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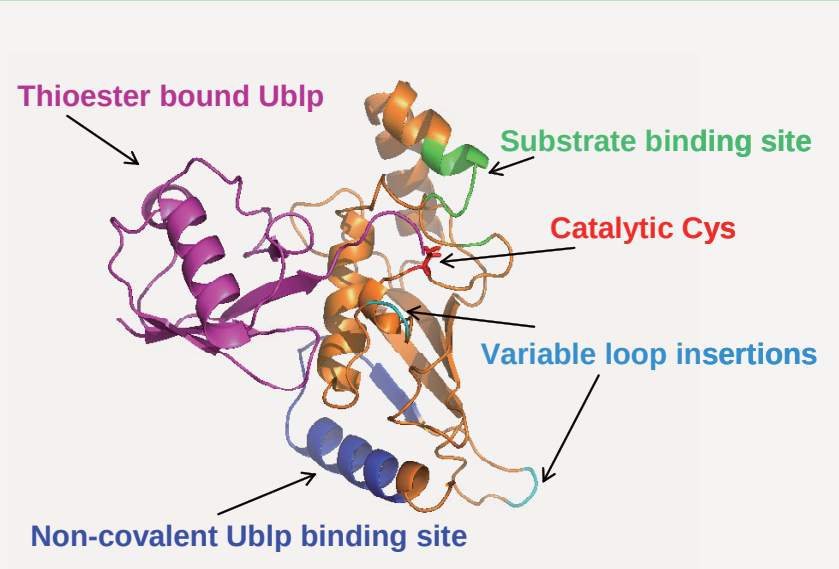

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Editorial

Masatoshi Makuuchi,
M.D., Ph.D.

President,
Japanese Red Cross Medical Center,
Tokyo, Japan.

I would like to invite the international scientific community to join me in welcoming this inaugural issue of *BioScience Trends*. Basic biological sciences and clinical practice, once considered to be entirely separate domains, are now converging toward the shared goal of streamlining the transition of scientific discovery from bench to bedside. We are simultaneously witnessing the globalization of this translational research effort. It is in the hope of fostering international dialogue aimed towards the common goal of improving human health worldwide that we offer *BioScience Trends* to encourage the rapid communication and dissemination of timely scientific and clinical research results between Asia and the West.

July 25, 2007

Masatoshi Makuuchi, M.D., Ph.D.

Editor-in-Chief
BioScience Trends

Editorial

Arthur D. RIGGS, Ph.D.

Director,
Beckman Research Institute of the City of Hope, Duarte, CA, USA.

It gives me great pleasure to introduce this inaugural issue of *BioScience Trends* to the worldwide scientific community. It is the hope of the editors that this new journal will provide an international forum for the exchange of ideas at the interface of biological and biochemical sciences and current clinical practice. Rapid improvements in biotechnology, completion of the various genome projects, and advances in proteomics and information sciences have propelled an information explosion of potentially unlimited benefit to biomedicine. The fervent hope is that these novel advances in basic biological sciences will translate into new and more efficacious medicines for the benefit of all humankind.

I have always held as an organizing principle for my own work that basic researchers should be informed about, and concerned about, human problems, including human disease. This was a major motivation behind our earlier efforts in the development of recombinant insulin and methods for the generation of humanized antibodies. Although it may seem more obvious now through the lens of modern experience, the power of basic research to tackle problems of immediate clinical relevance was not always appreciated. When Keiichi Itakura and I initially applied in 1976 to the United States National Institutes of Health for funding to express human proteins (somatostatin and then insulin) in bacteria using synthetic genes, our proposal was dismissed with the written critique including comments such as: "…the only possible outcome of this work would be to confirm that these manipulations can lead to the synthesis of a human peptide in *E. coli.* …. this appears as an academic exercise."

It is with this past history in mind that we welcome the arrival of *BioScience Trends* as an international medium to raise awareness of relevant clinical problems to the contemporary bench scientist, while simultaneously allowing the practicing clinician to keep abreast of relevant progress and discoveries in basic biology. It is our hope that this new resource will help foster the kind of interdisciplinary dialogue necessary to streamline the transition of discoveries at the basic research level to the realm of disease prevention and treatment, and to bring the promise of truly translational medicine to fruition.

Arthur D. RIGGS, Ph.D.
Co-Editor-in-Chief
BioScience Trends

Measles outbreak in Japan: Why now?

Kanako Masuno, Chushi Kuroiwa

Key Words: Measles, outbreak, Japan, vaccine, immunity

A significant number of schools and colleges in Japan have been closed for several weeks because of a measles outbreak this May. The National Institute of Infectious Diseases (NIID) reports a record 68 measles cases among individuals over the age of 15 in the period from the 14th to the 20th of May 2007, the highest recorded incidence since the reporting system was initiated in 1999. As of May 29th, the number of cumulative cases since January 2007 has reached as many as 286, of which 80% were individuals between the ages of 15 and 29. This outbreak has developed into a testament to the country's flawed vaccine policy.

In 2005, the NIID carried out a study targeting 5,614 populations that revealed that after the surge in measles antibody levels occurring in children between the ages of two and three years old the antibody levels in teenagers dropped remarkably, particularly between the ages of ten and fourteen. The proportion of individuals having antibody levels sufficient to prevent measles infection was around 80% within the vaccinated population and 50% within the non-vaccinated population of those from 10 to 14 years of age.

In developing countries, low antibody levels can be attributed to a limited cold chain and low vaccination coverage. Japan has developed an almost perfect infrastructure for the cold chain; however, immunization coverage from 1979 to 1994 was reported to be around 65%. During this period, the measles-mumps-rubella vaccine (MMR) had been introduced, followed by continuous reports of adverse effects such as aseptic meningitis, possibly due to MMR, resulting in a certain number of disabilities and deaths. The Government has offered compensations for those affected, as compulsory vaccination was being implemented under the law at that time. The Government paid bereaved families 43 million yen (about $353,000) and continues to pay each person affected 5.75 million yen (about $47,000) a year. This event could have made Japanese parents wary of vaccination, and the Government switched its immunization policy from a compulsory to a recommended regime in 1994. Significantly, the current outbreak has occurred among those who were born around this troubled period.

Another possible explanation for the low antibody levels in the population is the lack of opportunities to gain natural immunity. Some experts suggest that vaccine-induced immunity cannot be long sustained without natural infection. Because of the decrease in measles cases in Japan, younger generations might have fewer chances of acquiring natural immunity.

Because vaccination is now just a recommendation rather than a requirement in Japan, an individual's immunization history is not ascertained when entering school. Experts in the US criticized Japan's vaccination policy, claiming that Japan is exporting the measles overseas. Against this backdrop, the notoriously bureaucratic Japanese Ministry of Health, Labor, and Welfare in April 2006 finally adopted a policy calling for two doses of measles-rubella vaccine (MR), which was subsequently adjusted to one dose of measles vaccine. Hopes are that this policy will contribute to elevated and sustained immunity levels among Japanese children and minimize expected outbreaks to the extent possible.

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Dengue aggravation in developing countries in 2007

Xun Li, Ling-Zhong Xu

Key Words: Dengue fever, dengue hemorrhagic fever

According to Singapore's daily *Lianhe Zaobao*, dengue fever (DF) and dengue hemorrhagic fever (DHF) cases in 2007 may have hit a new high and represent a major public health problem.

The DF epidemic is not limited to Singapore, bringing calamity to most developing countries in the tropics. In Malaysia, for instance, 48 people died over the past five months as a result of DF, marking an increase of about 46% over last year. The DF peak has sounded the alarm bell for countries where dengue is endemic, but why is this epidemic conspicuously endemic to these countries? Why it is seldom seen in developed countries like the US, Japan, and nations in Europe? What measures should be taken for effective disease prevention and control in developing countries?

DF (or classic dengue; primary dengue) and DHF (or dengue shock syndrome, DSS; secondary dengue) are acute febrile diseases caused by four closely related virus serotypes of the genus virus, DEN-1, DEN-2, DEN-3, and DEN-4, that are transmitted to humans by the *Aedes aegypti* (and rarely *Aedes albopictus*) mosquito.

Tropical environments provide favorable conditions for mosquitos to breed and thus increase the risk of DF occurring. At the same time, global warming has made mosquitos more active; the geographic areas where they live have extended to both north and south of the equator, thus spreading DF more rapidly. Moreover, unremitting rainfall in the tropics may play an important role in dengue aggravation.

The reasons for the dramatic global emergence of DF/DHF are complex and not well understood; the natural environment is an important and inevitable factor, but more attention should be paid to several social factors.

First, major global demographic changes have occurred, the most important of which have been uncontrolled urbanization and concurrent population growth, especially in some developing countries. These demographic changes have resulted in substandard housing and inadequate water, sewer, and waste management systems, all of which increase *Aedes aegypti* population densities and facilitate transmission of *Aedes aegypti*-borne disease.

Second, the development of tourism in developing countries provides an ideal mechanism for infected human transport of dengue viruses between population centers in the tropics, resulting in a frequent exchange of dengue viruses and other pathogens.

Last, relatively poor hygiene in developing countries is another significant risk factor for dengue infection. Consequently, effective mosquito control is virtually nonexistent in most dengue-endemic countries. In other words, this epidemic has also exposed fatal flaws in these countries' systems of disease prevention and control.

Given that there is no dengue vaccine currently available, effective measures to control mosquitos are acceptable while awareness of hygeine is better, but the optimal solution to this problem is a sound system for disease prevention and control in accordance conditions in developing countries.

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Perspectives on liver transplantation in the People's Republic of China

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Key Words: Live donor, liver transplantation

The People's Republic of China (PRC), a country with rising geopolitical power, is also increasing its global presence in the field of liver transplantation.

The 2008 Beijing Olympics is just a little over a year away, and excitement and expectations are rising. Although in the 1932 Olympic Games there was a single entrant from mainland China under the rule of Chiang Kai-shek's Kuomintang nationalist party, the first official appearance of the PRC was at the Summer Games in 1984, officially known as the Games of the XXIII Olympiad, held in Los Angeles. The PRC had boycotted earlier games due to the Republic of China's presence as the Republic of China rather than as the PRC. In 1984, the Republic of China competed as Chinese Taipei and the PRC competed as China. Now, only a quarter of a century later, the country is hosting the international athletic celebration. The Olympics has fueled frenzies in many of the host cities in the past, and with less than 18 months to go before the games open in 2008, Beijing is no exception. China's economy is bright. As a whole, its growth is nearly as rapid as that of the capital: 11% last year compared with Beijing's 12% (its eighth consecutive year in double digits). The growth is clearly visible, as those who have recently paid a visit have observed the rapid changes and booming economic energy surge underlying this period of transformation. While there is some skepticism on the overall economic benefits the games will bring to the nation over the long-term, some calling it a mere temporary inflation caused by the Olympic spirit, there is little doubt that this is a time of increasing international exposure and an opportunity for positive cultural recognition.

The field of liver transplantation in China is not unaffected by this environment.

A group of pioneer surgeons, including Professors Qiu Fa-zu, Xia Shui-sheng, and others, led by Professor Lin Yan-zhen from Ruijin Hospital in Shanghai, made a heroic attempt in 1978, marking the initiation of liver transplant history in the PRC (*1*). Since then, although the field suffered a 10-year moratorium between 1983 and 1993 due to poor outcomes and relatively high costs, the introduction of better immunosuppression and refined surgical techniques has led to a current level of success in liver transplantation in the PRC comparable to that in Europe and North America. At present, liver transplantation is an accepted treatment for liver failure. There are approximately 10 transplant centers located in Tianjin, Guangzhou, Beijing, Hangzhou, Shanghai, Chengdu, and Wuhan. In each of these centers, more than 100 liver transplantations are performed annually. Apart from these major centers, more than 200 hospitals perform between 10 and 50 cases each year (*1*). On the whole, the total number of liver transplants reached 2,300 cases in 2004 and 3,500 in 2005, making the PRC the country with the second highest (next to the United States) rate of liver transplantations globally. The PRC is undoubtedly becoming a giant in this field.

Historically, this remarkable progress and achievement has, however, been tainted by two questionable practices: The use of executed prisoners as a source of deceased donor organs and the existence of so-called ''transplant tourism''.

Indeed, there is substantial published evidence that, sadly, the allegations of these practices, which have been condemned by human rights advocates, are not without grounds. In 1998, the Lancet reported the arrest of an "organ salesmen" in New York (*2*). The article reported that two Chinese citizens offering to sell human organs from prisoners executed in China were prosecuted, and further described that the event might be the tip of the iceberg, suggesting the practice of organ retrieval performed in the PRC against the Nuremberg Code and against the policy statement adopted by the Ethics Committee of The Transplantation Society in 1994. The event highlighted the serious need for the international community to confirm and to enter into discussions with the PRC regarding the internationally acceptable medical ethics standards of organ donation. Unfortunately, despite the recognized need, the matter was ignored until recently (*3*). As has been frequently reported, there is a tragic reality of an unbalanced supply and demand throughout the world. Transplant tourism involving paid living

donors has been reported in countries such as India, Philippines, Pakistan, and elsewhere. The effect of international condemnation and subsequent outlawing of such practices in these regions has only driaven these activities underground, where governmental agencies have little influence.

In the PRC, however, things seem to have changed for the better. Whether or not this is due to the exposure created by the Olympic Games, Chinese authorities and transplant centers are reacting positively. A recent statement by Dr. Jiefu Huang, Vice Minister of Health of PRC and Professor of Surgery of Peking Union Medical College in Beijing, that appeared in Liver Transplantation, an internationally acknowledged journal in the field, should be recognized as truly epoch - making in this sense. In the article, Huang reviews the historical background and ethical and legislative perspectives on liver transplantation in China. He describes that China is in the process of transition, and some socio-cultural beliefs and customs must be modernized to keep pace with social developments. With regard to the field of liver transplantation, he bravely admits that, "There is no doubt that Chinese medical ethics have not kept pace with rapidly changing technologies". He then introduces a major effort to push ahead with the revision of the current medical ethics instigated by the Ministry of Health. A draft for legislation involving the medical standards of brain death has been completed based on intensive consultation with national and international medical and ethics experts. Thereafter, the legislation was approved and came into effect May 1, 2007. It is true that in the midst of rapid technical developments in the PRC, medical ethics concerning organ donation and transplantation might continue to struggle. As Huang admits, "Even with anticipated national adoption of these guidelines, challenges will remain". Here, we must not be discouraged by the daunting burden the large country faces, but rather accept the positive message that has been expressed. The message is that the PRC has realized the problem, and that it is willing to cooperate with the rest of the world and honor the ethical commitment of the international society.

The problem of organ trafficking and solicitation, however, is far from being solved. The presence of the Olympic Games in the PRC, however, gains us a powerful ally. The exposure has resulted in the public admission of internal problems that liver transplant centers face in the PRC.

Laws have been enacted, which will give further opportunity to the international community to actively participate in helping the potentially largest transplant arena on the globe. We agree with the editors of the Liver Transplantation journal that we should remain optimistic that liver transplantation in the PRC will continue to progress and will soon adopt some, if not all, of the ethical principles that are recognized by the international community (*4*). With its emerging global presence and strong influence among the third world, we should anticipate its positive role in the future in all fields, including liver transplantation.

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Rapid progression of encephalopathy in a patient with hepatitis B infection

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SUMMARY **The mortality rate of fulminant hepatic failure was high until liver transplantation was presented as a potential therapy. We encountered a patient with hyperacute fulminant hepatic failure due to hepatitis B virus infection. Living donor liver transplantation was planned but abandoned because her brain edema progressed too rapidly to complete the donor evaluation. The present case reveals the limitation of living donor liver transplantation as a treatment for hyperacute fulminant hepatic failure.**

Key Words: Fulminant hepatic failure, brain edema, hepatic encephalopathy, hyperacute, fulminant hepatitis B

Introduction

Fulminant hepatic failure (FHF) is characterized by the acute onset of progressive jaundice, increased liver transaminase, prolonged prothrombin time, decreased liver size, and hepatic encephalopathy. The 1-year survival rate ranges from 65% to 92% in deceased donor liver transplantation (*1-4*) and 59% to 90% in living donor liver transplantation (LDLT, *5-7*). We encountered a patient with a rapid course of FHF and here discuss the indications of LDLT for FHF.

Case Report

A 22-year-old previously healthy woman felt general malaise on April 16th, 2004. Her body temperature became elevated 3 days after onset, and she was admitted to a hospital on April 21st. The patient was conscious and lucid; physical examination revealed no abnormalities except for mild conjunctival jaundice. Biochemical data were as follows: total bilirubin, 5.7 mg/dl (normal, 0.3-1.3 mg/dl); direct bilirubin, 3.5 mg/dl (0.0-0.2 mg/dl); serum aspartate aminotransferase, 6,090 IU/l (9-38 IU/l); serum alanine aminotransferase, 6,410 IU/l (4-36 IU/l); prothrombin time, 51.8 sec (10-13.5 sec); and ammonia, 111 μg/dl (< 90 μg/dl). Serologic analysis was positive for hepatitis B surface antigen, negative for hepatitis B surface antibody, positive for hepatitis B envelope antigen, negative for hepatitis B envelope antibody, and positive for IgM-hepatitis B core antibody.

Plasma exchange and hemodiafiltration were started. Methylprednisolone (1 g), interferon beta (3×10^6 U), and lamivudine (100 mg) were administrated. In spite of intensive medical care, the patient's consciousness was disturbed. She developed stage 2 encephalopathy (*3,8*, Table 1) 12 h after admission. She was diagnosed with hyperacute FHF due to hepatitis B infection (*9*).

She was transferred to our hospital on April 22nd for liver transplantation. On admission, her electroencephalogram showed diffuse slow waves. Computed tomography of the brain performed immediately after admission revealed mild brain edema. She was responsive only to noxious stimuli and her neurologic status had advanced to stage 4/grade 2. Corneal light reflex was preserved. Abdominal computed tomography revealed a total liver volume of 772 mL, corresponding to 80% of her standard liver volume (*10*).

Plasma exchange and hemodiafiltration were continued after admission to our hospital. Urgent transplantation was prepared although transplantation

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Table 1. Hepatic encephalopathy and coma classifications (*3,8*)

	Encephalopathy classification
Stage 1	Slowness of mentation and affect, euphoria
Stage 2	Drowsiness, inappropriate behavior, presence of asterixis
Stage 3	Incoherent words, marked confusion, reaction to vocal stimuli
Stage 4	Deep coma without vocal stimuli
	Coma sub-classification for encephalopathy stages 3 and 4
Grade 1	Uncoordinated reactivity to vocal stimuli
Grade 2	Absence of reactivity to vocal stimuli, coordinated response to nociceptive stimuli
Grade 3	Uncoordinated response to nociceptive stimuli
Grade 4	Brain death

was not indicated for the patient according to the criteria of the King's College group (*11*), Takahashi *et al.* (*12*), or Yoshiba *et al.* (*13*). The patient's 42-year-old mother was willing to donate part of her liver and we began the necessary physical, psychological, and biochemical examinations. During evaluation of the patient's mother as a potential donor, however, the patient's neurologic status progressed to stage 4 encephalopathy and grade 3 coma on April 23rd. An electroencephalogram showed electrocortical silence. Brain computed tomography showed that the sylvian fissures and cerebral sulcus had completely disappeared. LDLT was abandoned and the patient died 12 h after her arrival at our hospital (36 h after the onset of encephalopathy).

Discussion

In the present case, encephalopathy progressed rapidly. The time period between the appearance of jaundice and the development of encephalopathy was 36 h and the patient was classified as hyperacute (*9*). Evaluation and preparation of the potential living donor was not completed in time. Hattori *et al.* encountered two patients who suffered brain death within 3 days while awaiting LDLT (*14*). Donor safety must remain the first priority in high acuity situations, however, and the donor work-up is more difficult due to the time constraints (*15*). Careful screening for any conditions that represent an increased risk to the donor is essential. The same exclusion criteria that apply in elective situations must also apply in emergent cases, and no exceptions should be made to accommodate the needs of the recipient.

