieved in all patients according to plasma HCV RNA, the hepatitis status had advanced to liver cirrhosis in 13 patients (18.8%), indicating there might have some risk of HCC in these patients.

The first screening

NADM was detected in 4 of 69 participants (Table 2). Therefore, the prevalence of NADM was 5.8% (95% CI: 0.2-11.4%). The NADM was thyroid cancer in three cases and a neuroendocrine tumor in one. All of the NADM were detected with FDG-PET and then examined further. Two cases of thyroid cancer were confirmed with an echography-guided biopsy.

When the first case of papillary thyroid cancer was diagnosed on November 22, 2017, the tumor was 10 × 10 × 7 mm in size and lymph node metastasis was present. The tumor was surgically resected. The stage of the cancer was pT1aN1aM0. The patient's CD4 count was 484 cells/mm^3, and pVL was undetectable. The second case of follicular thyroid cancer was diagnosed on January 9, 2018, and surgical resection was performed on May 17, 2018. The tumor was 17 mm in size and metastasis was absent; the stage of the cancer was pT1N0M0. The patient's CD4 count was 1058 cells/mm^3, and pVL was undetectable at that time. However, echography revealed metastasis to the right cervical lymph node on April 2, 2019 (11 months after the first surgery), and a second surgery was performed on June 3, 2019. In the third case (CD4, 429 cells/mm^3; pVL, undetectable), echography strongly suggested papillary carcinoma, but the patient refused further examinations. This case has been included here as a case of thyroid cancer. The fourth case (CD4, 530 cells/mm^3; pVL, 41copies/mL) was definitively diagnosed as a neuroendocrine tumor in the pancreas based on abdominal CT and MRI and by performing an endoscopic ultrasound (EUS) with fine needle aspiration. The patient in this case underwent successful surgical resection without any metastasis to or invasion of the surrounding tissue on February 1, 2018. The tumor was 16 × 15 × 14 mm size, and the stage of the cancer was pT1N0M0.

In the first screening, FDG-PET was a very sensitive way to identify NADM. However, there were false-positive results in 12 cases (18.8%). Those patients had to undergo further examinations to rule out NADM (Table 3). Lung cancer was most commonly (in four cases) suspected in FDG-PET and excluded by chest CT. Each of Colon cancer

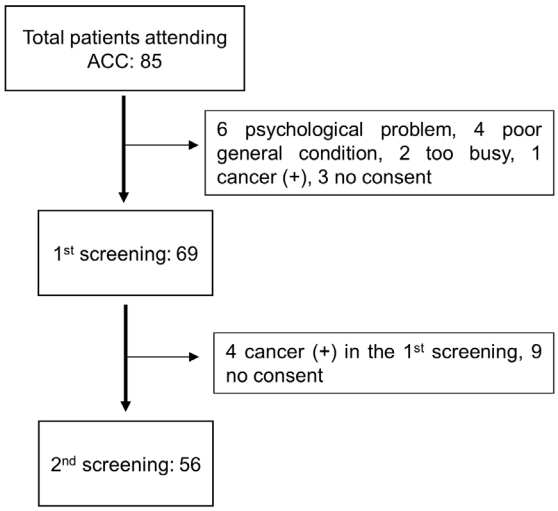

Figure 1. Flow of this study.

Table 2. Diagnosis of NADM

NADM	Age, year	Modalities performed	Tumor marker
1st screening			
1. Thyroid ca (papillary ca)	55	PET, echo+biopsy	CYFRA; 4.0 ng/mL [#2]
2. Thyroid ca (follicular ca)	44	PET, echo+biopsy	W.N.L
3. Thyroid ca[#1] (s/o papillary ca)	45	PET, echo	W.N.L
4. Neuroendocrine tumor, pancreas	39	PET, abd CT and MRI, EUS-FNA	W.N.L
2nd screening			
1. Pancreas ca (invasive ductal ca)	69	PET, abd CT and MRI, EUS	CYFRA; 5.1 ng/mL, DUPAN-2; 217 U/mL
2. Hepatocellular ca (moderately differentiated)	68	PET, abd CT and EOB-MRI	DUPAN-2; 197 U/mL