The incidence of neurologic death is 4% to 11% after deceased donor liver transplantation for FHF (*3,16*), suggesting that preoperative evaluation of the neurologic status or prediction of the neurologic results after transplantation is difficult. Whether LDLT should be performed for FHF with severe encephalopathy and brain edema is controversial. The Kyoto group treated a patient that developed widespread brain necrosis after LDLT with preoperatively diffuse brain edema (*14*), though the patient ultimately died of sepsis without neurologic recovery. Sterneck *et al.* reported three FHF patients that died of cerebral herniation after LDLT (*17*). Intracranial pressure is now monitored to evaluate brain edema (*18,19*) in patients with grade 3 or 4 coma. It is not used in our department, however, to avoid complications including hemorrhage and infection. The intracranial hemorrhage rate is 8% to 10%, which includes 2.7 % to 3.4% in fetal cases (*20,21*).

During donor evaluation, LDLT was contraindicated due to the advancement of the patient's encephalopathy. In high acuity situations, donor selection should be completed as soon as possible in the event of sudden progression of encephalopathy. The present case reveals the limitations of LDLT as a treatment for hyperacute FHF.

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Epidemiologic impact of invasion and post-invasion conflict in Iraq

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SUMMARY **There has been little systematic analysis of casualty data from the Iraq invasion and post-invasion conflict since 2003. Here we combine well known sources on military casualties and little known or understood sources on non-combatant mortality to identify major trends and impacts. This conflict is unique in many ways. It is associated with high risk of death to previously little affected groups – female and older adult soldiers. From the early days of combat, the conflict has resulted in a relatively high rate of death among soldiers, reversing a long term trend toward declining mortality among U.S. troops. Despite a high survival rate among those with serious injuries, it is the first conflict for which most deaths occurred after the end of major hostilities. Deaths among non-combatant groups are much higher, and have resulted in far more confusion regarding actual mortality rates. This is not surprising; in few of the major conflicts or humanitarian crisis have epidemiologic estimates been made. It is shown that pre-invasion projections regarding civilian casualties were uniformly mistaken regarding the major risks and risk levels to be faced. More research is needed to improve and standardize approaches to identifying mortality risk to major population groups.**

Key Words: Iraq, war, non-combatants, violence, soldiers

Introduction

The most contentious large-scale conflict in the last decade has been the coalition invasion, overthrow, and post-invasion military occupation of Iraq. Information on coalition troop casualties is relatively well publicized and widely known to the public, but information on casualties to other combatants and non-combatant groups is very limited and little known. Here we present an analytical summary of both kinds of information, during several different periods, through epidemiologic analysis. To do so we draw on published and unpublished reports, comparative analysis with prior conflicts, and personal knowledge of the health and information systems from years of work in that country prior to and following the 2003 invasion. The analysis follows evolving standards in epidemiologic analysis of intentional injury by identifying rates and risks among major relevant groups, starting with those for whom the most comprehensive information is available.

Coalition military casualties

Through December 31, 2006 there were 3,247 military deaths among non-Iraqi coalition forces (Table 1 and Figure 1) (*1*). Among these, 90 percent of all deaths occurred among U.S. troops. There were an estimated 9,200 deaths among Iraqi security forces, 133 deaths among Iraqi Kurdish coalition troops, at least 400 deaths among non-Iraqi contractors, and at least 92,000 deaths among suspected insurgents in Iraq.

About 2 percent of U.S. military deaths in Iraq have been among women. Though relatively small in number, these 60 deaths are greater than in any previous U.S. war. About 22 percent have occurred among reservists or members of the National Guard, and 25 percent among non-whites. Sixty percent of these deaths occurred among those under age 25, but the 12% of deaths among those over age 35 represent the largest proportion of deaths among older adults of any U.S. war.

The injured-to-dead ratio due to combat is 7:1 (*2*).

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Table 1. Timeline of war in Iraq

Coalition air attacks began.	(3/20/2003)
Saddam Hussein's statue pulled down.	(4/10/2003)
Bush declares major hostilities over.	(5/1/2003)
Economic sanctions on Iraq lifted.	(5/22/2003)
First meeting of the Iraqi Governing Council.	(7/13/2003)
Saddam Hussein's sons are killed.	(7/22/2003)
UN headquarters bombed.	(8/19/2003)
Madrid donors conference pledges about $2 billion per year for reconstruction.	(9/7/2003)
First helicopter downed.	(11/2/2003)
Oil for Food Program ends.	(11/21/2003)
Saddam Hussein captured.	(12/13/2003)
Interim constitution signed.	(3/8/2004)
Elections held for 275 seat Transitional National Assembly.	(1/20/2005)
New Iraqi government approved by parliament.	(4/6/2005)
National parliamentary elections and referendum on constitution.	(12/15/2005)
Saddam Hussein executed.	(12/30/2006)

In past wars, many more of the seriously injured died, producing a much lower ratio of 5:1 or 3:1. Among these injured, there an unprecedented low rate of 1.5 percent soldiers dying of wounds in 2003 (*2*). Like in Gulf War I, a high one-third of all deaths in the first months of the war occurred from non-hostile acts. This declined to around 15% of deaths within a year. After the first relatively peaceful months after the 2003 invasion, deaths resulting from improvised explosive devices grew to comprise more than a third of all deaths overall and more than half of all deaths in 2005. Body armor containing ceramic plates is partly responsible for the low rate of injuries to the torso. Of 598 soldiers treated at the 31st Combat Support Hospital in Baghdad, 14 percent suffered torso injuries. Among Iraqi prisoner patients, the rate was nearly twice that, at 27 percent (*3*).

One hundred nineteen U.S. military personnel and 61 other international Coalition forces were killed during the period of major hostilities through April 31, 2003. For the first time in any U.S. military engagement, most deaths occurred after the period of major hostilities ended. The total number of Coalition-troop deaths has also passed the 2,000 deaths among British soldiers to occur in the post-World War I occupation of Iraq.

Iraqi military casualties

No tracking system exists for deaths among Iraqi troops as the army was disbanded after the war. A total of 4,000 deaths among Iraqi soldiers is frequently used. In addition, a reported 5,500 soldiers were missing in action (MIA) at war's end. A higher figure of 9,200 plus 1,600 was estimated by the Defense Alternatives Project (*4*). Of these, the largest number, an estimated 2,878, died in the battle for Baghdad.

Non-military casualties

Internationals: Twelve international journalists, 24 relief workers and diplomats, and about 400 foreign insurgents during major hostilities in the first 6 weeks of the invasion. By 2006, the Iraq was had become the most deadly war for journalists, surpassing the Vietnam War (*5*).

Iraqi noncombatant casualties during major hostilities

In a change from policy during previous conflicts, the U.S. military has not provided information on deaths to non-combatants either during or after the period of major hostilities in Afghanistan or Iraq. General Tommy Franks and others have frequently repeated the new military refrain, "We don't do body counts." Yet on several occasions, the military has released just such civilian casualty figures. When asked about the contradiction, General Conway in a May 10, 2005, briefing responded: "You haven't heard me mention body counts....It does add perspective, but I don't think it's something we'll do as a matter of course" (*6*). He thus admitted, as military observers already knew, that indeed they do partial body counts, they just do not consider it frequently in their interests to report them.

During and immediately following the 2003 war, all-cause mortality was high. Baghdad hospitals reported 1,100 civilian deaths to the Ministry of Health during the war before the Hussein regime fell; among another

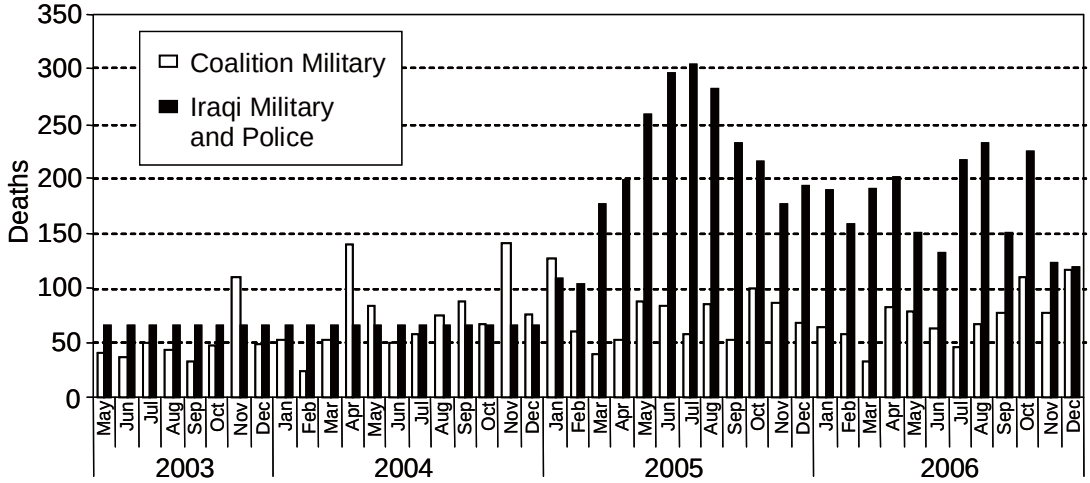

Figure 1. Coalition and Iraqi troop deaths, 3/2003 - 12/2006.

1,255 deaths it could not be determined if the dead were military or civilian (*4*). A review of hand-written reports from hospitals in the Baghdad area accounted for 1,700 war-related civilian deaths and 8,000 injuries by the time that major hostilities ended in late April (*7*). Hospitals throughout the country recorded about 2,000 war-related civilian deaths during April and May of 2003. Most Iraqi Shias are buried in the city of Najaf; there were about 2,000 extra burials during the war period (*8*). Only a fraction of these deaths were recorded the hospital system. Incident and press reports collected by Human Rights Watch account for about 700, representing about a third of these deaths. Taken together, the deaths recorded in hospitals and cemeteries exceed 4,000 deaths above normal levels during the 3 week period of major hostilities.

The Associated Press reviewed information from half of the hospitals in the country in June 2003 and accounted for a total of 3,200 civilian deaths (*9,10*). The Ministry of Health (MoH) statistical office attempted a more comprehensive accounting of civilian deaths starting in late 2003. These and subsequent efforts on civilian casualties have frequently met political interference (*11*). These data reflect that deaths recorded at hospitals may be as much as 80 percent of the total in normal times but only about half of all deaths during the period of major hostilities (*12*).

The problem is that press-based death reports are subject to undercount which cannot be estimated. As a similar evaluation in Guatemala found, the highest rate of deaths in that county's war occurred when press reports of deaths went down (*13*). Given the extremely high risk of death to journalists, press-based reports have progressively deteriorated in their ability to track a stable if small proportion of all deaths. Yet because they provide the only source for monthly monitoring of mortality trends among non-combatants, they are frequently used. The IBC database includes a minimum estimate of 3,480 deaths in March and 2,508 in April. This represents about 6,000 deaths among civilians during the period of major hostilities, or about twice the deaths recorded in hospitals and about 50 percent more deaths than were recorded in hospitals and cemeteries for this period.

Iraqi non-combatant casualties since the end of major hostilities

Excess deaths may occur from several causes. Intended or unintended victims of combat or sectarian operations are direct casualties. Many more excess deaths occur in some conflicts indirectly, as a result of injuries due to lawlessness, lack of health care, sanitation, water, or food, or inadequate access to essential goods and services due to a lack of security (*14,15*).

Apart from military engagements, there is steep rise in the number of homicides, especially those due to firearms. The average number of deaths recorded at the Baghdad morgue rose from around 200 per month before the war to 462 in May 2003, 626 in June, 751 in July, and reached 872 in August (*16*). They then leveled off at an average of around 600 per month for the following year (Figure 2). This rise was mostly the result of firearm injuries, which rose from 10 percent to more than 60 percent of the total. So called "accidental deaths" due to intoxication, burning, stabbing, road accidents, shooting, or drownings were the second most common cause, and also likely included some homicides.

Deaths recorded as homicides in the Baghdad morgue provide an indicator of trends in civil violence over time. The reported rate of homicides recorded there rose to a high of 185 per 100,000 in August 2003 (*17*). It then declined to about 100 per 100,000 in autumn and winter 2004. This represents a rate more than double that in the highest US cities and similar to rates in Colombia at its peak the early 1990s (*18*).

All count-based sources of information on non-combatant casualties are very incomplete. In 2006 the UN collected data from the central Baghdad morgue and reported hospital-based deaths due to violence, including crime (*19*). These data are about double the estimates produced by the Iraqi Body Count project's press-based reports (which exclude deaths known to be due to

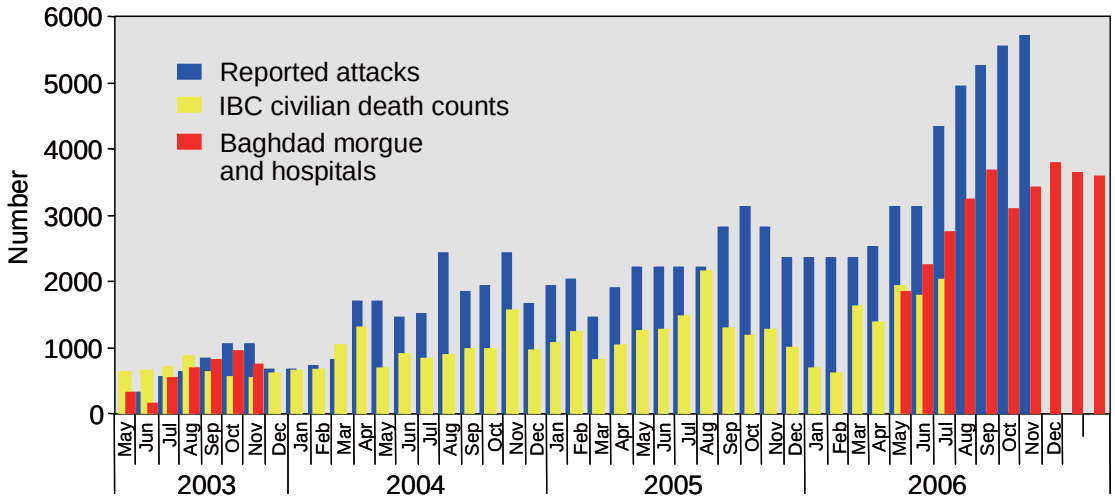

Figure 2. Count-based estimate of attacks and Iraqi non-combatant deaths.

crime (*20*)). But both sources fail to include data from many other morgues, hospitals that are not reporting, or people buried without presentation at a hospital or morgue. Two population-based sample surveys carried out interviews in 2004 to assess level of mortality. The first, the IMIRA survey carried out by the Norwegian Institute for Applied International Studies and the Central Statistical Organization (*21*) reached 21,000 homes in all 18 governorates during the April-June period using a two-stage randomization to collect a wide array of social indicator data. It reported a total of 24,000 conflict-related deaths (95%CI, 18,000-24,000) (*21*). The second involved researchers from US and Iraqi universities to assess levels and causes of mortality in the 15 months prior and the 18 months after invasion (*22*). It used a cluster sampling procedure to reach 7868 people representing 17 governorates in September and verified a sample of reported deaths by reviewing death certificates. It found a total of 98,000 excess deaths (95%CI, 8,000-194,000) of which 57,600 were deaths due to violence (Figure 3). Both studies underestimate deaths by not including households where no one has been left alive. The difference in results from these two sources is not surprising given their very different methods, training, and field supervision. But even their lowest calculated estimates are many times higher than the count-based sources.

A third survey, developed for a different purposes, interviewed returning US Army and Marine soldiers, 14 percent and 28 percent of whom reported, respectively, that they believed themselves to be responsible for the death of a noncombatant (*23*). Rates of deaths due to violence per day from these varied sources are summarized in Tables 2 and 4. None of the approaches to estimating mortality in post-invasion Iraq are able to reliably distinguish all combatant from noncombatant deaths or intentional from unintentional deaths. The social experience of excess mortality was well stated by one Iraqi observer: "We had hidden mass graves before. Now we have open mass graves," Al-Haili said. "Really, it is the same thing: We are losing our people" (*24*).

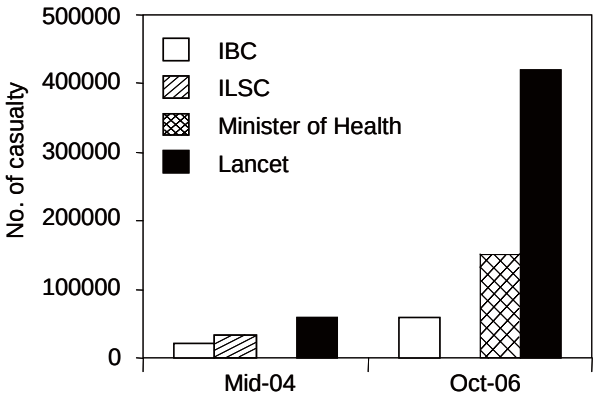

Figure 3. Civilian casualty estimates.
Sources: Iraq coalition casualty count. Accessed on December 13, 2006, at http://icasualties.org/oif/default.aspx.

The predominant cause of casualties through 2004 was actions by coalition troops. It then changed. Ministry of Health reporting during June 10-September 10, 2004, separated deaths by forces initiating violence. There were an average of 14 deaths per day by coalition-initiated actions and 6 from insurgent-initiated actions (*25*). Among this group, there were 59 deaths to children under age 12 in Anbar province, an area of major coalition attacks. In the same period there were 56 violent deaths among children in Baghdad, an area with five times more residents. The second household survey (*26*) also found that the most common cause of violent deaths in low-conflict communities was actions by coalition troops. Criminal activity and insurgent actions were the next most common causes. Far more injury deaths were reported in high-conflict Falluja in Anbar province, predominantly as a result of coalition actions.

By 2006 the level and pattern of non-combatant mortality had changed considerably. Using similar methods and a larger sample than the 2004 survey, a total of 651,000 excess deaths were estimated. This represents a level about ten times higher than the

Table 2. The state of Iraq: An update

	August, 2003	August, 2004	August, 2005	August, 2006
U.S. troop fatalities	36	65	90	63
U.S. troops wounded	181	891	608	641
Iraqi security force fatalities	65	65	282	233
Iraqi civilian deaths From violence	700	1,500	2,000	3,000
Multi-fatality bombings	4	13	27	52
Foreigners kidnapped	0	30	24	0
Internally displaced persons (since April 2003)	100,000	200,000	250,000	500,000
Attacks on oil assets	5	21	9	2
U.S./other Coalition troops in Iraq (in thousands)	139/22	140/24	138/23	140/19
Iraqi security forces (in thousands)	35	91	183	298
Iraqi security forces in top two readiness tiers(out of four; in thousands)	0	0	30	100
Oil production(in millions of barrels per day; prewar peak: 2.5)	1.4	2.1	2.2	2.2
Household and transport fuel supplies (as percentage of estimated need)	57	84	96	71
Average electricity production (in megawatts; prewar: 4,000)	3,300	4,700	4,000	4,400
Trained judges (estimated need: 1,500)	0	200	350	750
Registered cars (in millions; prewar: 1.5)	1.5	2.0	3.0	3.5
Children in school (in millions; prewar: 4.6)	4.6	4.8	5.1	5.2
Iraqis optimistic about the future (percent)	60	51	43	41

Source: Kamp N, O'Hanlon M, Unikewicz A. The State of Iraq: An Update. New York Times, October 1, 2006, p. 11.

cumulative count from the Iraqi Body Count and a shift to deaths primarily due to conflict between Iraqis rather than due to coalition forces.

Discussion

Mortality due to intentional injuries has been high since the 2003 invasion among both civilians and the military. The main cause of such deaths among civilians has changed from state-sponsored killings, to banditry and insurgency on the ground and air strikes from the air. The main cause of excess non-injury mortality has changed from lack of essential goods and infrastructure deterioration to reduced utilization and destruction of infrastructure resulting from insecurity.