Abbreviations: ca, cancer/carcinoma; NADM, non-AIDS-defining malignancies; s/o, suspected of; PET, 18F-fluorodeoxyglucose-positron emission tomography; echo, echography; abd, abdominal; CT, computed tomography; EOB-MRI, Gd-ethoxybenzyl-DTPA-enhanced magnetic resonance imaging; EUS, endoscopic ultrasound; FNA, fine needle aspiration; CYFRA, cytokeratin 19 fragment (<3.5 ng/mL); DUPAN-2, pancreatic cancer-associated antigen (<150 U/mL); W.N.L, within normal limits for all tumor markers examined. [#1], A definitive diagnosis was not reached in this case because this patient refused further examinations. However, thyroid cancer was strongly suspected based on several imaging examinations that were performed. This case was included as a case of cancer. [#2], Repeated CYRFA results were within normal values, indicating non-specific elevation during the first examination.

Table 3. False-positive results of FDG-PET in 12 cases

Case #	FDG uptake in	Suspected of	Further examinations
1	Rt lobe of thyroid	Thyroid ca	FNA under thyroid echo
2	Upper esophagus	Esophagus ca	GF, Chest CT
3	End ileum/Pancreas	Colon ca./Pancreas ca	GF, CF, abd CT, and echo
4	Rt lower lung	Lung ca	Chest CT
5	Pancreas	Pancreas ca	GF, MRCP
6	Rt lower lung S6	Lung ca	Chest CT
7	Rt middle lung	Lung ca	Chest CT
8	Rt upper lung	Lung ca	Chest CT
9	Duodenum	Duodenum ca	GF
10	Ascending colon	Colon ca	CF
11	Liver S4	Hepatocellular ca	Abd CT and EOB-MRI
12	Axillary lymph node	Metastasis ca	Abd echo and CT

Abbreviations: ca, cancer/carcinoma; FDG-PET, 18F-fluorodeoxyglucose-positron emission tomography; FNA, fine needle aspiration; echo, echography; GF, gastric fibroscopy; CF, colon fibroscopy; abd, abdominal; CT, computed tomography; MRCP, magnetic resonance cholangiopancreatography; EOB-MRI, Gd-ethoxybenzyl-DTPA-enhanced magnetic resonance imaging.

and pancreatic cancer was suspected in FDG-PET in two cases, respectively, but excluded by GF or colon fiberscope (CF), abdominal CT scan, or Magnetic resonance cholangiopancreatography (MRCP). Each of thyroid, esophagus, duodenum, hepatocellular, and metastatic cancer in axillary lymph node was suspected in one case, respectively, but excluded by further examinations. Tumor markers had no diagnostic value during the first screening.

The second screening

After a mean follow-up period of 1.2 years (67.2 PYs), cancer was diagnosed in 2 of the 56 study participants in the second screening, pancreatic cancer was diagnosed in one case and HCC was diagnosed in the other (Table 2). The incidence of NADM was 2.99/100 PYs in this population. Both of the aforementioned patients had already liver cirrhosis.

The patient with pancreatic cancer underwent the first screening with FDG-PET on January 26, 2017, and results were negative. The second screening was performed on May 31, 2018, revealing uptake of FDG in the pancreas. An abdominal CT was added on August 10, 2018, EUS was added on the August 20, and MRI was added on the August 21, resulted the strongly suspicion of pancreas cancer. In this case, however, a previous abdominal CT scan done on January 17, 2018 (4 months before the second FDG-PET) was negative for pancreatic cancer. The patient's CD4 count was 357 cells/mm^3, and pVL was undetectable during the second FDG-PET. The patient underwent surgical resection of pancreatic cancer on August 24th. The cancer was pT2N0M0, stage IB. The pathological diagnosis was invasive ductal carcinoma. The patient with HCC underwent the first screening with FDG-PET on June 22, 2017, and results were negative. The second screening was performed on August 22, 2018, revealing uptake of FDG in the liver. The patient's CD4 count was 238 cells/mm^3, and pVL was undetectable during the second FDG-PET. An abdominal CT scan performed on June 22, 2018 (2 months before the second FDG-PET) was negative, but a scan on September 21st (1 month after the second one) strongly suggested HCC. HCC was confirmed radiologically with Gd-ethoxybenzyl-DTPA-enhanced MRI (EOB-MRI) on October 16th, and the patient underwent surgical resection of HCC on October 25, 2018. The cancer was pT2N0M0, stage II. The pathological diagnosis was moderately differentiated HCC.