The potential humanitarian consequences of the 2003 war were widely discussed in the months prior to the war. Research reports claimed that hundreds of thousands of people might starve, be killed, become victims of weapons of mass destruction, or become refugees (*3,4*). All of these estimates greatly over-estimated the mortality impact of the war on Iraqi civilians, but very seriously underestimated excess deaths and indirect effects of conflict after major combat ended.

Even conservative estimates place the number of deaths among civilians at more than 20 times greater than the number of deaths among coalition military personnel.

The resurgence of injury deaths in 2003 is unprecedented both among civilians and the military in U.S. post-war occupations. In no other conflict in a century did killing continue at such high levels (Figure 4). By September 2003, for the first time in a U.S.-led war, the total number of deaths after major hostilities ended exceeded the number during major hostilities. By January 2005, more than 90 percent of all Coalition troop deaths occurred after the end of major hostilities.

The number of coalition soldiers dying during the period of major hostilities was at an unprecedented

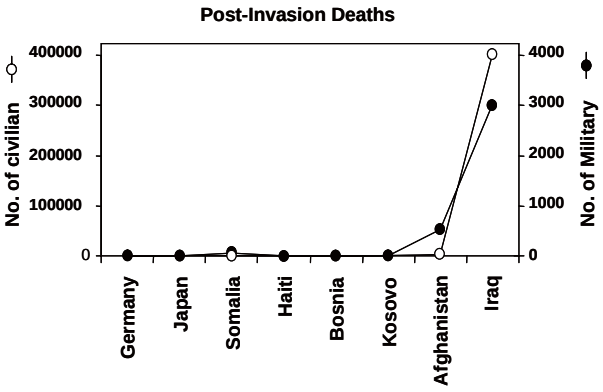

Figure 4. Deaths among civilians and military during eight occupations.

Table 3. Crude death rate among U.S. and other soldiers in various conflicts

Event	Deaths per 10,000 Soldiers Per Year	Total Deaths
World War I	142	106,700
World War II	68	407,300
Korean War	19	33,650
Vietnam War	8	58,100
Gulf War I, 1991	36	293
Gulf War II, 2003 (through 4/30/03)	119	171
Post-2003 Occupation - (4/30/03 - 12/31/04) U.S. Troops	48	1,353
Post 2003 Occupation - (4/30/03 - 12/31/04) Other Coalition Troops	40	140
Post-2003 Occupation - (4/30/03 - 12/31/04) Iraqi Security Forces	83	2,050

Sources: Garfield RM, Neugut A. Epidemiologic analysis of warfare: A historical review. JAMA 1991;266:688-692; Military casualty information http://web1.whs.osd.mil/mmid/causalty,castop,htm. Iraq coalition casualty count http://icasualties.org/oif/default.aspx, and http://www.brookings.edu/dybdocroot/fp/saban/iraq/index.pdf.

Table 4. Average number of estimated excess deaths per day, 2003 and 2004

Source	Dates Covered	Average Reported Excess Deaths per Day
Min of Health	4/5/2004 - 1/05/2004	20
Mass Bombings and Baghdad Morgue Deaths	2004	21
Interior Ministry	12/2003 - 5/2005	22
Iraqi Body Count	3/2003 - 12/31/2006	61
NGOs Committee in Iraq	Most of 2004	50
IMIRA (Norwegian)	3/1/2003 - 5/30/2004	56 (95%CI 42-69)
UN Assistance Mission for Iraq	1/1/2006 - 10/31/2006	92
Minister of Health	3/2003 - 11/2006	100
Roberts *et al.*	3/1/2003 - 9/21/2004	173 (95%CI 14-341)
of which, violent deaths		101
NEJM Mental Health Study	2003 - 2004	133
People's Kifah	3/2003 - 10/2003	152
Burham, *et al.*	3/1/2003 - 6/2006	510 (95%CI 306-740)

low in both 1991 and 2003 Iraq wars. This low number of deaths masked a resurgence in the mortality rate among U.S. soldiers. The mortality rate among soldiers since the end of major hostilities similarly exceeds that of prior U.S. wars in the last 50 years. The death rate among Iraqi security forces is about twice that of coalition troops (Table 3).

The quick response, careful assessment, and policy changes in response to 22 suicides among U.S. troops in Iraq was excellent. By contrast, there has been almost no attention to acute or chronic mental health needs of 27 million Iraqis. Following decades of political terror, family separation, repression and politicization of the very limited mental health services, the needs among Iraqis must be enormous. They remain, 3 years after the end of major hostilities, largely unknown.

A battle over the counting and representation of civilians deaths began in 2004 and has continued ever since. Non-combatant deaths seem to have become the most widely used indicator of the well-being of Iraqis in public policy debates. Unfortunately, the coalition appears to have put more effort into "spin and damage control" over the perception of civilian deaths than actions in the field to reduce these deaths. In 2005, the Bush administration started providing estimates, apparently based on IBC data, to systematically under-represent Iraqi non-combatant deaths.

The conflict in Iraq highlights the changing nature of conflict and difficulties with the definitions we use to analyze it. It is a war with no end in sight and for which no definition of an end exists. It is an undeclared war which was proclaimed to be over within weeks of the coalition invasion. It is the first war in which most of the coalition troop deaths occurred after the end of major hostilities. It represents a rising death rate compared to U.S. wars since WWII, and the highest rates of death among older adult and female coalition troops ever.

Asymmetric conflict between the world's only superpower and a little understood insurgency has generated tactics which continue to cause very high civilian casualties. New approaches and understandings for this war on terror are needed if we are to do a better job.

Acknowledgements

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The enzymes in ubiquitin-like post-translational modifications

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SUMMARY **Ubiquitin and at least ten ubiquitin-like proteins are important post-translational modifiers that regulate nearly every aspect of cellular function. These modifications require several chemical reactions that are catalyzed by at least three enzymes. Significant progress has been made in the structure-function analysis of these enzymes. This review describes new advancements in an understanding of the mechanisms of the enzymes catalyzing ubiquitin-like modifications, and highlights the important problems that remain to be addressed.**

Key Words: SUMO, ubiquitin, enzymes, X-ray crystallography, NMR spectroscopy

Introduction

The discovery that ubiquitin can conjugate to target proteins to regulate their cellular life spans and functions has revolutionized our understanding of eukaryotic regulation (*1*,*2*). Ubiquitin belongs to a family of at least ten homologous protein modifiers that conjugate to cellular target proteins using a similar biochemical mechanism (*3*,*4*). Post-translational modifications with these ubiquitin-like modifiers regulate nearly every aspect of cellular functions including immune response, viral and bacterial infection, gene transcription, RNA processing, DNA-repair, cell cycle progression, and intracellular trafficking.

Ubiquitin-like modifications are different from other post-translational modifications in that they require multiple enzymes to catalyze several sequential reactions (*5*). The chemical reactions leading to ubiquitination are adenylation, thioester formation, transesterification and isopeptide bond formation. Catalysis is strictly necessary for the adenylation step; the other reactions can occur in the absence of enzymes, but at much slower rates. For example, an intein-based method for protein ligation *in vitro*, which proceeds via similar chemistry as ubiquitination, can take as long as overnight to complete at room temperature (*6*). Other intracellular processes, such as protein lipidation and non-ribosomal peptide synthesis also use similar, catalyzed chemical mechanisms (*7*,*8*). The enzymes in all of these processes dramatically accelerate the reactions by mechanisms that are not yet well understood.

The ubiquitin-like modifications universally require at least four types of enzymes referred to generally as isopeptidase, E1 (activation enzyme), E2 (conjugation enzyme) and E3 (ligase) (Figure 1). A ubiquitin-like protein (Ublp) is usually synthesized as a precursor, which is matured by an **isopeptidase** to remove C-terminal residues and expose the Gly-Gly motif. Conjugation of a Ublp to target proteins then begins with **E1**, which catalyzes the adenylation of Ublp's C-terminal COOH group. The adenylated Ublp binds E1 non-covalently, and a thioester bond is formed between the SH group of a Cys residue on E1 and the C-terminal -COOH group of Ublp. Ublp is then transferred to a conjugation enzyme **E2**, where it forms a thioester bond with the -SH group of the catalytic Cys residue of the E2. In the final step, Ublp is attached to target proteins by the formation of an isopeptide bond between its C-terminal -COOH group and the ε-amino group of a Lys residue on the target protein. This step generally requires **E3 ligase**, although additional protein factors referred to as **E4** may also be involved in poly-ubiquitination of some proteins (*9*). The E1, E2 and E3 enzymes are commonly involved in nearly all ubiquitin-like modifications. Isopeptidases also remove ubiquitin-like proteins from modified targets, and thus regulate the levels of these modifications.

The ubiquitin-like post-translational modifications are dependent on protein-protein interactions between the enzymes, protein substrates, and Ublp to accomplish each step of the reactions leading to the modifications. The protein associations in these processes are dynamic, and a stable complex of all components does not exist (*10*). Characterizing the molecular mechanism

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of these multi-enzymatic processes is important to our understanding of how multi-protein machineries carry out macromolecular chemistry. This review will focus on the recent structural and enzymological studies of the enzymes in ubiquitin-like post-translational modifications.

The E1 enzyme and the transfer of Ublp from E1 to E2

A single and unique E1 enzyme is responsible for activating each ubiquitin-like modification (*11*). Both the ubiquitin and SUMO E1 enzymes are essential genes in yeast (*12,13*). The three-dimensional structures of the NEDD8 and SUMO E1 enzymes as well as several of their complexes have been solved (*14,15*). The structure of a small domain of ubiquitin E1 has also been solved (*16*). These E1 enzymes contain regions that resemble the bacterial proteins ThioS and MoeB (*17-20*). Both the SUMO and NEDD8 E1 enzymes are tight heterodimers of two polypeptides, which are homologous to the N-terminal and C-terminal portions of the ubiquitin E1, respectively. The overall structure of E1 contains three domains (Figure 2A). One domain contains the ATP-binding site and catalyzes the adenylation of Ublp. Another domain contains the catalytic Cys residue which forms a covalent thioester bond with the C-terminus of a Ublp. The third domain has a three-dimensional fold similar to that of ubiquitin in the absence of any sequence similarity, and is known as the Ubl domain.

Recently structural studies using both X-ray crystallography and NMR spectroscopy have greatly advanced our understanding of the mechanism of Ublp's transfer from E1 to E2. The Ubl domain, Cys domain and adenylation domain of E1 all participate in recruiting E2 for the transfer of Ublp from E1 to E2 (*21,22*) (Figure 2C). The Ubl domain has the highest affinity for E2 among the three E1 domains (*23*). It also has the flexibility to undergo a large scale rotation to properly position E2 (Figures 2B and 2C) (*22*). In addition, our NMR study has shown that the SUMO E2 has an intrinsic affinity for the Cys domain of its cognate E1 (Figure 2D) (*21*). The affinity between the E2 and the Cys domain of E1 is not high, but is important for the guided translocation of E2 to the catalytic Cys residue of E1 and for properly positioning the E2 for the transfer of SUMO from E1 to E2. The affinity between E2 and the adenylation domain is also expected to be weak, based on the small contact interface. The multiple low affinity binding sites on E1 for E2 provide an effective high affinity between the two enzymes to ensure efficient catalysis at low protein concentrations. At the same time, the low affinity of each site allows rapid protein association and dissociation for efficient catalysis.

The mechanism of how an Ublp translocates from the adenylation active site to catalytic Cys on E1 remains unclear. The structure of NEDD8 E1 in complex with NEDD8 and ATP, and the structure of SUMO E1 in complex with SUMO-1 and ATP have shown similar features of how the adenylated Ublps bind to their cognate E1 (*14,15*). The C-termini of both NEDD8 and SUMO are buried deeply within their cognate E1 enzymes near the adenylation active site. However, this site is distal (approximately 30 Å away) from the Cys residues with which it forms the thioester bonds in the subsequent step of the conjugation pathway (Figure 2E). The structures raise the question of how Ublp transfers with high efficiency and specificity between the two catalytic active sites of E1.

The E2 enzyme and its recognition of substrates and Ublp

Multiple E2 enzymes have been identified for ubiquitination of different target proteins (*1*), but only one specific E2 appears to be required by each of the ubiquitin-like proteins NEDD8 (*24*) and ISG15 (*25*). Additionally, a single E2 called Ubc9 serves all SUMO paralogues (*26*). The E2 enzymes are of variable sizes, but they all contain a core catalytic domain of approximately 150 amino acid residues (Figure 3). Some E2s consist of just the core catalytic domain, whereas others contain N- and/or C-terminal extensions of variable lengths beyond their core catalytic domains (*27*). There are also E2-like proteins involved in ubiquitin-dependent processes that lack the catalytic Cys residues and are catalytically inactive, such as the Tsg101 UEV domain and Mms2 (*28,29*).

The core catalytic domains of E2 enzymes have a highly conserved three-dimensional structure, with the biggest differences manifested in two surface loops of variable lengths (*30-33*). One of the variable loop insertions in E2 is located adjacent to the catalytic Cys residue (Figure 3). Mutations in this loop of Ubc9 affected the transfer of SUMO from E1 to E2 (*34*). Enzyme kinetic analysis indicates that this loop is also important for substrate recognition by the E2. A conserved Asn residue near the active site Cys has been identified as the catalytic residue that stabilizes the transition state oxyanion during the transfer of Ublp from E2 to target proteins (*35*).

Characterization of the SUMO modification pathway has provided a clear indication of direct substrate-E2 interaction. A consensus sequence, ψKxE (where ψ represents a bulky hydrophobic residue, K is a Lys, x is any amino acid, and E is a Glu), occurs in most SUMO-1 substrate proteins (*36*), although modifications at non-consensus sequences may occur much less frequently (*37*). This consensus amino acid sequence in substrates is a Ubc9 binding motif, and binds specifically to a region of Ubc9 near the active site Cys93 (Figure 3) (*38,39*). The substrate-binding surface

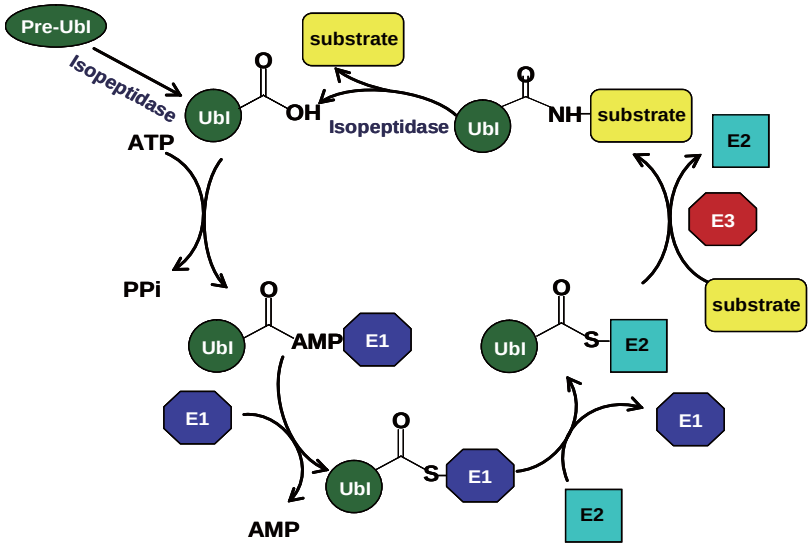

Figure 1. Schematic diagram of the chemical reactions involved in ubiquitin-like modifications.

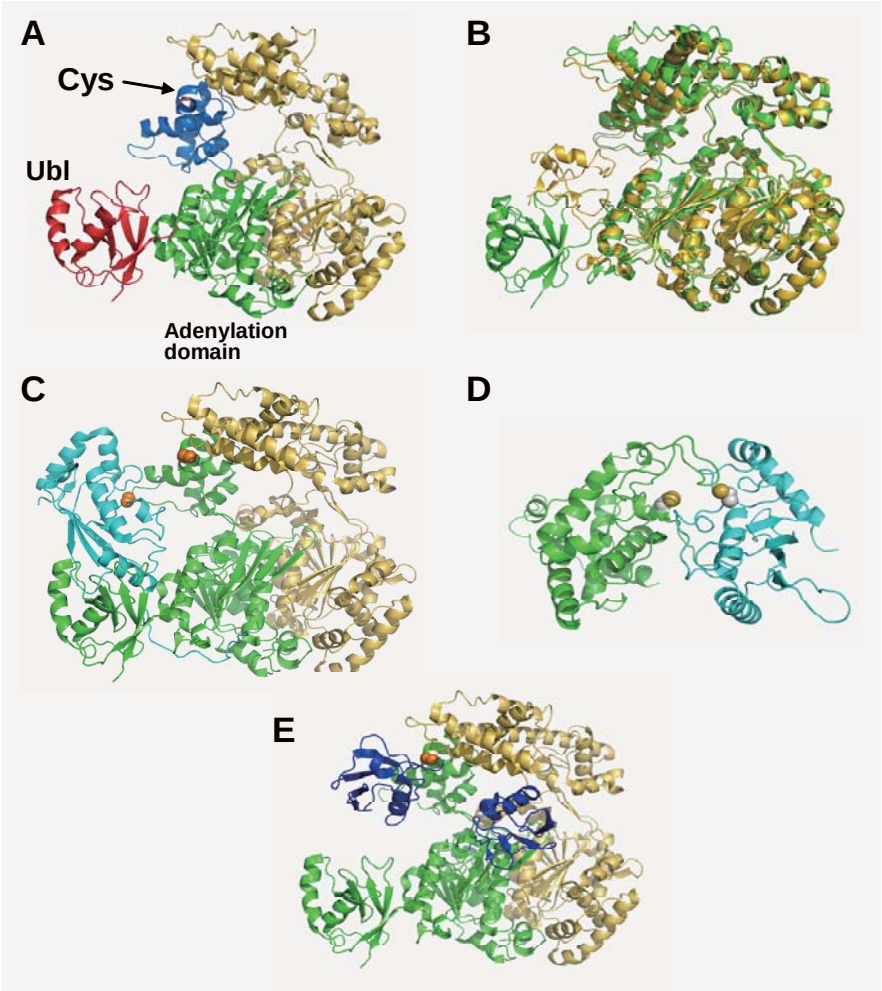

Figure 2. Summary of structural mechanism of E1. (A) The structure of NEDD8 E1 with different domains color as red, blue and green corresponding to the Ubl, Cys, and adenylation domains, respectively. The catalytic Cys is indicated in orange on the Cys domain. The APPBP1 subunit of the E1, which does not contain any catalytic active site, is indicated in gold. (B) Superimposed E1 structures from two different complexes to demonstrate the flexibility of the Ubl domain. (C) The complex of E2 and E1. E2 is colored in light blue. The two subunits of the NEDD8 E1 are colored with green and gold, respectively. The catalytic Cys residues of both E1 and E2 are shown with their sidechains in orange. (D) The interaction of the SUMO E2 (Ubc9, in blue) with the Cys domain of the SUMO E1 (green). The catalytic Cys residues of both enzymes are shown with their sidechains. (E) The structure of NEDD8 E1 in complex with two NEDD8, one non-covalently bond to the adenylation active site with the C-terminus deeply buried inside, and another NEDD8 covalently bond to the catalytic Cys. The two catalytic sites on E1 are distal from each other.

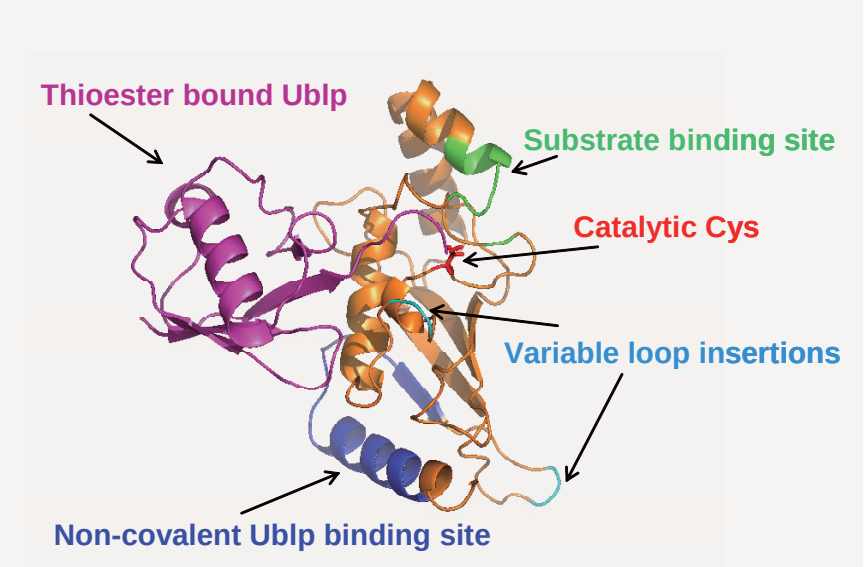

Figure 3. Summary of current knowledge of key functional sites of E2. The structure of Ubc9, shown in orange, is a representative of the conserved catalytic core structure of E2. The different functional sites are indicated in the figure. The thioester bound Ublp is shown in a position analogous to that in an ubiquitin-E2 thioester.

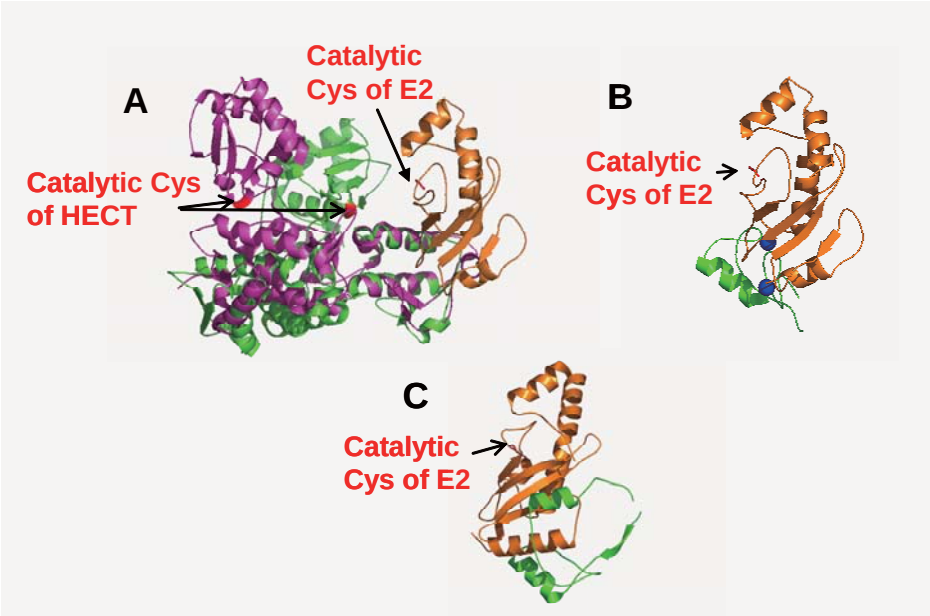

Figure 4. E3 structures and regulation. (A) The interaction between E2 and HECT motif. E2 is shown in orange and one HECT domain is shown in green. Another HECT domain is also shown in magenta in order to illustrate the conformational flexibility of HECT domains. The catalytic Cys residues of HECT domains are indicated in red. (B) The interaction between E2 and the RING motif. E2 is shown in orange and the RING domain is shown in green. (C) A complex between RanBP2 (green) and Ubc9 (orange).

has been previously shown to include residues that demonstrate significant dynamics on the microsecond to millisecond time scale (*40*). The conformational flexibility in E2 appears to be conserved, because the region of Ubc7 that is equivalent to Ubc9's substrate binding site also has one of the largest structural variations between crystal structures of the same protein determined from different complexes (*41,42*). The Lys residue where the modification occurs on substrates is found in a groove formed by the backbone atoms of residues Asp127, Pro128, Ala129 and the sidechain atoms of residues Asp127 and Tyr87 of Ubc9. In this position, the ε-amino group of the substrate Lys is 2.6 Å away from the Sγ atom of Ubc9's Cys93. Among the four residues that form important contacts with the Lys at the modification site, only the Pro and Ala are relatively conserved in E2 enzymes catalyzing ubiquitination. It remains to be established that a similar substrate binding mechanism is involved in the ubiquitination pathway; however, ubiquitination sites of target proteins do not have a consensus sequence. The direct interaction of substrates and Ubc9 may account for the lack of HECT-domain containing E3s in SUMO modifications.

Ubiquitin E2 enzymes have been shown to form homo and heterodimers. For example, Ubc3 (CDC34)

forms a heterodimer with the DNA repair related Ubc2 (Rad6) (*43*) and also forms a homodimer facilitated by the formation of ubiquitin thioester (*44*). These dimers are required for the degradation of many key regulators of cell cycle progression through the proteosome pathway. For example, a Ubc6 and Ubc7 heterodimer is responsible for the turnover of Matα2 transcription factor in yeast (*45*). Dimerization of E2s may be responsible for the formation of poly-ubiquitin chains in some cases; however, dimerization does not appear to be a general requirement for poly-ubiquitination. For example, Ubc5 does not appear to form homo- or hetero-dimers *in vitro* or *in vivo* (*40*), but Ubc5 can catalyze the formation of poly-ubiquitin chains (*45*).

Ublp-E2 thioester is an important intermediate in ubiquitin-like conjugation. Biochemical studies have shown that different ubiquitin-E2 thioester have different stabilities (*46*). Several structural characterizations of ubiquitin-E2 thioester covalent complexes have been carried out using NMR methods. In one study, the catalytic Cys residue was mutated to a Ser to produce an Ubc2b-ubiquitin oxyester, which has a more stable covalent linkage than thioester, and the oxyester was purified for NMR characterization (*47*). In other studies, productive thioesters were made in the NMR tube by the addition of E1 and ATP (*48,49*). These studies have shown that thioester bound ubiquitin contacts the surface of E2 that is centered on the second α-helix, which is adjacent to the active site Cys (Figure 3). The binding interface on ubiquitin is located on the β-sheet. The first study also demonstrated that the oxyester-bonded ubiquitin has linewidths that are similar to that of the free protein, and this suggests that ubiquitin moves somewhat independently from the covalently bound E2.

A specific and conserved non-covalent interaction between Ublp and E2 has been observed across the ubiquitin-like proteins (*23,47,49-51*). The non-covalent interaction between SUMO-1 and Ubc9 involves interfaces that are structurally conserved in Ublps and E2 enzymes. The binding site on Ubc9 covers the N-terminal helix, following β-strand, and the loop between them (Figure 3), and the binding surface on SUMO-1 is located on the main β-sheet (*50*). This interaction is conserved across SUMO paralogues, and is both enthalpically and entropically driven (*26*). Similar interactions have been observed between an E2 and ubiquitin, between the E2-like protein Mms2 and ubiquitin (*47,49,51*). However, the ubiquitin-like domains of the NEDD8 and SUMO E1 enzymes bind to their E2 in a similar, but clearly distinct manner (*23,52*). The functions of the non-covalent Ublp-E2 interaction are unclear. Mutations in human Ubc9 that disrupt the interaction with SUMO were shown to significantly reduce the transfer rate of SUMO-1 from E1 to E2 without affecting the transfer of SUMO from E2 to substrates (*26*). Similar amino acid substitutions on ubiquitin E2 hindered its catalysis of poly-ubiquitin chains (*53*). Further studies are necessary in order to understand the role of Ublp-E2 interaction.

The E3 and E4 enzymes

E3 ligases have received the most attention among the three enzymes involved in ubiquitin-like modifications, because many E3s are key players in essential cellular processes, and/or are prominent oncogenes and tumor suppressor genes. For example, one of the proteins frequently mutated in breast and ovarian cancers, BRCA1, has E3 ligase activities for both ubiquitination and sumoylation (*54*). The SCF (Skp1-Cullin-F-box protein) complexes and APC (anaphase-promoting complex), which are critical for cell-cycle progression, are ubiquitin E3 ligases (*55-58*). The host ubiquitin-like modification systems can be hijacked for pathogen infections. For example, the human papilloma virus (HPV) encoded E6 protein and E6-AP (E6 associated protein) form a complex that functions as an E3 ligase to reduce the level of the tumor suppressor protein p53 in HPV positive tumors (*59,60*). Knowledge about E3 ligases has enabled the development of research tools. For example, over-expression of specific ubiquitin E3 in cells can achieve specific and sustained knockout of only a subset of a target protein (*61*). Most E3s that catalyze ubiquitin-like modifications contain either the HECT or the RING domain (*62*). Both the HECT and RING domains are E2 recognition motifs (*41,42,63-65*).

HECT containing E3s receive ubiquitin from E2 to form thioester bonds with ubiquitin before transferring it to substrate proteins. The three-dimensional structures of several HECT domains in complex with E2s have been determined by X-ray crystallography (*42,66,67*). The HECT domain contains three subdomains; the N-terminal subdomain contacts E2 directly, the C-terminal subdomain contains the catalytic Cys residue, and the middle subdomain separates the two (Figure 4A). The linkers connecting the different subdomains have considerable flexibility, which allows the subdomains to move quite independently. Such domain movement can change the distance between the catalytic Cys residues on HECT and on E2 from 40 Å apart to close enough that the transesterification reaction can happen efficiently (Figure 4A). There is likely a general base to stabilize the transition state in which the HECT thioester bond is broken and the substrate isopeptide bond is formed. However, such a residue has not been identified in the HECT motif.

The RING-containing E3s constitute the largest family of E3 and can be divided into two sub-families, Zn-binding RING and Zn-independent RING-like fold formed by the U-box motif (*65,68*). RING motifs bind to the same site of E2 as the HECT domain despite the absence of sequence and structural similarity to the HECT domain (Figure 4B). Some U-box containing

proteins have been shown to function as E4s, which recognize mono-ubiquitinated proteins and catalyze the formation of long poly-ubiquitin chains (*9*). RING-containing E3s do not form thioester intermediates with ubiquitin, but bind to both E2 and substrate proteins. Both E3 recognition of substrate proteins and E2 enzymes are critical for catalyzing substrate modifications. For example, E3s in the N-end rule pathways recognize specific N-terminal residues of their target proteins (*2*). Most SUMO E3 enzymes contain RING domains and belong to the Siz/PIAS family of proteins (*69-72*). These SUMO E3s are similar to RING containing ubiquitin E3s in that they use the RING domains for binding E2 and also contain separate domains for binding target proteins.

Biochemical and structural studies have not provided a clear understanding of how RING-containing E3 ligases activate the transfer of Ublp from E2 to substrates. Because substrate and E2 binding activities are both required for the function of most of these ligases, the RING-containing E3s are thought of as adaptors that bring substrate and E2 together. Structural studies of a SCF complex indicate that the substrate and E2 binding sites on this E3 are surprisingly distal (as far apart as 50 Å) (*41,42,63,73-76*). Such a wide distance between the substrate and E2 was contrary to the efficiency of the E3 (*77*). In one study, a flexible linker that was engineered in the Cullin protein of a SCF complex destroyed the E3's enzymatic activity, indicating that the rigidity of SCF complexes is important for their ligase activity (*63*). This is clearly different from the catalytic mechanism employed by HECT domains. Additionally, there are increasing biochemical data contradicting the theory that these E3s are merely adaptors. For example, it has been shown that a small subunit in APC containing only the RING domain, but not the substrate binding domain, could enhance APC specific ubiquitination (*78,79*). Another study also showed that simply bringing an E2 and a substrate close together by fusing a substrate protein to the C-terminus of an E2 was not sufficient to bypass E3 to activate ubiquitination of the substrate protein (*80*). It has also been shown that E3 interacts with E2 differently from interacting with E2-ubiquitin thioester (*81,82*). However, the structural details of the interaction between E2-ubiquitin thioester and an E3 have not been characterized.

Another mystery in the E3 mechanism is the way in which poly-ubiquitin chains are formed. Poly-ubiquitin chains can form on E2 first and are then transferred to substrates, or ubiquitin is added one by one from an E2 to substrate proteins (*83*). Thus, the E3 catalyzed reactions have substrate "flexibility", which is either the target protein or the ubiquitin moiety of the ubiquitinated target protein. On the other hand, the reaction has clear "specificity"; in most cases, the poly-ubiquitin chains are formed on a very specific Lys residue (*e.g.* Lys48 or Lys63) on ubiquitin, instead of randomly on any Lys residue. The substrate flexibility and specificity in ubiquitination is distinct from most well characterized enzymatic reactions. In some cases, an E3 ligase only catalyzes mono-ubiquitination, and then an E4 takes over to add poly-ubiquitin chains to target proteins (*9*). However, in most *in vitro* reactions, poly-ubiquitination readily occurs with the addition of E3 and in the absence of E4.

Compared to the E1 and E2 enzymes, the E3 enzymes are much less conserved. For example, a SUMO E3 RanBP2 (also known as Nup358) (*84*) do not contain HECT, RING or U-box domains (*84*). Unlike other characterized E3s, RanBP2 does not form detectable non-covalent complexes with target proteins, nor does it form thioester bonds with SUMO-1 (*84*). The three-dimensional structure of the region of RanBP2 that has the E3 ligase function in complex with Ubc9 and sumoylated RanGAP1 has been solved by X-ray crystallography (*85*), which shows that the interaction between Ubc9 and RanBP2 is distinct from previously characterized E2-E3 interactions (Figure 4C). In this case, the E2-E3 interaction involves a surface of E2 that is different from that involved in binding the HECT or RING containing E3s (*86*). In general, it is not clear how the structurally distinct RING-containing E3s and RanBP2 achieve similar functions in the absence of sequence and structural conservation.

An interesting finding from the RanBP2-related studies is that the interaction between SUMO and E3 is critical for the E3's ligase activity (*85*). The M-IR2 domain of RanBP2, which binds specifically to SUMO-1 and not to SUMO-2, can catalyze SUMO-1 but not SUMO-2 modifications (*86*). This is the first E3 ligase in which the interaction between an E3 ligase and the ubiquitin-like protein has been shown to be important for the ligase activity, although it has been known for more than 20 years that some ubiquitin E3 ligases bind ubiquitin non-covalently (*10*).

Enzyme kinetic analysis of the conjugation process

Quantitative enzyme kinetic analysis is necessary to provide insights into reaction mechanisms that would be difficult to obtain otherwise. Quantitative enzyme kinetic approaches have been developed to examine every step of the reactions that lead to ubiquitin-like modifications. An elegant quantitative framework has been developed to characterize each step of E1 catalyzed reactions (*87*). Steady-state methods can be used to obtain enzyme kinetic information for the E2 and E3 enzymes (*39,82*). These approaches take advantage of the fact that in a reaction requiring multiple enzymes, kinetic information on a particular enzyme can be extracted when this enzyme limits the overall reaction rate. For example, in sumoylation reactions, the conditions can be set so that E2 limits the rate of the

overall reactions. Therefore, the kinetic parameters obtained reflect the properties of E2 (*39*). A non-steady-state kinetic approach (*34*) was also developed based on the transfer experiments pioneered in studies of the ubiquitin modification pathway (*10*). The advantage of the non-steady-state kinetic analysis is that one can extract individual kinetic constants, such as on-rate and off-rate of the enzyme-substrate association, and the catalytic rate constant in order to gain detailed insights into each step of the reaction. Fluorescence labeled protein substrates are extremely useful for evaluating the reaction rates (*88*), particularly in quantitating the heterogeneous products of ubiquitination reactions (*89*).

The deconjugation enzyme

The deconjugation enzymes in the ubiquitin and homologous pathways have two functions: to activate the Ublp and to remove Ublp from target proteins (*90*). As mentioned above, the first step in Ublp activation is the cleavage of the C-terminal residues by deconjugation enzymes to expose the Gly-Gly motif. The deconjugation enzymes are also important in regulating Ublp's modifications by removing these modifications.

The deconjugation enzymes can be classified into different families: the ubiquitin C-terminal hydrolases (UCH) that remove small peptides from the C-terminus of ubiquitin (*91*), ubiquitin-specific processing proteases (UBP) that remove mono- and poly-ubiquitin modifications (*92*), the ubiquitin-like proteases (ULP) that act on SUMO and NEDD8 (*93-96*), the JAMM motif-containing metalloproteases that act on ubiquitin and NEDD8 (*97*), and members of the ovarian tumor (OTU) superfamily (*98*). The different families of deconjugation enzymes do not share significant sequence similarities and overall folds. Except for the JAMM motif-containing metalloprotease family, the other four families of deconjugation enzymes have conserved geometry of the catalytic triad formed by a His, an Asn and a Cys (*99-104*). They belong to the cysteine protease superfamily. Therefore, inhibitors of the cysteine proteases can also inhibit these deconjugation enzymes. The JAMM motif-containing proteases have diverse functions, including deubiquitination and degradation by the 26S proteasome, and deubiquitination of proteins at the endosome (*105*).

The deconjugation enzymes present excellent targets for developing research tools and therapeutics because they are critical for regulating these post-translational modifications, and thus directly interfere with related biological functions. For example, the deubiquitination activity of CYLD, which encodes an UBP and has UBP activity, has been shown to play a role in regulating the activity of NF-κB (*106*). Mutations in CYLD are linked to familial cylindromatosis, which is an autosomal dominant predisposition to tumors of skin appendages. Most cancer associated mutations of CYLD result in truncations or frameshift alterations of the UBP domain. Herpes virus associated ubiquitin-specific protease (HAUSP or USP7) is responsible for deubiquitination of ubiquitinated p53 and promotion of p53 dependent cell growth arrest and apoptosis (*107*). Dysfunction in UCH has been associated with neurodegeneration and cancer (*108*). In the human genome, there are many uncharacterized proteins that resemble the sequences of deconjugation enzymes. Their functions as deconjugation enzymes for ubiquitin or ubiquitin-like modifications remain to be established.

Interplay between different post-translational modifications

Ubiquitin-like modifications are regulated by each other and by other post-translational modifications. For example, the ubiquitin-like protein NEDD8 modifies the cullin family of proteins, which are subunits of the SCF family of ubiquitin E3 ligases (*55*). Sumoylation and ubiquitination sometimes occur at the same Lys residue and antagonize each other (*109*). The oncogene MDM2, which contains a RING motif, has the ligase activity for both NEDD8 and ubiquitin modifications of the tumor suppressor protein p53 (*110*). Phosphorylation and dephosphorylation of target proteins can regulate the interactions of target proteins with E3 ligases (reviewed in (*62*)). Sumoylation of a transcription factor or histone triggers recruitment of histone deacetylases (HDAC) to remove histone acetylation and thus leads to transcriptional repression (*111,112*). Ubiquitination of histone H2B regulates its methylation and leads to gene silencing (*113*).

Concluding remarks

Significant progress has been made in elucidating the molecular mechanism of the enzymes involved in ubiquitin and ubiquitin-like modifications. Ubiquitin-like modifications are similar to other intracellular macromolecular chemical reactions, such as DNA repair, gene transcription, and RNA processing, in that they require multiple proteins to catalyze multiple reactions. The common theme in these processes is that the protein-protein interactions often involve multiple, medium to low affinity binding sites. The multi-valent, medium to low affinity interactions allow for the rapid turn over of the enzymes and the efficient catalysis at low enzyme and substrate concentrations. Similarly, low affinity, multi-valent protein-protein interactions are also common in Ublp-dependent protein-protein interactions. Ubiquitin-like proteins provide additional docking sites for protein-protein interactions. However, the affinities of ubiquitin-binding motifs for ubiquitin or the SUMO-binding motif for SUMO were not high with the K_d in

the 10-100 μM range (*114*). Despite the low affinities, these interactions play critical roles in the presence of other low or medium affinity interactions, and offer a mechanism to turn protein-protein interactions on and off quickly by conjugation and deconjugation of Ublps. Ubiquitin-like modifications are important in nearly every aspect of cellular function. Therefore, understanding the mechanism of the enzymes catalyzing these modifications will lead to the development of strategies to manipulate them for developing research tools and novel therapeutic approaches.