Discussion

This is the first prospective longitudinal study of cancer screening in HIV-1-infeceted Japanese hemophiliacs. This study determined the prevalence and incidence of NADM in this population. In the light of the mean age of this study population (48.9 years), the prevalence (5.8%) and incidence (2.99/100PY) of NADM might be high (*9*). HIV infection was very well controlled in this population, and the mean CD4 count was 575 cells/mm^3. The CD4 count in patients who had an NADM during the first screening was higher than 400 cells/mm^3, suggesting that their immune function was sufficient to avoid opportunistic infections related to AIDS. However, the recovery of their immune system was not complete enough to mitigate the development of an NADM. The number of survivors among HIV-1-infected hemophiliacs was reported to be 718 patients as of March 2018 (*18*). Presumably there might be around the 40 cases of undiagnosed NADM currently and the 20 cases of new NADM annually in this population in Japan.

The methods of cancer screening in this study included whole body FDG-PET, chest CT, GF, measurement of tumor markers, and 2 tests for occult blood in stool because a retrospective study by the current authors found that gastric cancer, colon cancer,

lung cancer, and liver cancer were predominant forms of NADM (*9*). FDG-PET was used because FDG-PET is a very sensitive way to detect occult cancer (*19*) as well as dementia (*20*). Then, we aimed to make co-screening of NADM and HAND with FDG-PET in the original protocol. Since FDG-PET was used as a screening tool, the types of cancer detected in this study differed from those detected in the previous retrospective study (*9*). FDG-PET is reported to be highly sensitive at detecting colon/rectum, thyroid, and lung cancers but relatively less sensitive at detecting prostate and gastric cancers (*19*). In the current study, FDG-PET revealed three cases of incident thyroid cancer. There are some arguments that as the natural course of the thyroid cancer is very slow and the prognosis is not poor in most cases, then, finding the early stage thyroid cancer is not appropriate by using the sensitive method such as FDG-PET in general population (*21,22*). In HIV-1-infected patients, however, progression of some diseases, such as hepatitis C, is reported to be faster than that in the general population (*14*). Progression of thyroid cancer in HIV-1-infecetd patients is unclear and must be carefully followed in the future. In two of the current patients with thyroid cancer, the disease was relatively advanced, and patients had to undergo surgical resection. However, the cancer was found during the first screening, so disease progression in those cases was unclear. We should observe the third case with careful and close monitoring.

There are no guidelines regarding NADM screening in HIV-1-infected patients. Based on the current findings, NADM screening should be considered for those patients. However, the method of screening should be based on epidemiological data indicating how frequently NADM develop in HIV-1-infected patients (*9*). The current study used FDG-PET because this study sought to co-screen for dementia. However, FDG-PET is not inappropriate as a screening tool because FDG-PET yielded false-positive results in 12 cases; the patients in those cases had to undergo further examinations (including invasive procedures) to rule out an NADM (Table 3). Moreover, if it was listed, it is impossible to perform NADM screening in most of hospitals in Japan. We have a plan to conduct NADM screening again in the same population with chest and abdominal CT including the thyroid and prostate, GF, tests for occult blood in stool, and measurement of the tumor markers CEA, AFP, and PSA. This next screening would provide more accurate data on the incidence of NADM in HIV-1-infected hemophiliacs and indicate the usefulness of screening methods in the future.

This study had several limitations. First, this was a single-center study with a small number of patients, even though all patients seen by the ACC were approached and all eligible patients were included. There might be some institutional bias. Second, the mean observation period of 1.2 years was too short. The incidence certainly tended to be high, indicating the possible overestimation of NADM in this population. The current authors plan to address this issue by continuing to conduct cancer screening to obtain more accurate data. Third, this study did not include HIV-1-infected non-hemophiliacs or non-HIV-1-infected hemophiliacs who were age-matched to study participants, so the data on prevalence and incidence cannot be compared to data from other populations. Accordingly, whether data obtained in this study were specific to HIV-1-infected hemophiliacs or not it is unclear.

In conclusion, this study found that the prevalence and the incidence of NADM might be unexpectedly high in HIV-1-infected hemophiliacs under the mean age of 48.9 years, suggesting the NADM screening for this population in other hospitals should be strongly recommended.

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Long-term outcomes in patients undergoing resection, ablation, and trans-arterial chemoembolization of hepatocellular carcinoma in the United States: a national cancer database analysis

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Abstract: In the United States, hepatocellular carcinoma (HCC) incidence rates were approximately three times higher in over 30 years. To investigate the long-term outcomes of patients who underwent resection, ablation, and trans-arterial chemoembolization (TACE) of HCC, we analyzed the National Cancer Data Base (NCDB), which is a nationwide oncology outcomes database and covers approximately 70% of new cancer cases in the United States. A total of 56,512 patients with HCC in the NCDB during 2004-2013 were retrospectively analyzed. Results showed that liver resection (48.5%) and ablation (57.0%) were performed more frequently than TACE (31.5%) in patients with AJCC stage I HCC. The 5-year overall survival (OS) was significantly higher in patients undergoing resection (52.4%) than in patients undergoing ablation (40.5%; $P < 0.001$) and patients undergoing TACE (36.1%; $P < 0.001$). For patients with AJCC stage I, the 5-year OS of patients undergoing resection (51.6%; $P < 0.001$) and patients undergoing ablation (51.1%, $P = 0.005$) remains significantly better than patients undergoing TACE (40.0%). However, the 5-year OS did not differ significantly between patients undergoing resection and patients undergoing ablation ($P = 0.486$). Additionally, the findings of our study confirm that the sub-stratification of T1 category by HCC diameter in the AJCC staging eighth edition (*i.e.*, T1a, HCC diameter ≤ 2 cm and T1b, HCC diameter > 2 cm) is valid, with a 5-year OS of 54.1% and 50.4%, respectively ($P = 0.031$).

Keywords: Hepatocellular carcinoma, liver resection, ablation, trans-arterial chemoembolization, long-term outcome, United States of America

Introduction

Liver cancer is predicted to be the sixth most common cancer and the fourth leading cause of cancer-related death in 2018, worldwide (*1*). For males, rates of incidence and mortality are approximately 2-3 times higher than for females, with liver cancer being the fifth most common cancer and the second leading cause of death (*1*). Hepatocellular carcinoma (HCC) is the most common primary liver cancer and accounts for 75-85% of diagnoses, followed by intrahepatic cholangiocarcinoma (10-15%), and other rare liver histologies. The major epidemiological risks of HCC are chronic viral infections with hepatitis B virus and/or hepatitis C, alcoholic hepatitis, and non-alcoholic steatohepatitis (*1,2*).

In the United States, HCC incidence is increasing, and age-adjusted incidence rates of HCC were approximately three times higher in 2005 than in 1975 (*3*). HCC has several treatment options including liver resection, transplantation, ablation, trans-arterial chemoembolization (TACE), and systemic therapy. Herein, we sought to evaluate long-term outcomes of patients who underwent resection, ablation, and TACE for HCC using the National Cancer Database (NCDB).

Methods

Data source

The NCDB is a joint project of the Commission on Cancer of the American College of Surgeons and the American Cancer Society. The NCDB is a nationwide oncology outcomes database, which covers approximately 70% of new cancer cases in the USA and has more than 34 million patient records (*4,5*). This analysis of a publically available deidentified data set was exempt from the institutional review board.

Study cohort and variables analyzed

From NCDB during 2004-2013, HCC patients were

identified using primary site code C22 and histology code 8170. According to the primary surgery recode, patients with the code 20-90 were defined as those who underwent resection, and patients with the code 11-17 were defined as those who underwent ablation. A variable, RX_SUMM_CHEMO in the NCDB, was used for defined patients who underwent TACE. Patients who underwent 'single agent' (code 2) or 'multi-agent' (code 3) of chemotherapy variables as the first treatment were defined as those who underwent TACE. Age, sex, Charlson-Deyo score,7 years of diagnosis, largest diameter of HCC, the American Joint Committee on Cancer (AJCC) stage (6th and 7th editions), and survival were assessed.