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High-resolution mapping of copy number aberrations and identification of target genes in hepatocellular carcinoma

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SUMMARY **Hepatocarcinogenesis involves complex combinations of molecular events, such as genetic aberrations, epigenetic changes, and alterations in gene expression. To elucidate the mechanism of hepatocarcinogenesis, it is necessary to reconstruct these molecular events at each level. This article presents a review of copy number analyses of hepatocellular carcinoma (HCC) using traditional comparative genomic hybridization (CGH), array-based CGH (aCGH), and single nucleotide polymorphism (SNP) arrays. A number of studies have applied CGH technology for copy number analysis of HCC and have indicated the significance of correlations of frequent genomic aberrations with various clinicopathological parameters, prediction of recurrence and prognosis, and treatment selection, followed by comprehensive genomic analysis using aCGH with much higher resolution. Furthermore, we present our data regarding genomic aberrations of HCC obtained using the Genome Imbalance Map (GIM) algorithm, which simultaneously detects DNA copy number alterations and loss of heterozygosity using SNP arrays, and the Expression Imbalance Map (EIM) algorithm, which detects mRNA expression imbalance correlated with chromosomal regions. Using these two algorithms, we integrated the expression profiles, locus information, and genomic aberrations in a systematic manner, which is effective for detecting structural genomic abnormalities, such as chromosomal gains and losses, and showed that gene expression profiles are subject to chromosomal bias.**

Key Words: Liver cancer, karyotyping analysis, high-resolution mapping, copy number alterations

Introduction

Cancer is a genetic disease of somatic cells arising from accumulation of genetic changes, and abnormalities of suppressor genes and oncogenes are frequently associated with carcinogenesis. To stratify patients and select the most appropriate treatment options for hepatocellular carcinoma (HCC), many staging systems from the standpoint of clinical information and pathological classification have been proposed (*1*,*2*). However, despite improvements in these trials, prognostic predictions for HCC are still not fully acceptable for selection of individualized treatments (*3*). Therefore, there has been a great deal of effort using molecular biological technologies to establish prognostic models for HCC.

Many researchers have reported genomic decoding regarding carcinogenesis, invasion, and metastasis of liver cancer (*4-14*). Furthermore, considering the complexity of carcinogenesis, many other genes may be involved in both the initiation and progression of cancer, and comprehensive expression analysis using microarray technology has great potential for the discovery of new genes involved in carcinogenesis (*15*).

In addition to identification of novel candidate genes for biomarkers and the discovery of therapeutic targets, which are helpful for improvement of clinical diagnosis and treatment (*16*,*17*), classification and selection of predictor genes for HCC using genome-wide expression analysis have been reported (*18*). Okabe *et al*. reported gene expression profiling analysis

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of liver cancer etiology, including hepatitis B and hepatitis C viral infection (*19*). Using comprehensive expression analysis, gene prediction sets for anti-cancer drug sensitivity (*20*) or intrahepatic recurrence (*21*) were demonstrated. Thus, comprehensive expression analysis has enabled us to perform clustering analysis based on clinicopathological features, identification of candidate genes for therapy, and diagnosis, and selection of predictor genes for tailor-made therapy.

On the other hand, by integration of expression profiles with gene loci, it has been shown that gene expression profiles are subject to chromosomal bias (*22-26*). In addition, genes in regions of chromosomal aberration with altered gene expression levels are more likely to represent oncogenes or tumor suppressor genes. Therefore, it is necessary to investigate the copy number information in addition to expression profile in the same samples. Comparative genomic hybridization (CGH) has been used extensively to detect genome-wide copy number alterations in various types of cancer and to determine the localization of expression of many oncogenes and tumor suppressor genes (*27*), and there have been a number of reports of chromosomal analysis using CGH in HCC (*28-40*). These previous studies investigated the associations between chromosomal alterations and various clinicopathological factors, such as tumor progression (*29,30,32,36,37,40*), prognosis (*35*), and viral infection (*31,33*) in liver cancer.

Recently, array-based CGH (aCGH) using genomic DNA or cDNA clones has been developed and provided much higher resolution detection of copy number alterations than conventional CGH. Therefore, accurate identification of genes with DNA copy number changes in carcinogenesis is now possible (*41-43*). Using aCGH, high-resolution mapping of copy number aberrations in HCC has been reported, especially in measuring high-level amplification and homozygous deletion (*44-48*).

Single nucleotide polymorphism (SNP) arrays, which were originally designed for high-throughput SNP analysis (*49,50*), can provide high-resolution analyses of loss of heterozygosity (LOH) in a genome-wide fashion (*51-55*). We and other groups have developed novel algorithms for global and high-resolution analysis of copy number changes using SNP arrays (*56-59*). In comparison to aCGH, the newly developed Genome Imbalance Map (GIM) algorithm (*56*) has advantages for detecting not only copy number aberrations but also allelic imbalance, including LOH and uniparental disomy (UPD) (*24*).

This article presents a review of the outcomes of copy number analysis for HCC through a literature search of published reports, especially with regard to identification of candidate genes for oncogenes and tumor suppressor genes using aCGH and SNP arrays. Furthermore, we propose an algorithm for integration of expression data with gene loci, and discuss the chromosomal bias of gene expression and pitfalls of gene clustering.

Molecular karyotyping analysis for hepatocellular carcinoma using conventional comparative genomic hybridization

A number of studies of chromosomal alterations in HCC using conventional CGH have been reported (*28-44*), which were summarized according to etiology, histological grade, and tumor stage (*60,61*). A meta-analysis based on 31 CGH analyses of 785 HCC nodules showed that gains on chromosome arms were observed on 1q (57.1%), 8q (46.6%), 6p (23.3%), and 17q (22.2%), while losses were detected on 8p (38%), 16q (35.9%), 4q (34.3%), 17p (32.1%), and 13q (26.2%) (*60*). Through this meta-analysis, Moinzadeh *et al*. further classified chromosomal alterations according to clinicopathological parameters, including hepatitis virus infection (*31,33*), tumor differentiation grade (*32*), and tumor progression (*30,36,37,62*). Comparison between HBV-positive and -negative cases indicated that losses at 4q, 8p, 13q, and 16q were positively correlated with HBV-positive HCC, whereas only 8p loss was more frequent in HCV-positive cases. With regard to tumor histological grade, chromosomal losses at 4q and 13q were significantly associated with tumor dedifferentiation. Although the number of dysplastic nodules analyzed by CGH was low, 1q gains were characteristic of the initiation of hepatocarcinogenesis (*60*). In addition to the clinical features described above, Pang *et al*. reported that gains at 1q and 6p were independent factors for liver cancer invasion (*29*).

If copy number analysis can predict the recurrence of HCC after resection, individualized therapy may be possible. Kusano *et al*. reported that recurrence was linked to loss at 13q, which was a variable independent of other factors on multivariate analysis (*35*). Furthermore, Poon *et al*. reported a tumor progression model for HCC using bioinformatics analyses using the self-organizing tree algorithm (SOTA) in a large-scale study. Based on the patterns of significant chromosomal aberrations derived, they identified 4 HCC classes at 3 different evolution levels, one group of which had poorer recurrence-free survival than the other 3 groups. They also showed that patients with 3q22-24 gain have both poorer recurrence-free and overall survival rates (*40*).

Thus, CGH analysis can make it possible not only to classify the clinicopathological parameters of the tumor but also to predict the prognosis of HCC patients, which will facilitate individualized therapy.

Comparative genomic hybridization for determination of liver cancer clonality

Multifocal cancer growth of HCC is due to either intrahepatic metastasis or multicentric origin, which

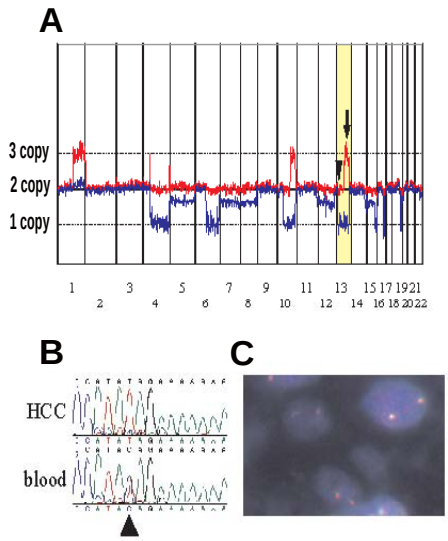

Figure 1. Genome Imbalance Map (GIM) of a representative hepatocellular carcinoma (HCC) sample. GIM can detect not only genome dosage but also allelic imbalance status more precisely than aCGH analysis. (A) Allelic dosage analysis across the whole genome showed uniparental disomy in 13q31.2-34; (B, C) Fluorescence in situ hybridization and loss of heterozygosity analysis for validation of allelic imbalance in 13q. (Modified from Reference 24 with permission)

is clinically significant. However, methods for clinicopathological and morphological discrimination have not been sufficiently reliable for physicians to determine the appropriate treatment for patients with multiple HCC. To differentiate intrahepatic metastasis from multicentric origin in HCC, it has been shown to be useful to compare the clonalities of multifocal HCC using molecular methods, such as CGH analysis

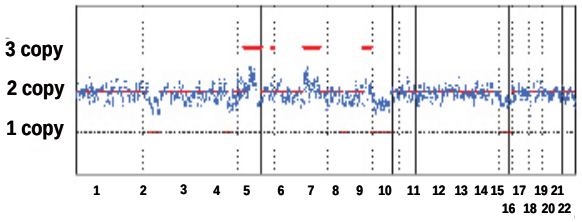

Figure 3. Comparison of genomic alteration and gene expression status. Total gene dosage and expression analysis across the whole genome of a patient. Dots represent HCC/liver expression intensity ratio and the continuous lines indicate copy numbers. Gene expression levels changed in accordance with genomic alterations. (Modified from reference 24 with permission)

(*38*,*39*,*63*), DNA fingerprinting by LOH assay (*64-66*), and hepatitis B virus integration pattern (*67-69*), as the recurrent neoplasm inherits the same altered genome from the initial HCC.

Chen *et al*. applied CGH for 31 primary and the corresponding recurrent liver tumors and calculated the clonal relationships, by which they could distinguish truly relapsed from second primary HCC in 22 of 31 cases (*39*). On the other hand, using all of the 3 molecular methods described above, Ng *et al*. succeeded in complete determination of the clonal relationships of 25 nodules from 11 patients (*38*).

Thus, evaluation of clonality of multifocal HCC using molecular methods is useful for physicians to allow precise determination of the treatment for multiple HCC. CGH is the most powerful and most readily available tool for this purpose.

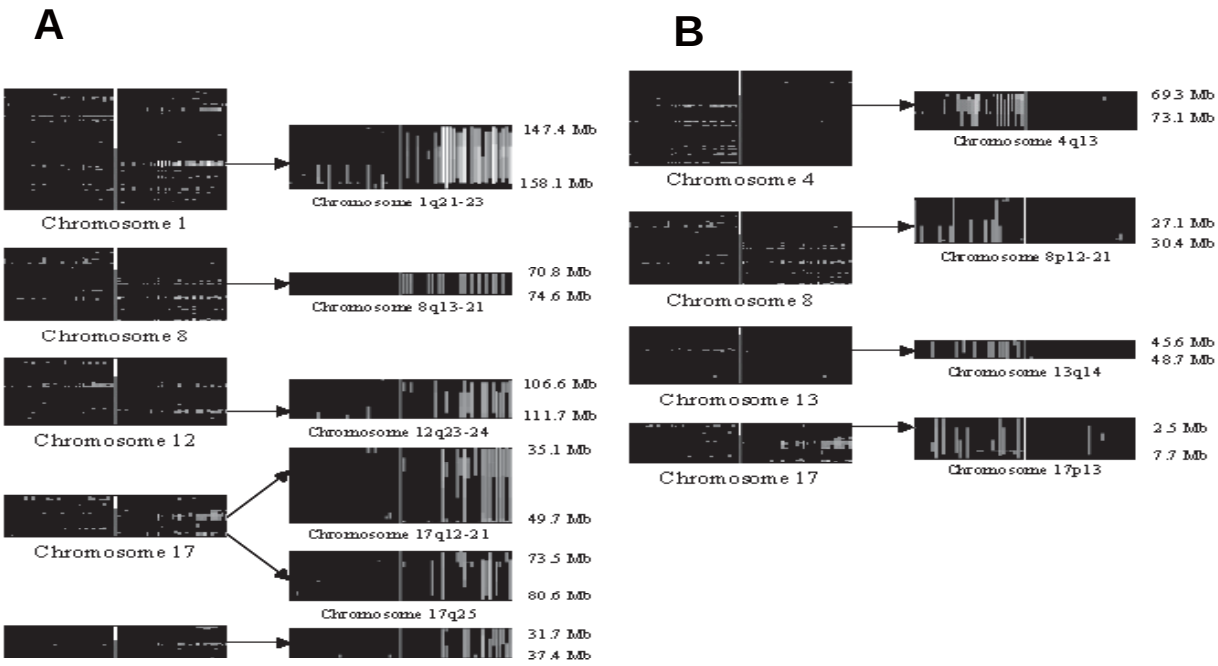

Figure 2. Expression Imbalance Map (EIM) for detecting expression imbalance region in hepatocellular carcinoma (HCC). EIM enables identification of many more genes by referring to the expanded area with lower luminance. (A) Expression imbalance region at an E value > 2 and a range of expression gain > 3 Mb. (B) Expression imbalance region at an E value > 2 and a range of expression loss > 3 Mb. (Modified from reference 23 with permission)

High-resolution mapping of copy number aberrations and identification of target genes in hepatocellular carcinoma

In comparison to traditional CGH, aCGH can detect chromosomal aberrations with high resolution. By comparison between conventional CGH and aCGH in 19 HCC samples, Hashimoto *et al*. demonstrated that 80% of the target clones identified by aCGH were included in CGH analysis, while copy number alterations for FGR/SRC2, HRAS, THRA, and GSCL, of which clones were detected by aCGH, were not found by conventional CGH (*46*).

Using aCGH, the significance of correlations of frequent chromosomal aberrations with various clinicopathological features were investigated, and Katoh *et al*. demonstrated that chromosomal loss on 17p13.3 and gain of 8q11 were independent prognostic indicators by multivariate analysis (*44*). Among the various clinicopathological features, differentiation grade is one of the best indicators of malignancy of liver cancer (*70*), and 4q and 13q were shown to be correlated with dedifferentiation of HCC (*48*).

In addition to high-resolution mapping of chromosomal aberrations, aCGH is available for identification of candidate genes correlated with DNA copy number alterations for narrowing the list of oncogenes and tumor suppressor genes. Integrating the correlation between copy number alterations and gene expression profile, Patil *et al*. identified Jab1 as a target for 8q gain, which was suggested to have a potential role in the development of HCC by functional analysis (*47*).

Molecular karyotyping analysis of hepatocellular carcinoma using single nucleotide polymorphism arrays

The detection of genome-wide LOH is possible by comparing the calls for normal control and tumor samples using SNP arrays (*51,53-55*). The accuracy of this method was validated by comparison to PCR-based microsatellite analysis by Hoque *et al*. (*52*). In addition to LOH, we and other groups have developed algorithms for detecting copy number alterations and allelic imbalance simultaneously using SNP arrays (*56-59*).

Our method, named GIM analysis (Figure 1), was applied to 36 HCC samples and recurrent chromosomal aberrations in liver cancer were analyzed (*24*). That is, even fractional copy number, suggesting heterogeneity of cancer cells, was detected, and validated by fluorescence in situ hybridization. In this study, in addition to the gains of 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q, and LOH of 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, and 17p, which were significantly associated with HCC, we identified UPD and UPT on 13 regions, suggesting that genome dosage analysis misses many LOH regions with normal copy number. For example, on 6q24-25, which contained imprinting gene clusters and UPD regions in our data, we observed reduced levels of *PLAGL1* expression due to loss of the unmethylated allele. Thus, high-resolution GIM analysis can accurately determine the localizations of genomic regions with allelic imbalance, and when integrated with epigenetic information, a mechanistic basis for inactivation of tumor suppressor genes in HCC was elucidated.

Furthermore, using much higher-density arrays, it will soon be possible to elucidate micro-homozygous deletion and chromosome amplification, and boundary regions suggesting breakpoints in liver cancer.

Systematic integration of expression profiles with gene loci

We have integrated gene expression data and gene locus information, and the regions in which the numbers of up-regulated and down-regulated genes were significantly concentrated were mapped on the chromosome (*22*). This method for detection of regions of mRNA expression imbalance is called Expression Imbalance Map (EIM), and we applied EIM analysis to gene expression data from 31 HCC tissues (*23*). Our data revealed that expression gains of 1q21-23, 8q13-21, 12q23-24, 17q12-21, 17q25, and 20q11, and losses of 4q13, 8p12-21, 13q14, and 17p13 were significantly associated with HCC (Figure 2), consistent with previous reports using CGH in liver cancer (*28,32,36,37,67,71-75*). Furthermore, more poorly differentiated liver cancer contains larger numbers of chromosomal alterations, which are accumulated in a stepwise manner in the course of HCC progression.

If not only gene expression but also cytogenetic data can be obtained from the same sample, integration of expression profile with chromosomal loci will enable comparison of gene expression with gene dosage. Pollack *et al*. measured parallel mRNA levels by microarray analysis and DNA copy number alterations by aCGH in breast cancer cells, and they reported that 62% of highly amplified genes show elevated expression and that DNA copy number influences gene expression across a wide range of DNA copy number alterations (*26*).

In liver cancer tissues, we and other groups reached the same conclusions as Pollack *et al*. Furge *et al*. obtained regional expression biases (REBs) from a multiple span moving binomial test and demonstrated that REBs overlapped genetic abnormalities identified using aCGH in HCC (*25*). We have also demonstrated the effects of genome imbalance on the transcriptome by direct comparison with expression data from the same samples (*24*) (Figure 3).

On the other hand, Huang *et al*. investigated the relationship between genomic DNA copy number

changes and transcriptional levels, and found that DNA copy number alterations appeared not to parallel the corresponding gene expression profiles in either HCC specimens or cell lines (*45*).

Thus, gene expression profiles are subject to chromosomal bias and EIM can correlate gene expression to gene loci with high resolution and sensitivity.

Conclusions

Microarray analysis has contributed to identification of candidate genes and has been shown to be available for clinical application. In addition, clustering analysis of expression data and selection of predictor genes based on clinicopathological features could have been performed. However, bioinformatics technology indicated that gene expression profile is subject to chromosomal bias, *i.e.*, clustering analysis involves the risk of being affected by gene structural abnormalities. To resolve this problem, combined and well-organized reconstruction of different molecular levels, including genetic aberrations, epigenetic changes, and expression alterations, is required to narrow the candidates responsible for cancer.

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Household risk factors associated with dengue-like illness, Republic of Palau, 2000-2001

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SUMMARY **The authors investigated the association between household hygiene status and the occurrence of dengue-like illness (DLI) during the 2000-2001 outbreak in the Republic of Palau. Hygiene status was compared between 55 households with DLI patients and 55 households without DLI patients during the period of the outbreak. Shower facilities with hygienic problems and potential breeding sites of mosquitoes were more frequently found in the lots of households with DLI patients than in those without (OR = 3.1, 95%CI = 1.3-7.2; OR = 3.2, 95%CI = 1.02-9.4, respectively). The total number of hygienic problems was higher for households with DLI patients than for those without (*t*-test, $p = 0.016$). Results indicated that the overall household hygiene status, and particularly the existence of inappropriate shower facilities and mosquito breeding sites, was the predictor of the prevalence of DLI during the outbreak; this status will be considered in the prevention of future outbreaks of DLI in Palau and in other tropical island nations as well.**

Key Words: Dengue-like illness, republic of Palau, hygiene status

Introduction

During the period between September 2000 and December 2001, 887 patients were diagnosed as having "dengue-like illness (DLI)" in the Republic of Palau. This was the third outbreak after the re-emergence of DLI in Palau in 1988 and 1995. DLI re-emerged after a long period that started in 1944; during this period there were no reported incidences of the disease (*1*).