AJCC Staging Manual, 6th, 7th, and 8th Editions

Recently, the AJCC has released the new staging manual, 8th edition (*6*) (Table 1), which has several changes to the T category from the 7th edition (*7*) (Table 2). The newest 8th edition staging system divided T1 category (solitary tumor in the seventh edition) into T1a category (solitary tumor ≤ 2 cm) and T1b category (solitary tumors > 2 cm without vascular invasion). The AJCC stage of our study is based on the 7th edition for patients treated from 2010-2013 and based on the 6th edition (*8*)

Table 1. AJCC staging Manual, 8th edition for HCC*

Primary tumor (T)				Regional lymph nodes (N)		Distant metastases (M)	
T1a	Solitary tumor ≤ 2 cm with/without vascular invasion			Nx	Regional lymph nodes cannot be assessed	M0	No distant metastasis
T1b	Solitary tumor > 2 cm without vascular invasion			N0	No regional lymph node metastasis	M1	Distant metastasis
T2	Solitary tumor >2 cm with vascular invasion or multifocal tumors, none >5 cm			N1	Regional lymph node metastasis		
T3	Multifocal tumors at least one of which is >5 cm						
T4	Single tumor or multifocal tumors of any size involving a major branch of the portal vein or hepatic vein or tumor(s) with direct invasion of adjacent organs other than the gallbladder or with perforation of visceral peritoneum						
Stage							
IA	T1a	N0	M0				
IB	T1b	N0	M0				
II	T2	N0	M0				
IIIA	T3	N0	M0				
IIIB	T4	N0	M0				
IVA	Any T	N1	M0				
IVB	Any T	Any N	M1				

HCC, hepatocellular carcinoma. *According to the *AJCC Cancer Stating Manual*, 8th editions (*6*).

Table 2. AJCC staging Manual, 7th edition for HCC*

Primary tumor (T)				Regional lymph nodes (N)		Distant metastases (M)	
T1	Solitary tumor without vascular invasion			Nx	Regional lymph nodes cannot be assessed	M0	No distant metastasis
T2	Solitary tumor with vascular invasion, or multifocal tumors, none >5 cm			N0	No regional lymph node metastasis	M1	Distant metastasis
T3a	Multifocal tumors at least one of which is >5 cm			N1	Regional lymph node metastasis		
T3b	Single tumor or multifocal tumors of any size involving a major branch of the portal vein or hepatic vein						
T4	Tumor with direct invasion of adjacent organs other than the gallbladder or with perforation of the visceral peritoneum						
Stage							
IA	T1	N0	M0				
IB	T2	N0	M0				
II	T3a	N0	M0				
IIIA	T3b	N0	M0				
IIIB	T4	N0	M0				
IVA	Any T	N1	M0				
IVB	Any T	Any N	M1				

HCC, hepatocellular carcinoma. *According to the *AJCC Cancer Stating Manual*, 7th editions (*7*).

(Table 3) for patients treated from 2004-2009.

Statistical analysis

Categorical variables are expressed in numerical figures and percentages and were compared among groups using Fisher's exact test or χ^2 test, as appropriate. Continuous variables were expressed as median values with the interquartile range (IQR) and were compared using the Kruskal-Wallis test. Overall survival (OS) was estimated using the Kaplan-Meier method. $P \leq 0.05$ was considered to indicate statistical significance, and all tests were two-sided. Statistical analysis was conducted with SAS (SAS Institute, Cary, NC).

Results

Study population

A total of 56,512 patients with HCC who underwent resection, ablation, and TACE was found in the NCDB from 2004-2013. Demographic characteristics are shown in Table 4. Median (IQR) age was 61 (55-69) years, and female sex was 24.4% of the cohort. There

Table 3. AJCC staging Manual, 6th edition for liver (including intrahepatic bile duct cancer)*

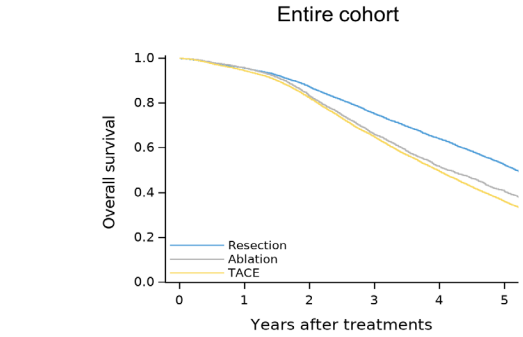
Primary tumor (T)				Regional lymph nodes (N)		Distant metastases (M)	
TX	Primary tumor cannot be assessed			Nx	Regional lymph nodes cannot be assessed	MX	Distant metastasis cannot be assessed
T0	No evidence of primary tumor			N0	No regional lymph node metastasis	M0	No distant metastasis
T1	Solitary tumor without vascular invasion			N1	Regional lymph node metastasis	M1	Distant metastasis
T2	Solitary tumor with vascular invasion, or multiple tumors none more than 5 cm						
T3	Multiple tumors more than 5 cm or tumor involving a major branch of the porta or hepatic vein(s)						
T4	Tumor(s) with direct invasion of adjacent organs other than the gallbladder or with perforation of the visceral peritoneum						
Stage							
I	T1	N0	M0				
II	T2	N0	M0				
IIIA	T3	N0	M0				
IIIB	T4	N0	M0				
IIC	Any T	N1	M0				
IV	Any T	Any N	M1				

*According to the *AJCC Cancer Stating Manual*, 6th editions (*8*).

Table 4. Demographic characteristics by treatments

Characteristic	All	Resection	Ablation	TACE	*P*
Number of patients	56,512	14,663	6,323	35,526	
Age, median (IQR), yr	61 (55–69)	61 (54-69)	62 (56-71)	61 (55-69)	< 0.001
Sex, n (%)					< 0.001
Male	42,700 (75.6)	10,732 (73.2)	4,611 (72.9)	27,357 (77.0)	
Female	13,812 (24.4)	3,931 (26.8)	1,712 (27.1)	8,169 (23.0)	
Charlson-Deyo Score (*9*)					< 0.001
0	26,148 (46.3)	6,710 (45.8)	2,733 (43.2)	16,705 (47.0)	
1	16,176 (28.6)	4,326 (29.5)	1,810 (28.6)	10,040 (28.3)	
2	14,188 (25.1)	3,627 (24.7)	1,780 (28.2)	8,781 (24.7)	
Year of diagnosis					< 0.001
2004-2006	9,891 (17.5)	3,867 (26.4)	1,416 (22.4)	4,608 (13.0)	
2006-2009	16,030 (28.4)	4,582 (31.3)	1,646 (26.0)	9,802 (27.6)	
2009-2013	30,591 (54.1)	6,214 (42.4)	3,261 (51.6)	21,116 (59.4)	
Largest diameter of HCC, cm	4.0 (2.5-7.0)	3.7 (2.2-6.7)	2.7 (2.0-3.8)	4.5 (2.8-7.5)	
AJCC stage,* *n* (%)					< 0.001
Stage I	6,219 (43.6)	4,471 (48.5)	354 (57.0)	1,394 (31.5)	
Stage II	4,714 (33.1)	3,015 (32.7)	201 (32.4)	1,498 (33.9)	
Stage III	2,213 (15.5)	1,502 (16.3)	42 (6.8)	669 (15.1)	
Stage IV	1,111 (7.8)	225 (2.4)	24 (3.9)	862 (19.5)	
Unavailable	42,255	5,450	5,702	31,103	

TACE, trans-arterial chemoembolization; IQR, interquartile range; HCC, hepatocellular carcinoma. *According to the *AJCC Cancer Stating Manual*, sixth and seventh editions (*7,8*).



Median survival (95% CI), yr	
Resection	5.2 (5.1–5.3)
Ablation	4.2 (4.0–4.3)
TACE	4.0 (3.9–4.0)

Patients at risk						
Resection	14663	11621	9185	6965	5315	3922
Ablation	6323	4790	3185	1929	1177	748
TACE	35526	20402	11996	7150	4469	2830

5-year OS (95% CI)	HR (95% CI)	*P* value
52.4% (51.4%–53.5%)	0.68 (0.66–0.70)	< 0.001
40.5% (38.6%–42.4%)	0.87 (0.83–0.91)	< 0.001
36.1% (35.1%–37.0%)	1 (reference)	–
Resection vs. Ablation	0.77 (0.74–0.81)	< 0.001

Figure 1. OS of the entire cohort. OS, overall survival.