DLI patients, who were diagnosed by medical doctors in a national hospital in Palau on the basis of diagnostic criteria (for details, see Methods), were presumably treated as dengue fever patients. The antibody testing of 338 DLI patients by IgM capture ELISA in the 1995 outbreak revealed that 75% of patients were positive for dengue viruses (*1*). In Palau or other Pacific nations where dengue fever is a major health concern but serological test kits were not always available for diagnosis of dengue fever, a reasonable approach is to study risk factors for DLI by assuming that most DLI cases were caused by dengue viruses.

Dengue fever is primarily a disease of the tropics, and responsible viruses are maintained in a cycle that involves humans and some species of *Aedes* mosquito. The distribution of dengue has expanded and an estimated 2.5 billion people live in areas at risk of epidemic transmission (*2*). According to a survey in Palau during the DLI outbreak in 1995 (*1*), the prevalent mosquitoes were *Ae. aegypti*, *Ae. albopictus*, *Ae. hensilli*, *Ae. palauensis*, and *Ae. scuttelaris*; the first two species were not reported in the survey by Bogart in the 1950s (*3*) and thus were probably introduced between the 1950s and 1995. These species of mosquitoes are sedentary and prefer to feed on humans.

In the present study, the authors hypothesized that household hygienic problems related to the breeding of mosquitoes were associated with the occurrence of DLI in the household. The authors compared the hygiene status of households that had at least one DLI patient

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during the outbreak period to that of households that did not have any DLI patients. The objectives were to investigate the association between overall household hygienic status and DLI occurrence and to identify specific household environmental factors that influence the risk of DLI occurrence.

Methods

The Republic of Palau comprises the western-most group of the Caroline Islands in Micronesia (Figure 1). Of the 340 islands in the Republic, Babeldaob, which occupies 70% of the total land area of the country (488 km^2), is the largest. The capital city is in Koror State located southwest of Babeldaob. More than 70% of the total national population inhabited the capital with an area of 12 km (*i.e.*, the total area of populated islands in Koror State) in 2000 (national population, 19,129; population in Koror, 13,303) (*4*). The country is administratively divided into 16 states, each of which is further divided into hamlets. Most administrative functions and economic sectors are concentrated in Koror. The national hospital and two private clinics are also located in Koror.

According to the database of DLI surveillance of the Division of Environmental Health (DEH), 887 people were diagnosed as having DLI by medical doctors in a national hospital between September 2000 and December 2001. The incidence of DLI during the above period exceeded the estimated number of patients diagnosed as having the viral syndrome during the non-outbreak period in 1993 and 1994 (*i.e.*, 4.5 per week throughout Palau) (*1*). The incidence of DLI peaked in January 2001 (*i.e.*, 212 DLI patients diagnosed in Palau) and gradually decreased to the level of the non-outbreak period in January 2002.

Patients showing febrile illness in Koror and Airai States visited a national hospital or two private clinics

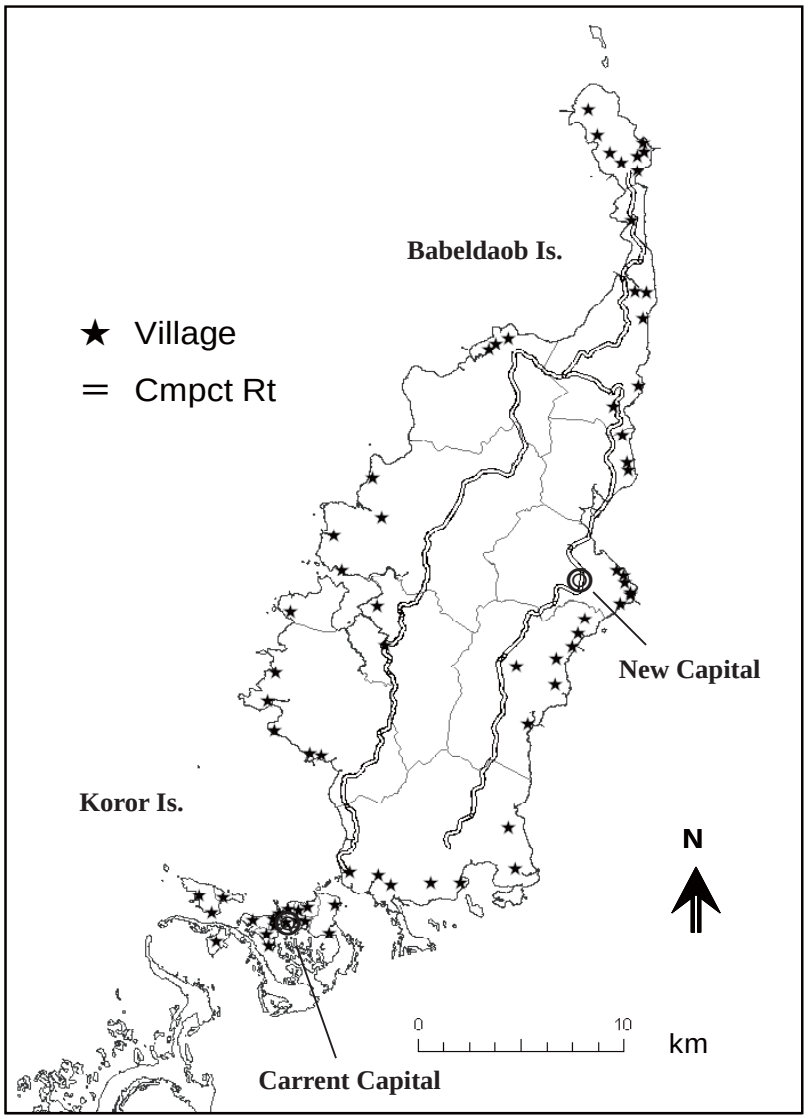

Figure 1. Koror and Babeldaob Islands in Republic of Palau. The locations of the new capital and new highways are indicated. The administrative boundaries shown on the map correspond to those of states.

Table 1. Comparison of 55 households with at least one dengue-like illness (DLI) patient and 55 households without (control) in terms of household hygiene problems between September 2000 and December 2001, Republic of Palau.

Category	Problems	Number of households with problems (% total) DLI *n* = 55	Control *n* = 55	OR	95%CI
Existence of breeding sites					
Mosquitoes	Actual	9 (16.4)	6 (10.9)	1.6	0.5 - 4.8
	Potential	13 (23.6)	5 (9.1)	3.1	1.02 - 9.4
Rats	Actual	8 (14.5)	4 (7.3)	2.2	0.6 - 7.7
	Potential	9 (16.4)	13 (23.6)	0.6	0.2 - 1.6
Flies	Actual	12 (21.8)	6 (10.9)	2.3	0.8 - 6.6
	Potential	6 (10.9)	5 (9.1)	1.2	0.4 - 4.3
Problems with facilities					
Shower	Poor drainage	23 (41.8)	11 (20.0)	2.9	1.2 - 6.7
	No proper building	7 (12.7)	1 (1.8)	7.9	0.9 - 66.0
Kitchen	Poor drainage	18 (32.7)	11 (20.0)	1.9	0.8 - 4.6
	Attracts flies and rats	5 (9.1)	2 (3.6)	2.7	0.5 - 14.3
Toilet	Lacking	2 (3.6)	0	NA	
	Full pit	1 (1.8)	0	NA	
	Screen on holes /no screen	14 (25.5)	11 (20.0)	1.4	0.6 - 3.3
	Door not self-closing	7 (12.7)	8 (14.5)	0.9	0.3 - 2.6
Trash site	Trash container without lid	0	1 (1.8)		
	Improper trash dump	8 (14.5)	6 (10.9)	1.4	0.4 - 4.3
Water tank	Without lid or stand	0	1 (1.8)	NA	
Conditions of plot					
Pigsty	No cesspool or septic tank	4 (7.3)	1 (1.8)	4.2	0.5 - 39.2
Yard	Overgrowth of grass	9 (16.4)	7 (12.7)	1.3	0.5 - 3.9
	Trash/litters found	21 (38.2)	17 (30.9)	1.4	0.6 - 3.0
Total number of problems					
	Mean (SD)	3.2 (2.6)	2.1 (2.0)	$p = 0.017$ (*t*-test)	

Table 2. Comparison of 55 households with at least one dengue-like illness (DLI) patient and 55 households without (control) in terms of household hygiene status by category between September 2000 and December 2001, Republic of Palau.

Category	Number of households with problems (% total) DLI *n* = 55	Control *n* = 55	OR	95%CI
Mosquito	18 (32.7)	10 (18.2)	2.2	0.9 - 5.3
Rat	17 (30.9)	17 (30.9)	1	0.4 - 2.2
Flies	18 (32.7)	10 (18.2)	2.2	0.9 - 5.3
Shower	24 (43.6)	11 (20.0)	3.1	1.3 - 7.2
Kitchen	20 (36.4)	11 (20.0)	2.3	0.97 - 5.4
Toilet	19 (34.5)	14 (25.5)	1.5	0.7 - 3.5
Trash	8 (14.5)	7 (12.7)	1.2	0.4 - 3.5
Water	0	1 (1.8)	NA	
Pig	4 (7.3)	1 (1.8)	4.2	0.5 - 39.2
Yard	25 (45.5)	20 (36.4)	1.5	0.7 - 3.1
Total number of categories in which problems were found;				
Mean (SD)	2.8 (2.2)	1.9 (1.7)	$p = 0.016$ (*t*-test)	
Total number of categories (except for shower) in which problems were found;				
Mean (SD)	2.3 (2.0)	1.7 (1.6)	$p = 0.046$ (*t*-test)	

for diagnosis, whereas those living outside Koror and Airai visited local health clinics. Each health clinic is obligated to transfer suspected DLI patients to a national hospital for diagnosis and treatment by medical doctors. The diagnostic criteria in the hospital included fever (38.5°C) with body or joint aches, headache, myalgia, asthenia and rashes. Since September 2000, the staff of DEH have checked the national hospital and

private clinics daily and recorded the name, sex, age, and place of residence of DLI patients.

In 2001-2002, the DEH staff inspected the hygiene status of all households in 13 states (those located outside Koror and Airai). The status, as indicated by 10 categories (mosquito breeding sites, rat breeding sites, fly breeding sites, shower facilities, kitchen, toilet, trash site, water tank, pig sty, and yard), was investigated by a pair of trained inspectors (one inspector evaluated the state of the problem and the other inspector recorded findings after confirming the state) on the basis of a structured checklist (20 hygiene problems in total) (see Table 1 for the hygiene problems evaluated). Abandoned tires, cans, shells of giant clams, cooler boxes, coconut shells, flower pots, boats, and puddles on the ground were regarded as the breeding sites of mosquitoes, whereas holes on the ground and the leavings of a meal or dung were those of rats and flies, respectively. Those breeding sites were examined for evidence of breeding ("actual" breeding sites) or not ("potential" breeding sites). Evidence of breeding was the existence of larvae or eggs for mosquitoes, excrement for rats, and maggots for flies.

Of the 887 DLI patients, 71 resided in 13 states where household hygiene data were available. The DLI patients were members of 55 households (one household had four patients, three households had three and seven households had two). For each household with a DLI patient (a case, hereafter referred to as a DLI household), a control household was randomly selected from households (**1**) in the same neighborhood (**2**) that had no DLI patients during the 2000-2001 outbreak period and (**3**) that had a member whose age matched (within 5 years) that of the DLI patient in the case household. Household hygiene data for the 55 DLI households and the control households were compared.

Results

In Table 1, household hygiene status of the 55 DLI households and the 55 control households was compared for each of the 20 hygiene problems. For most household hygiene problems, the odds of having problems were higher in the DLI households than in the control households. The lower limit of the 95% confidential interval (CI) of the odds ratio for having problems (reference = control households) exceeded 1 for poor drainage of shower facilities (OR = 2.9, CI: 1.2-6.7) and existence of potential breeding sites for mosquitoes (OR = 3.1, CI: 1.02-9.4). The total number of hygiene problems was statistically significantly higher in the DLI households than in the control households (*t*-test, $p = 0.017$).

Table 2 shows the number of households that have one or more hygienic problem in each category. The odds of having problems were higher in the DLI households than in the control households for most categories. The lower limit of the 95%CI of the odds ratio (reference = control households) exceeded 1 for having problems with shower facilities (OR = 3.1, CI: 1.3-7.2). The total number of categories in which problems were found was statistically significantly higher in the DLI households than in the control households (*t*-test, $p = 0.016$). This tendency did not change even when the problems with shower facilities were not included (*t*-test, $p = 0.046$), which indicated that overall household hygienic status predicted the risk of DLI occurrence.

Discussion

The present analysis of the association of household risk factors with DLI revealed that poor drainage and shower facilities and the presence of potential mosquito breeding sites were significant risk factors for DLI occurrence. In the analysis of the 1995 DLI outbreak in Palau, Ashford *et al*. found that DLI was associated with the existence of food and water pans for animals on the property and taro farming but not with the existence of potential breeding sites for mosquitoes (tires, cans or buckets) (*1*) The lack of the expected association was explained by the high prevalence of mosquito breeding sites in both affected and non-affected households. In the present study, potential mosquito breeding sites were found in only 24% of the DLI households and 9% of the control households. This may reflect the effect of measures to control DLI infection (such as a public education campaign to reduce vector breeding sites and improved solid waste disposal) implemented after the 1995 outbreak. The association between dengue infection and potential mosquito breeding sites became apparent during the period from 1995 to 2001.

Poor drainage of shower facilities was also a significant risk factor for dengue infection. According to the 2000 Census of Population and Housing (*4*), 48% of households outside Koror did not have appropriate waste disposal facilities, whereas 49% connected their sewage to a septic tank or cesspool. Water from the former households usually flows into open drains, which often form puddles suitable for breeding of mosquitoes. Larvae and pupae were usually observed in such places.

Previous studies in other countries commonly indicate that the environmental conditions of residential areas are frequently associated with the risk of dengue infection (*5-7*); individual risk factors varied among the populations because of differences in vector species and breeding site. In a case-control study in Brazil, Heukelbach *et al*. showed that receptacles in the garden or courtyard, plants with temporary water pools on the property, gutters in which rainwater collected, and uncovered water storage areas are significant risk factors for dengue infection (*5*). Koopman *et al*.

emphasized that the characteristics of a community (*i.e.*, the proportion of households with larvae on the premises and the proportion of households with uncovered water containers) are associated with the proportion of the individuals infected (*7*). The present study showed that, in Palau, the risk factors for DLI (presumably dengue fever) during the outbreak period were the overall household hygiene status, existence of mosquito breeding sites, and poor drainage of shower facilities.

The WHO's Healthy Islands initiatives introduced by the Palau government in 1999 were directed toward improving household hygiene by educational campaigns in the communities and practical demonstration of clean drainage and septic tanks using a participatory approach. These initiatives may have improved the people's knowledge, attitude and practice, which are essential for the achievement of improved hygiene. Further implementation will reduce the risk of DLI infection among the populace. Environmental risk factors have effects beyond individual household and individuals infected with DLI create risks for others. The organisation of control measures must be at the community level (*7*).

The Republic of Palau is now in the process of relocating its capital from Koror to the centre of Babeldaob. Road networks linking Koror, the new capital, and other states in Babeldaob are currently under construction. This change in social infrastructure may induce either the migration of Koror residents to their home villages in Babeldaob (*i.e.*, they will commute from their home villages to Koror) or frequent visits of Koror residents to their home villages (every weekend or so), both of which may change the risk of dengue infection for Palauans (*8,9*). Frequent monitoring of both dengue cases as well as household hygiene status are fundamental strategies for the prevention of future outbreaks of dengue fever in the Republic of Palau. This is also the case for other Pacific nations that have recently experienced dengue outbreaks (*e.g.*, Fiji in 1998, Cook Island in 1997, French Polynesia in 2001 and Samoa in 1996) (*10*) or that have yet to experience such outbreaks but share similar physical and social environments and lifestyles.

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Are health inequalities increasing in Japan? The trends of 1955 to 2000

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SUMMARY **This study aimed to elucidate trends in socioeconomic inequalities in health during the past half century in Japan. Association of life expectancy and age-adjusted mortality with per capita income was examined using data on prefectures and municipalities in Japan of 1955 to 2000 via the slope index of inequality (SII) and Poisson regression. Although there were a few differences among health indicators and sex, health inequalities by prefecture, measured by the SII, decreased from 1955 to 1995. However, health inequalities increased from 1995 to 2000 both for life expectancy and mortality. Similar trends were found in municipal analyses: the association between income and mortality, measured by the rate ratio from Poisson regression, decreased until 1995 but increased from 1995 to 2000. In the past half century, and especially until 1995, geographical health inequalities decreased in Japan, while from 1995 to 2000 health inequalities appeared to increase. Recent social conditions including the possible increase in social inequalities may have contributed to this increase. Careful monitoring and elimination of social and health inequalities should be encouraged.**

Key Words: Health inequalities, socioeconomic factors, life expectancy, ecological study

Introduction

Elimination of health inequalities has been a great challenge in international and domestic public health policy. A large number of studies have demonstrated health inequalities attributable to socioeconomic conditions, including income, educational attainment, social class, and other factors (*1-4*). The degree of socioeconomic inequalities in society is closely linked to the health of the population (*5,6*).

Japan has shown marked improvement in the health of the population in the past half century. Major health indicators such as life expectancy and infant mortality have been ranked as some of the world's highest (*7*). In addition to economic growth and improved living standards, decreased socioeconomic inequalities and an egalitarian social system are considered to contribute to the health improvement of Japanese (*6,8-11*).

This egalitarian society, however, may be changing. Researchers in the fields of economics, sociology, and education are extremely concerned about increasing socioeconomic inequalities in Japan, and especially in the past decade (*12-14*). Although more discussion is needed, the social conditions underlying the increasing inequalities include economic recession and recent economic, taxation, and social security policies (*12-14*). Little is known about the influence of the possible increase in socioeconomic inequalities in health, leading to the question of if health inequalities are increasing in Japan.

This study elucidated the trends in health inequalities during the past half century in Japan. To this end, an ecological approach was taken at the prefectural and municipal levels to gather data in order to facilitate further debate on health inequalities.

Methods

Populations studied and observation period

The populations studied were prefectures and municipalities. These are basic administrative divisions in Japan: the prefecture is the higher level and consists of municipalities. There are currently 47 prefectures, an increase from 46 after 1972 with the reversion of

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Table 1. Mean and coefficient of variance (CV) of per capita income, life expectancy, and age-adjusted mortality of 46 prefectures in Japan, 1955 to 2000

Year	Per capita income (thousand yen)		Life expectancy (years)				Age-adjusted mortality (per 100,000)			
			Men		Women		Men		Women	
	Mean	(CV)	Mean	(CV)	Mean	(CV)	Mean	(CV)	Mean	(CV)
1955	68.7	(22.0)	62.95	(2.03)	67.13	(1.93)	-		-	
1960	112.2	(26.6)	65.19	(1.66)	70.07	(1.37)	-		-	
1965	215.5	(23.4)	67.49	(1.44)	72.88	(1.01)	632.0	(9.31)	375.9	(6.37)
1970	495.4	(21.7)	69.41	(1.35)	75.05	(0.71)	566.7	(10.64)	321.8	(5.84)
1975	1019.1	(14.2)	71.44	(1.06)	76.84	(0.64)	459.1	(9.65)	255.0	(5.50)
1980	1575.0	(12.7)	73.34	(0.92)	78.92	(0.54)	402.0	(9.92)	206.2	(6.06)
1985	1960.9	(14.6)	74.82	(0.80)	80.73	(0.53)	365.2	(9.50)	176.6	(6.46)
1990	2634.7	(15.9)	75.96	(0.78)	82.10	(0.47)	335.5	(9.01)	155.2	(5.57)
1995	2865.5	(13.2)	76.66	(0.76)	83.30	(0.54)	319.9	(10.38)	148.9	(9.12)
2000	2867.6	(12.4)	77.62	(0.75)	84.70	(0.48)	297.7	(8.82)	135.6	(6.28)

CV = Standard deviation/Mean × 100

Okinawa Prefecture to Japan. Okinawa Prefecture was excluded from the current analyses to ensure comparability of time trends.

Municipalities include cities ("shi"), towns ("machi"), villages ("mura"), and wards ("ku"). The number of municipalities fluctuated and numbered almost 3350 during the observed periods because of mergers and dissolutions of municipalities.

The entire observation period was 1955 to 2000, but the analytical period depended upon variables because of limited data availability.

Data

Health indicators were life expectancy (LE) and mortality. In prefectural analyses, LE and age-adjusted mortality among populations aged 20 to 64 years were used. The data were obtained from the Prefectural Life Table and Vital Statistics (*15-18*).

In municipal analyses, the observed number of deaths was obtained from Vital Statistics (*19,20*) and aggregated in intervals of five consecutive years (1973-77, 1978-82, 1983-87, 1988-92, 1993-97, and 1998-2000). The expected number of deaths was estimated using the age-specific population of the municipality and age-specific mortality of the entire country (*19-21*). Analysed municipalities numbered 3346, 3348, 3356, 3346, 3361, and 3356, respectively, for the five observation periods.

Per capita income served as a socioeconomic indicator. Per capita income by prefecture and municipality was obtained from a published database (*21,22*).

Analyses

In prefectural analyses, the slope index of inequality (SII) served as a measure of the association between health indicators and income. The SII is estimated from the slope of the linear regression line between income ranking and health indicator and the mean of the health indicator (*23*). Since the SII is independent of absolute values of health and its predictive variables, it is useful for comparison of the magnitude of health inequalities, and especially for comparing time trends and different indicators (*23*).

First, the prefectures were ranked according to per capita income, and ranking values ranging from 0 (lowest income) to 1 (highest income) were assigned to prefectures. Then, the linear regression line was fitted with the health indicator as the dependent variable and the ranking value as the explanatory variable. The SII was estimated by dividing the slope by the mean of the heath indicators and presented as a percentage (× 100). The observation period was 1955 to 2000 for LE and 1965 to 2000 for mortality.

In municipal analyses, Poisson regression was used with the number of observed and expected deaths and per capita income, and the rate ratio (RR) of income for mortality was estimated. Two income variables were separately introduced. First, per capita income was introduced as a continuous variable (in units of a million yen). Second, an ordinal variable was used: the lowest decile = 0.05 to the highest decile = 0.95. The observation period was 1975 to 2000. Municipal per capita income in 1975 was not available, so that in 1980 was used for the 1975 analysis.

SPSS 15.0J was used for linear regression analysis and MLwiN 2.02 for Poisson regression analysis.

Results

Table 1 summarizes the health indicators and per capita income of 46 prefectures from 1955 to 2000. Income markedly increased, and especially until 1990, with narrowing of the variation in accordance with the coefficient of variance (CV). LE continuously increased with narrowing variation. Age-adjusted mortality continuously decreased but was not accompanied by narrowing variation.

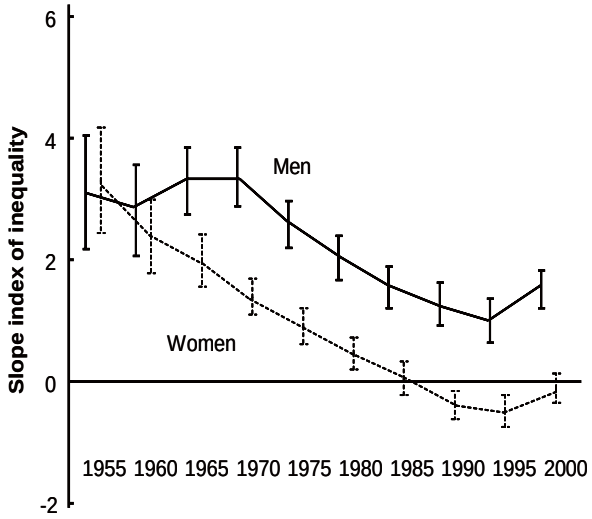

Figure 1. Slope index of inequality for income and life expectancy for 46 prefectures in Japan, 1955 to 2000. Vertical lines show the 95% confidence interval.

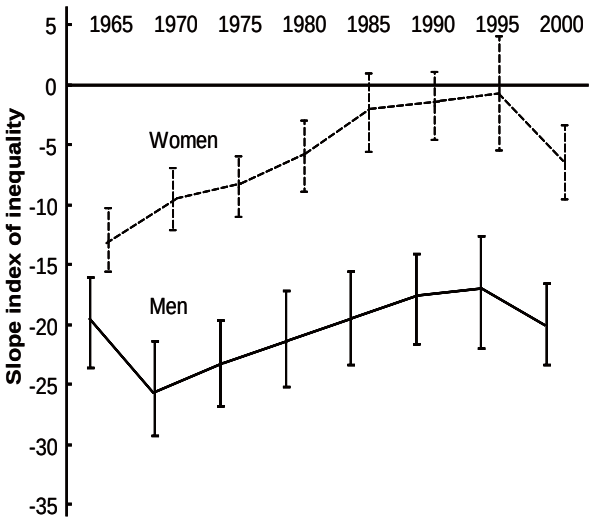

Figure 2. Slope index of inequality for income and age-adjusted mortality for 46 prefectures in Japan, 1965 to 2000. Vertical lines show the 95% confidence interval.

Figure 1 shows the SII for LE and per capita income in prefectural analyses from 1955 to 2000. A positive sign means that a prefecture with higher incomes had a longer LE. The SII decreased during 1955 to 1995 for women and during 1970 to 1995 for men. In contrast, the SII increased for both men and women from 1995 to 2000.

The SII for age-adjusted mortality and per capita income from 1965 to 2000 is shown in Figure 2. A negative sign means that a prefecture with higher incomes had a lower mortality rate. According to the SII, the association between income and mortality decreased until 1995 but increased from 1995 to 2000.

Table 2 shows the results of municipal analyses, demonstrating the RR of per capita income for mortality. An RR of less than 1.0 means that municipalities with higher incomes had a lower mortality rate. There were some differences in absolute values between continuous and categorical variables due to the different units, but the time trends were similar. The association between income and mortality decreased until 1995. For women in 1990 and 1995, the RR was more than 1.0, showing that municipalities with higher incomes had a higher mortality rate. From 1995 to 2000, RR increased in both men and women.

Discussion

This paper demonstrated the possibility of health inequalities increasing from 1995 to 2000 in Japan. An increase in health inequalities over the past few decades has been found in other industrial countries (*24-26*). The current findings offer the first suggestion of a recent increase in health inequalities in Japan.

Until 1995, the association between income and health indicators decreased, as shown in previous studies (*8,9,27*). The decrease in health inequalities was accompanied by significant LE extension and a decline in mortality. Possible contributors to the improved health of the Japanese population have been noted. An egalitarian social system and culture appears to contribute substantially through compulsory education, universal health insurance coverage, public health services, income adjustment policy, and strong social relationships (*6,8-10*). This is considered to be a good indication that fewer socioeconomic inequalities improve the health of the population (*6,11*).

The association of income and health indicators

Table 2. Results of poisson regression of per capita income and mortality by municipal level: rate ratio with 95% confidence interval

Year	Men		Women	
	Continuous[a]	Categorical[b]	Continuous[a]	Categorical[b]
1975	0.779 (0.766, 0.793)	0.861 (0.851, 0.872)	0.877 (0.861, 0.893)	0.945 (0.933, 0.958)
1980	0.855 (0.841, 0.870)	0.920 (0.909, 0.931)	0.938 (0.921, 0.955)	0.987 (0.975, 1.000)
1985	0.876 (0.865, 0.887)	0.913 (0.902, 0.924)	0.959 (0.946, 0.972)	0.998 (0.878, 1.134)
1990	0.950 (0.943, 0.957)	0.941 (0.940, 0.942)	1.004 (0.996, 1.012)	1.042 (1.030, 1.055)
1995	0.944 (0.936, 0.951)	0.935 (0.925, 0.945)	1.012 (1.003, 1.020)	1.035 (1.023, 1.048)
2000	0.886 (0.883, 0.889)	0.853 (0.849, 0.857)	0.975 (0.972, 0.979)	0.972 (0.967, 0.977)

[a]Per capita income was used as the continuous variable in units of a million yen.
[b]Per capita income was used as the ordinal variable: from the lowest decile of 0.05 to the highest decile of 0.95.

increased from 1995 to 2000. This increase was consistent regardless of different health indicators and different geographic levels for both men and women. The recent data invite several warnings about Japanese health status. LE of some occupational classes declined in the past few years (*28*). LE of all Japanese men also declined from 2004 to 2005 (*29*). This is not conclusive, but the increase in health inequalities may be linked to the deterioration of the health of the population.

Although the explanation for the possible increase in health inequalities in recent years is beyond the scope of this study, increasing socioeconomic inequalities are a potential contributor to increasing health inequalities. Some measures such as the Gini coefficient suggest a widening of income distribution in Japan (*14,30*). The economic recession after the collapse of the bubble economy in the early 1990s and the subsequent policies on economics, taxation, and social security might have contributed to increased socioeconomic inequalities (*12-14*). Crumbling of the lifetime employment system found in Japanese companies, the increase in unstable employment, and the increase in social security costs might have also accelerated worries about increasing socioeconomic inequalities (*12-14*).

The health care system in Japan is considered among the best in the world in terms of fairness of financial contribution, health outcomes, and other indicators (*31*). The system is believed to contribute to the healthy status of the Japanese population (*9*). However, recent figures suggest an increase in inequality in access to and use of health care in Japan. Geographic disproportions in health care, such as in the number of obstetricians, gynecologists and pediatricians, and cancer care resources are increasingly receiving attention (*32,33*). A previous ecological study showed that the lack of resources for maternal and child health is associated with higher infant mortality in Japan (*34*). Other studies have noted that the postgraduate medical training system and recent health policies, mainly in relation to the postgraduate medical training system and control of health care expenditures, might trigger geographic disproportions and widening inequalities in health care (*35*). In addition, an increasing number of people who cannot afford insurance premiums appears to be endangering universal health insurance coverage (*36*). Circumstances concerning health care may widen health inequalities and consequently threaten improvements in the health of the Japanese population.

Analyses of health inequalities often suffer from methodological problems. The selection of both health indicators and socioeconomic variables and methods of analyzing their association are critical (*37*). The findings of this study were obtained using sophisticated methods with reliable health and socioeconomic variables at two different levels. Nonetheless, a few limitations are acknowledged below.

First, the observation period is too short to conclude that health inequalities were increasing until 1995, and health inequalities should be continuously monitored. Second, another combination of health indicators and socioeconomic variables could demonstrate a different pattern from that of this study. More specified health indicators, such as cause-specific mortality, will elucidate more detailed situations including an explanation for increasing health inequalities. Area indicators representing socioeconomic conditions are critical in area-based analyses. Agreed-on area indicators have not been established in Japan, unlike in some countries where indicators such as deprivation indices have been applied (*38*). The development of area-based socioeconomic indicators is an urgent challenge for the study of health inequalities in Japan. Lastly, but of equal importance, ecological studies have methodological limitations, including confounding factors and the ecological fallacy (*39*). Nonetheless, geographical data can yield meaningful evidence on health inequalities, especially in the long term, since individual-level data are generally of limited use for such analyses (*40*). Further studies with individual-level analyses based on a system to monitor individual-level inequalities should be encouraged in order to provide more conclusive evidence.

In conclusion, this study showed a possible increase in socioeconomic inequalities in life expectancy and mortality from 1955 to 2000, following a decrease in inequalities from 1955 to 1995. Although conclusions should be carefully drawn from further studies and future monitoring, Japan's marked health improvement in the past half century may not enjoy an equal parallel in the future.

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Factors affecting routine immunization coverage among children aged 12-59 months in Lao PDR after regional polio eradication in Western Pacific Region

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SUMMARY **The global poliomyelitis eradication programme had a great impact on routine immunization coverage in Lao PDR: DPT3 increased 23% in 1992 to 56% in 1999; OPV3 27% to 64%. However, after the achievement of regional eradication, coverage became stagnant in accordance with the withdrawal of various sources of financial supports. In place of the former funds, a public-private global partnership began to support EPI. We aim to explore factors affecting routine immunization coverage. From February to March of 2005, a cross-sectional questionnaire survey was conducted, targeting 341 mothers living in two districts where immunization coverage was the lowest and the middle in Oudomxay province. DPT3 coverage was 72%, higher than the national target of 65%; however, the drop-out rate was 21%. Influential factors on fully immunized child was distance, literacy, possession of livestock; mothers knowledge of immunization target diseases, measles immunization schedule; and mother's willingness to pay for immunization. In total, 98% of all mothers lived within a 30-minute walk of the immunization site. Household visits increased the immunization status among mothers who were illiterate, utilizing an outreach site for immunization, not willing to pay for immunization, receiving home delivery, and without health education attendance. The much higher routine immunization coverage especially in a district of poor EPI activities suggests a well-designed primary health care approach under the district strategy, the zone-zero social mobilization strategy and good lines of communications; it also points to the benefits of the polio eradication initiative. Household visits were found to be effective for people living with difficulties in such as education, living location, and finance. An equally shared funding system for the basic health as well as international policy for respecting the existing system in poor country is important.**

Key Words: Polio eradication, stagnation of vaccine coverage, routine immunization, campaign, primary health care

Introduction

In Lao PDR, the expanded programme on immunization (EPI) was initiated in 1979. By 1982, EPI was operating in only two of 18 provinces and in only 10 of the then 121 districts, but it had expanded to cover 97 districts by 1992 (*1*). In 1991, Lao PDR initiated the global polio eradication programme (*2*), which made substantial progress by its mass oral polio vaccination campaign and acute flaccid paralysis surveillance (*3*). This global programme greatly contributed to the progress of basic routine immunization coverage: BCG coverage in 1992, 1995, and 1999 was 34%, 62%, and 63% in 1992, 1995, and 1999; three doses of diphtheria-

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pertussis-tetanus toxoid vaccine (DPT3) was 23%, 54%, and 56%; three doses of oral polio vaccine (OPV3) was 27%, 64%, and 64%; measles vaccine 46%, 68%, and 71%, respectively (*4*).

Mass polio vaccination campaign for children < 5 years of age was very useful strategy for the country with difficulty in routine immunization service due to limited infrastructure. The Western Pacific Region achieved regional polio eradication in 2000 (*5*).

However, after this historic achievement, immunization coverage in Lao PDR became stagnant: BCG coverage was in 2000, 2002, and 2004 was 69%, 65%, and 60%; DPT3 was 53%, 55%, and 45%; OPV3 was 57%, 55%, and 46%; measles was 42%, 55%, and 36%, respectively (*4*). This trend might have been caused by the cessation of the intensive vertical approach in response to the achievement (*6,7*). The withdrawal of international partners' support was also a factor in this declining coverage: The Japan International Cooperation Agency (JICA) project terminated its technical support for general EPI activities as well as polio eradication, despite the continuation of its free provision of all basic EPI vaccines; the United Nations Children Fund (UNICEF) has shifted its priority back to community-based activities targeting mothers and children; and The Australian Government's overseas aid program (AusAID) withdrew its activities from Lao PDR. However, the Global Alliance for Vaccines and Immunization (GAVI), a public-private global partnership, was created in 1999 to enable even the poorest countries to provide vaccines to all children (*8,9*), and GAVI started to provide Lao PDR with combination of Hepatitis B-DPT vaccines and auto-disable syringes in 2002 under the three-year plan (*10*).

The stagnations of vaccination coverage have been observed at the global level after substantial progress towards universal immunization in the 1980s, reaching over 70% coverage for children globally (*11*). Coverage for DPT3, the benchmark indicator of annual routine immunization coverage, was less than 50% in 10 countries, and Lao PDR was one of them (*12*).

Lao PDR is categorized as one of the least developed countries with a GDP per capita of $491 in 2005 (*13*) and these days donors tend to ask for concrete the achievements for the sake of accountability to contributors of funding and in order to make judgments as to whether or not to continue support, as seen in case such as GAVI (*14*). However, in the country, no paper exploring authentic immunization status and factors affecting immunization coverage has been presented for publication to international academic journals to date, a paper which might help to attract international or bilateral partners to attain the more stable financial support essential to immunization services. National EPI staff also claimed that official coverage was underestimated because the real target population or denominator must have been lower than the official estimated population due to the progress of family planning.

As shown in previous studies in resource limited settings, good immunization coverage has been achieved by the efforts of a robust primary health care approach (*15*), mothers' knowledge (*16-18*) and the provision of immunization information (*17,19,20*), results which encouraged us. This study aimed to investigate true immunization coverage in two rural districts in Oudomxay province by field study, and to explore factors affecting routine immunization coverage.

Materials and Methods

District strategy and zone zero social mobilization for immunization service

Since much of the country is mountainous and the transportation network is underdeveloped nationwide, a district strategy developed for universal access to immunization services in 1991 (*21*). In this strategy the district was considered as the operational unit with the capacity to plan and manage an immunization programme which would deliver services through health facilities and outreach activities to villages. The catchment area for a health facility was divided into four zones: 1) zone 0 containing villages within 3 kilometers of a health facility for immunization delivery; 2) zone 1, villages to which a vaccinator can walk or ride by bicycle; 3) zone 2, villages to which a vaccinator can reach with mechanized transport, conduct a vaccination session and return within one day; and 4) zone 3, villages which require more than one day for travel and to conduct an immunization session. The strategy also proposed that each village should be visited at least four times a year and community should be actively involved in supporting delivery of immunization services.

In 1996, zone zero social mobilization, encouraging villagers residing nearby a health facility to visit the health facility without waiting for the visit of an outreach team, was adopted, and villagers were expected to get additional health services as well as immunization service at the health facility (*22*).

The Ministry of Health set a 2004 national goal of fully immunized children (FIC) coverage 60%, and basic immunization coverage of BCG, DPT3, OPV3, and measles vaccine of 75%, 65%, 65%, and 60%, respectively (*23*).

Site and subjects

From February to March of 2005, a community based cross-sectional questionnaire survey was conducted in Oudomxay province located in northern part of the country, targeting mothers living in the Xay and Beng districts where immunization coverage was the lowest and at intermediate levels, respectively, among a total of

7 districts. In 2004 provincial reported official coverage of BCG, DPT3, OPV3, and measles vaccine was 67%, 54%, 59%, and 44%, respectively. In Xay district, 2004 official coverage was 51%, 27%, 26%, and 21%; in Beng district, it was 94%, 75%, 74%, and 58% (*24*).

The province is mountainous, and consisted of 7 districts with 867 villages. The area is 15,370 square kilometers, and total population was 247,385. There are 189 villages in Xay district and 108 in Beng, and two villages were randomly selected from each zone (0 to 3) in each district. Thus, 8 villages were selected from Beng district and 8 from Xay, containing a total of 1134 households. By visiting all households, we selected 341 mothers with children from 12 to 59 months old. The ethnic groups are predominant: Lao Loum (major ethnic group) and Lao Theung (minor ethnic group).

Measurement and analysis

The research team was composed of a national EPI staff member, two district EPI staff members, two trained interviewers, and a Japanese researcher. A face-to-face interview with mothers at the target households was conducted using a structured questionnaire, developed by the EPI experts from both Lao PDR and Japan and revised after the pre-survey conducted in the capital city of Vientiane. The questionnaire was originally developed in English and translated into the Lao language by the national EPI manager, then translated back into English by Laotian researchers in the team.

The dependent variable was child immunization status, obtained from an immunization card. When the card was not available, the mother was asked orally about the child's immunization history by the EPI staff. Child immunization status was categorized into two groups: 1) fully immunized children who received all doses of BCG, DPT, OPV, and measles vaccine; and 2) partially or not immunized children who missed any dose of the vaccines. Independent variables were socio-economic characteristics, mother's KAP (knowledge, attitude and practice) on vaccination, and sources of information on vaccination.

Ethical approval was obtained from the National Ethics Committee for Health Research of the Ministry of Health in Lao PDR, and the Ethics Committee of the University of Tokyo. Informed consent was obtained from all respondents before the interview.