were 14,663 patients (25.9%) who underwent resection (resection group), 6,323 (11.2%) who underwent ablation (ablation group), and 35,526 (62.9%) who underwent TACE (TACE group). Demographic characteristics were significantly different between the three groups (Table 4). Median largest diameter of HCC was significantly different between the three groups: resection group, 3.7 (IQR, 2.2-6.7) *vs*. ablation group, 2.7 (IQR, 2.0-3.8) *vs*. 4.5 (2.8-7.5), $P < 0.001$. Liver resection (48.5%) and ablation (57.0%) were performed more frequently than TACE (31.5%) in patients with AJCC stage I HCC.

OS of the entire cohort

For the entire cohort, the 5-year OS was significantly better in the resection group (52.4%) than the ablation group (40.5%; $P < 0.001$) and TACE (36.1%; $P < 0.001$) group and higher in the ablation group than the TACE group ($P < 0.001$) (Figure 1).

OS of patients with AJCC stage I

For the cohort including patients with AJCC stage I, the 5-year OS of the resection group (51.6%; $P < 0.001$) and ablation group (51.1%, $P = 0.005$) remains significantly better than the TACE group (40.0%) (Figure 2A). However, the 5-year OS was not significantly different between the resection and ablation groups ($P = 0.486$). OS curve of the resection group was further stratified by HCC diameter. Within the resection group, patients with HCC diameter ≤ 2 cm were significantly associated with better survival than patients with HCC diameter > 2 cm, with 5-year OS, 54.1% *vs*. 50.4%, $P = 0.031$ (Figure 2B).

Discussion

This large retrospective cohort study from a large nationally representative dataset, showed that resection and ablation were performed more frequently in patients with lower AJCC stage. When local therapy was chosen, TACE was more often selected for patients with higher AJCC stage. The 5-year OS survival of patients with HCC is 52.4% after resection, 40.5% after ablation, and 36.1% after TACE.

A recent report based on the Surveillance, Epidemiology, and End Results Program demonstrated that the 5-year relative survival rates for liver cancer in the United States between 2008-2014, was 31% for localized disease and 2% for distant metastases (*10*). In the current NCDB study, patients undergoing resection were associated with better survival, more than 50% at 5 years, although confounders among the three groups (resection, ablation, and TACE) were not adjusted. Additionally, the findings of our study confirm that the sub-stratification of T1 tumors by diameter in the AJCC staging 8th edition (*i.e*., T1a, HCC diameter ≤ 2 cm *vs*. T1b, HCC diameter > 2 cm) is valid. Similar to our results, a recent meta-analysis showed that ablation was associated with worse overall survival at 5 years than resection for patients with a single HCC of any size and up to 3 tumors all less than 3 cm (hazard ratio, 1.91; $P = 0.001$) (*11*). Another meta-analysis showed that resection had significantly better OS than TACE for patients with multiple tumors (hazard ratio, 0.65; $P < 0.001$) (*12*).

Liver transplantation has the advantage of removing the diseased liver together with HCC and it is regarded as an ideal treatment option for HCC patients associated with chronic liver diseases if donors are available. Liver transplantation is generally recommended in patients within the Milan criteria (single lesion ≤ 5 cm or up to three separate lesions, none larger than 3 cm) (*13*). Studies reported that the 5-year OS is similar between patents who have HCC within the Milan criteria and patients who had other indications. As a result, the Milan criteria is included in the Barcelona Clinic Liver

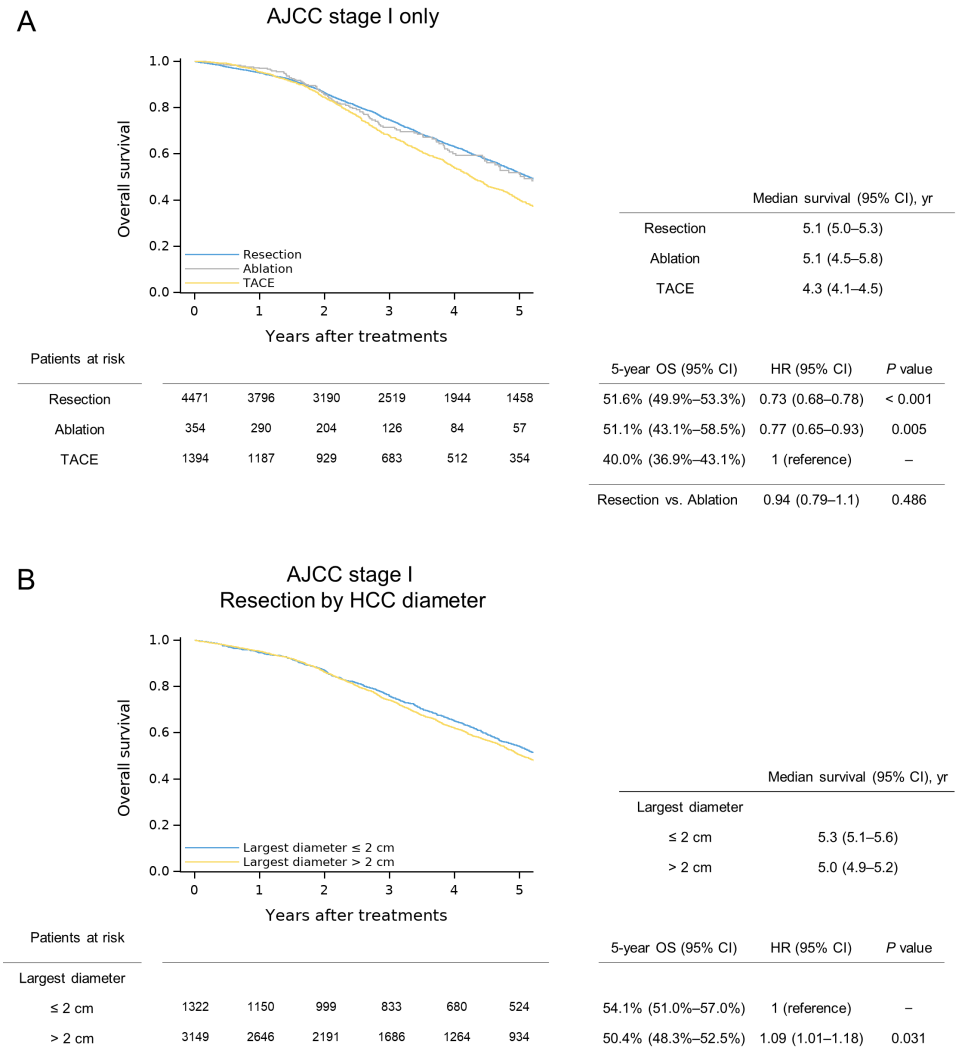


Figure 2. OS of patients with AJCC stage I. (A) By resection, ablation, and TACE; **(B)** Resection by HCC diameter ≤ 2 cm *vs.* > 2 cm. OS, overall survival; TACE, trans-arterial chemoembolization; HCC, hepatocellular carcinoma.

Cancer system and the American Association for the Study of Liver Diseases guideline. The 5-year survival of patients who underwent liver transplantation within Milan criteria is approximately 65-75% (*14,15*). Many studies reported the expanded Milan criteria and showed that patients within their criteria had comparable survival to patients within the Milan criteria. The long-term outcomes in patients beyond the Milan criteria need to be compared not only with patients undergoing liver transplantation within the Milan criteria but also with patients undergoing liver resection. Additionally, donor shortage, cultural limitations on deceased donors, and organ allocation remain unresolved barriers for unlimited deceased-donor and living-donor liver transplantations.

Potential limitations of this study are the direct result of using a national dataset. There are inherent limitations on data granularity such as knowing the surgical quality and understanding the reasons patients were triaged to one treatment modality over another. However, this limitation is counterbalanced by the tremendous statistical power available in a national dataset that no institutional study can offer. Within these confines, this study provides a contemporary overview of current treatment practices for HCC and modern prognostic expectations by stage and treatment.

In conclusion, based on this NCDB study, demographic and clinicopathologic characteristics were different between patients who underwent resection, ablation, and TACE, likely reflecting patient selection. Survival was better in patients undergoing liver resection *vs*. ablation and TACE. Further evaluation is needed to compare long-term outcomes between patients undergoing resection and patients undergoing liver transplantation beyond Milan criteria.

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