To assess factors associated with immunization status, univariate, bivariate and multivariate analyses were conducted. Univariate analysis was employed to clarify frequencies and distributions of each variable. Chi-square test and stratified analysis were used for bivariate analyses. From the results of univariate analysis, multivariate logistic regression analysis was conducted to determine the predictors of fully immunized children. A p-value < 0.05 was considered to indicate statistical significance. To perform statistical analysis, SPSS 11.0 for Windows was used.

Results

There was no significant difference in immunization coverage between Xay and Beng district (Table 1). The fully immunized children coverage of 60% was equal to the national target, and only 12 children (3.5%) were not immunized. The coverage of BCG, DPT3, OPV3, and measles vaccine in Xay district was 92%, 75%, 74%, and 69%, while official coverage in 2004 was 51%, 27%, 26%, and 21%, respectively. The coverage in Beng district was 86%, 70%, 73%, and 75%, while official coverage 94%, 75%, 74%, and 58%, respectively. The average of DPT1 coverage was as high as 93%; however, drop-out rate was 21% (DPT1-DPT3).

Table 2 shows the association between socio-economic characteristics and immunization status. Zone of residence, ethnic group, literacy, and livestock possession were significantly associated with immunization status. However, household income, place of delivery, and history of anti-neonatal care (ANC)

Table 1. Child immunization status (n = 341)

	Xay		Beng		TOTAL	(%)
	N	(%)	N	(%)		
Immunization status						
Fully immunized	93	(66.3)	122	(57.7)	205	(60.1)
artially immunized	51	(34.7)	73	(37.6)	124	(36.4)
Not immunized	3	(2.0)	9	(4.6)	12	(3.5)
Own immunization card ("Yellow "card)						
Yes					248	(72.7)
No					93	(27.3)
BCG	135	(91.8)	167	(86.1)	302	(88.6)
DPT1	139	(94.6)	177	(91.2)	316	(92.7)
DPT2	126	(85.7)	163	(84.0)	289	(84.8)
DPT3	110	(74.8)	136	(70.1)	246	(72.1)
OPV1	130	(88.4)	170	(87.6)	300	(88.0)
OPV2	121	(82.3)	167	(86.1)	288	(84.5)
OPV3	109	(74.1)	142	(73.2)	251	(73.6)
Measles	102	(69.4)	145	(74.7)	247	(72.4)
Drop-out (DPT1-DPT3)	29	(19.8)	41	(21.1)	70	(20.6)

Table 2. Socio-economic characteristics and immunization status (n = 341)

		Fully (n = 205)		Partially or Not (n = 136)		P value
		N	(%)	N	(%)	
Zone of residence						
	0-1	130	(63.4)	44	(32.4)	< 0.001
	2-3	75	(36.6)	92	(67.6)	
Ethnic group						
	Lao Lum	92	(44.9)	29	(21.3)	< 0.001
	Lao Theung	113	(55.1)	107	(78.7)	
Age of mother (years)						
	< 20	14	(6.9)	16	(11.9)	
	20-29	125	(61.3)	78	(57.8)	
	30-39	55	(27.0)	28	(20.7)	
	≥ 40	10	(4.9)	13	(9.6)	
	N/A†	1		1		
Gender of child						
	Male	97	(49.5)	67	(52.8)	
	Female	99	(50.5)	60	(47.2)	
	N/A	9		9		
Literacy						
	Literate	79	(40.7)	29	(22.3)	< 0.01
	Illiterate	115	(59.3)	101	(77.7)	
	N/A	11		6		
Household income/month (Kip)						
	≤ 100,000 (≥ 10 USD)	102	(50.8)	68	(52.3)	
	> 100,000 (< 10 USD)	100	(49.5)	62	(47.7)	
	N/A	3		6		
Livestock						
	Yes	158	(77.1)	88	(64.7)	< 0.05
	No	47	(22.9)	48	(35.3)	
Place of Delivery						
	Health facility	58	(28.3)	32	(23.7)	
	Other (Home delivery)	147	(71.7)	103	(76.3)	
	N/A	0		1		
History of ANC attendance						
	Yes	128	(62.4)	72	(52.9)	
	No	77	(37.6)	64	(47.1)	

† Not available

attendance were not associated with immunization status.

The association between KAP of mothers and immunization status is shown in Table 3. Mothers who knew the target diseases of immunization, knew the schedule for measles immunization, and knew the number of times to visit the immunization site, and mothers who had willingness to pay for immunization had significantly increased chances of having fully immunized children. The place to get immunization (outreach site or health facility) and means of access to immunization site were not associated with immunization status.

Table 4 shows the effect of the source of information on immunization affecting on immunization status. Household visit as a source of information for immunization day was associated with a significant increase in the rate of fully immunized children (p < 0.05). Although there was no significant association observed to support the observed result, as many as 63% of the mothers of the fully immunized children were encouraged by village heads to bring their children to an immunization site. Mothers who obtained information on immunization before the delivery significantly influenced the rate of fully immunized children (p < 0.01). Health education attendance, village meeting attendance and birth registration after delivery were not associated with immunization status.

In Table 5, we divided the subjects into two groups of mothers who had received a household visit and mothers who had received no household visit, and explored factors affecting immunization status. Household visits significantly influenced the rate of fully immunized children among mothers who were illiterate, were utilizing an outreach site for immunization, had no willingness to pay for immunization, had delivered at sites other than health facilities (*e.g.*, home delivery, and reported no health education attendance (p < 0.05).

To control confounding factors, the data were analyzed by a multivariate logistic regression model (Table 6). Three factors significantly increased the rate of fully immunized children: zone of residence (OR = 2.40, CI = 1.13-5.13); mothers' knowledge of schedule for measles immunization (OR = 3.35, CI = 1.49-7.69); and willingness to pay for immunization (OR = 5.40, CI = 1.48-19.73).

Discussion

Our study revealed remarkably higher immunization coverage compared with official coverage in Xay

Table 3. KAP of mothers and immunization status (n = 341)

		Fully (n = 205)		Partially or Not (n = 136)		P value
		N	(%)	N	(%)	
Knowledge:						
Target diseases of immunization						
	Know	144	(70.2)	75	(55.1)	< 0.01
	Unknown	61	(29.8)	61	(44.9)	
Cause of measles						
	Know	21	(10.2)	7	(5.1)	
	Unknown	184	(89.8)	129	(94.9)	
Benefit of immunization						
	Know	142	(69.3)	86	(63.2)	
	Unknown	63	(30.7)	49	(36.0)	
	N/A†	0		1		
Number of doses of DPT						
	Know	82	(40.0)	40	(63.9)	
	Unknown	123	(60.0)	96	(70.6)	
Symptoms of measles						
	Know	139	(67.8)	99	(72.8)	
	Unknown	66	(32.2)	37	(27.2)	
Schedule for measles immunization						
	Know	70	(34.1)	13	(9.9)	< 0.001
	Unknown	135	(65.9)	118	(90.1)	
	N/A	0		5		
Times visiting immunization site to complete the immunization schedule						
	Know	163	(79.5)	86	(63.2)	< 0.01
	Unknown	42	(20.5)	50	(36.8)	
Attitude:						
Will you take your child for getting immunization if you have to pay for it?						
	Yes	195	(95.1)	112	(83.6)	< 0.01
	No	10	(4.9)	22	(16.4)	
	N/A	0		2		
Practice:						
Place where immunization is received						
	Outreach site	122	(59.5)	91	(66.9)	
	Health facility	83	(40.5)	45	(33.1)	
Means of access to immunization site						
	On foot	164	(80.0)	113	(87.9)	
	Bicycle, Motorcycle, *etc.*	40	(20.0)	16	(12.4)	

† Not available

Table 4. Source of information on immunization and immunization status (n = 341)

	Fully (n = 205)		Partially or Not (n = 136)		P value
	N	(%)	N	(%)	
Persons encouraging mother to bring child to immunization site					
Village head	126	(63.0)	86	(63.7)	
Hospital staff (Doctor/Nurse)	49	(24.5)	35	(25.9)	
Others (Relatives *etc.*)	23	(11.5)	11	(8.1)	
None	2	(1.0)	3	(2.2)	
N/A†	5		1		
Household visit for informing of immunization day					
Yes	71	(35.3)	31	(23.7)	< 0.05
No	130	(64.7)	100	(76.3)	
N/A	4		5		
Obtained information on immunization before delivery					
Yes	145	(85.8)	88	(68.8)	< 0.01
No	24	(14.2)	40	(31.3)	
N/A	36		8		
Health education attendance in the past year					
Yes	158	(79.8)	107	(84.3)	
No	40	(20.2)	20	(15.7)	
N/A	7		9		
Village meeting attendance in the past year					
Yes	183	(90.1)	121	(91.0)	
No	20	(9.9)	2	(9.0)	
N/A	2		3		
Birth registration after delivery					
Yes	120	(58.8)	79	(58.1)	
No	84	(41.2)	57	(41.9)	
N/A	1		0		

† Not available

Table 5. Household visit for information and immunization status (n = 332[§])

			Household visit				P value
			Yes (n = 102)		No (n = 230)		
			N	(%)	N	(%)	
Literacy							
	Literate	Full	23	(79.3)	55	(70.5)	
		Partially or Not	6	(20.7)	23	(29.5)	
	Illiterate	Full	44	(64.7)	68	(48.6)	< 0.05
		Partially or Not	24	(35.3)	72	(51.4)	
	N/A[†]		5		12		
Type of immunization site							
	Outreach site	Full	47	(68.1)	72	(51.8)	< 0.05
		Partially or Not	22	(31.9)	67	(48.2)	
	Health facility	Full	24	(72.7)	58	(63.7)	
		Partially or Not	9	(27.3)	33	(36.3)	
Willingness to pay for immunization							
	Yes	Full	63	(71.6)	128	(59.8)	
		Partially or Not	25	(28.2)	86	(40.2)	
	No	Full	8	(61.5)	2	(12.5)	< 0.05
		Partially or Not	5	(38.5)	14	(87.5)	
	N/A		1		0		
Place of Delivery							
	Health facility	Full	20	(69.0)	36	(62.1)	
		Partially or Not	9	(30.1)	22	(37.9)	
	Others	Full	51	(69.9)	94	(55.0)	< 0.05
		Partially or Not	22	(30.1)	77	(45.0)	
	N/A		0		1		
Health education attendance in the past							
	Yes	Full	57	(65.5)	97	(56.4)	
		Partially or Not	30	(34.5)	75	(43.6)	
	No	Full	11	(100)	29	(61.7)	< 0.05
		Partially or Not	0	(0)	18	(38.3)	
	N/A		4		11		

[§] Nine Questionnaires were not available
[†] Not available

Table 6. Factors affecting rate of fully immunized children (n = 341)

Independent variables			OR	95%CI	P value
Socio-demographic characteristics:					
	Zone of residence (Living Zone)				
		0-1	2.40	[1.13-5.13]	< 0.05
	Ethnic group				
		Lao Lum	1.36	[0.58-3.17]	
	Literacy				
		Literate	1.25	[0.90-1.75]	
	Property (Livestock)				
		Yes	1.83	[0.97-3.45]	
KAP:					
	Target disease of immunization				
		Know	1.23	[0.66-2.30]	
	Number of doses of DPT				
		Know	1.09	[0.56-2.12]	
	Times visiting immunization site to complete the immunization schedule				
		Know	1.21	[0.62-2.35]	
	Schedule for measles immunization				
		Know	3.35	[1.46-7.69]	< 0.01
	Willingness to pay for immunization				
		Yes	5.40	[1.48-19.73]	< 0.05
Information:					
	Information on immunization before delivery				
		Yes	1.40	[0.69-2.84]	
	Household visit by some informants				
		Yes	1.31	[0.67-2.53]	

district, the district with the poorest EPI activities in the province. This suggests that the real target population in the district might be smaller than the estimated population based on the 1995 census for calculating coverage, probably due to the success of family planning as pointed out by national EPI staff. The total fertility rate fell from 5.6 in 1995 to 4.9 in 2000 by UNFPA report (*25*). The higher coverage could be expected at cities in Lao PDR where family planning has been successful, and the demonstration of the additional immunization coverage based on the new census conducted in 2005 next to the current official coverage would be useful to obtain a real picture of coverage. The wide gap between our findings and official data might be attributable to incomplete reporting: if some villages do not report, the numerator becomes smaller, resulting in lower coverage (*26*). In contrast, Beng district, demonstrating the intermediate performance in EPI, exibited coverage closer to the official report, suggesting good activities in reporting and recording as well as in immunization services. This indicates the necessity of continuous training especially in weak EPI management area, even after the achievement of regional polio eradication. A previous paper describing health workers in Ghana reported that continuing professional education is required to ensure homogenous provision of appropriate quality of services (*27*). The high drop-out rate of about 20% indicates that there is a chance to increase the coverage from around 70% to more than 90% by overcoming associated factors detected in this study.

This study revealed a successful primary health care approach to immunization in a limited infrastructure setting. Residences near health facilities or zone 0-1 (by multivariate logistic regression), and the major ethnic group of Lao Loum must have great benefits from geographical advantage and presumably better chances of education and communication, which would be attributable to increasing the coverage. Papers from Bangladesh, Nepal, and China also reported the benefits of short distance to immunization site for coverage (*19,28,20*). However, the efforts made to offer an equal opportunity of utilize immunization services for those residing in zone 2-3 and the ethnic group of Lao Theung who live in highland were also revealed: 335 (98.2%) of the total of 341 mothers lived within a 30-minitue walk from immunization sites, suggesting outreach activities enabled people living in remote areas to access to immunization service. Bishai *et al.* also reported outreach services can reduce socioeconomic differentials in vaccine receipt (*29*).

Literate mothers perhaps had basic knowledge of EPI, such as target diseases, times of immunization, and especially schedule for measles immunization (multivariate logistic regression), which contributed to the rate of fully immunized children. The schedule for measles must have been strongly influenced by the intensive measles elimination programme instituted after regional polio eradication was achieved. Mothers education was positively correlated with immunization status elsewhere (*16,17,30*). However, more advanced knowledge such as cause of measles or benefits of immunization did not influence immunization status, suggesting the necessity of more advanced education for mothers. A recent Indian study concluded that increasing women's literacy at the community level, in addition to mother's access to higher education such as matriculation and beyond were effective development tools for child's complete immunization status (*18*). More willingness to pay on the part of the mother was a strong predictor for full immunization and 90% [(195 + 112)/341] of all mothers reported willingness to pay, which show quite positive on immunization in the areas, and additional higher education could enhance immunization coverage more. Although encouragement by village head did not affect the immunization status statistically, as much as 63% of the mothers have been encouraged to obtain full immunization for their children, suggesting that village head education remains a possible key for improving immunization coverage.

We revealed that household visits and receipt of information before delivery influenced the number of fully immunized children. Further analysis revealed that household visits contributed to higher rate of immunization, especially among classes of marginalized mothers such as those with the indicators of illiterate, use of outreach site, no willingness to pay for immunization, delivery other than health facility, and no health education attendance, which indicates the remarkable achievement of universal access, one of the main principles of primary health care (*31*) in countries with limited human resources and funds. Previous studies have shown that direct communication through household visits was clearly effective in improving immunization coverage (*17,19*). The policy of the district strategy for immunization service in Lao PDR has been developed based on a primary health care approach for universal access dating back to 1991 (*21*), and our findings indicate the strategy was well-designed and implemented efficiently by the efforts of local staff in accordance with the progress of the polio eradication initiative. A similar strategy, the Reaching Every District (RED) strategy has been initiated especially in African countries since 2002 to improve stagnated routine immunization coverage (*32*), and the RED strategy implemented in five African countries by WHO and UNICEF showed good results due to outreach services and micro-planning (*33*).

As suggested in a USAID report, even the best-designed and carefully implemented communication interventions in support of immunization will deliver few results if not properly funded (*11*), and EPI in Lao PDR faces serious funding problems as reported by Save the Children Australia: only 26% of children in

a western province of Lao PDR were fully immunized in 2004 due to vaccine supply problems and a lack of adequate funds (*34*). After the regional polio eradication in 2000, main donors priorities have shifted from EPI to another priority: The Japanese government started considering the termination of basic vaccines which have supplied since 1989; UNICEF reduced operational costs; and the WHO extra budget seems too small to adequately support vaccine procurement. Under these circumstances, GAVI started to provide a combination of Hepatitis B-DPT vaccines together with auto-disable syringes in 2002. However, the support will be terminated soon, as the outcome did not meet the target set by GAVI. Since Lao PDR is a country with limited budgetary resources and a human development index ranking of 135 (*35*), a stable supply of the resources necessary for basic public health should be provided free of charge through the international society rather than through a single bilateral donor or ad-hoc private funding. Private involvement in public health in developing countries has been controversial: Lu C *et al.* reported GAVI's successful funding of immunization support in countries with baseline DPT3 coverage of 65% or less (*14*) and some have argued that GAVI might build health services (*36*), while some (*37-41*) have expressed concerns that GAVI investments could distort national priorities and lead to reduction in the delivery of other health services. In Lao PDR, at the time of introduction of GAVI, there had been no discussion of which donors would succeed the fund after termination of its support. From the perspective of sustainability and environmental preservation, as pointed out in the previous studies conducted in African countries (*37,38*), the international society should share the fund equally for basic human needs in developing countries.

Our study was conducted in two districts, which are not necessarily representative of the country as a whole. However, the data were obtained from typical rural districts and villages with low and intermediate immunization coverage by a scientific approach. Routine immunization coverage was higher than or closer to the official reports, suggesting a well-designed primary health care approach under the district strategy, zone-zero social mobilization strategy and good communications as well as the benefits of the polio eradication initiative. Household visits were found to be effective for people living with difficulties in such as education, living location, and finance. As equally shared funding system for basic health, as well as an international policy for respecting the existing system in poor countries is important.

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Nucleotide sequence context influences HIV replication fidelity by modulating reverse transcriptase binding and product release

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SUMMARY **An RNA template/DNA primer (T/P) complex derived from the env gene of HIV-1 was used to examine the kinetic effects of specific basepair substitutions on dNTP incorporation and RNase H cleavage by HIV-1 reverse transcriptase (RT). Single basepair substitutions 2 or 6 nucleotides upstream from a defined polymerization site (denoted -2 and -6) were engineered by oligonucleotide synthesis to provide 7 T/P substrates for kinetic analysis. A -6 A/T substitution in the wild type sequence resulted in 14- and 7-fold increases in the apparent second order rate constants (k_{2app}) for U/A and U/G basepair formation. The k_{2app} for U/A formation was relatively unchanged for all other T/P basepair changes. The -6A/T substitution also uniquely lowered the RNase H cleavage rate by 3-fold. Combined kinetic and thermodynamic analyses indicated that these effects were due almost exclusively to increases in the K_D (k_{off}/k_{on}) of initial binding of RT to the T/P and the rate of product release. The data suggest that certain sequence contexts may influence RT fidelity by modulating enzyme binding/dissociation rather than by altering dNTP binding affinity or the rate of the bond forming step.**

Key Words: Reverse transcriptase, polymerase, RNase H, sequence context, steady and pre-steady state kinetics

Introduction

The relatively low fidelity of HIV-1 RT (~1 in 10^4 errors/replication cycle (*1-4*); coupled to rapid viral turnover (~10^{10} virions/day (*5*); contributes to the phenomenon of retroviral hypermutagenesis. This results in the evolution of drug resistance and escape from immune system surveillance, persistent obstacles in the treatment of AIDS. Other biochemical factors that influence HIV replication fidelity include the effects of sequence context (*6,7*), RNA secondary structure (*8,9*), accessory proteins (*10-12*) and the presence of modified bases in the RNA template and dNTP precursor pool (*13-15*). In particular, the sequence composition of the T/P stem alone appears to exert large effects on the processivity and fidelity of HIV-1 RT. For example, polymerase termination frequency can be influenced by single base substitutions upstream of the dNTP incorporation site (*6,7*). The incorporation efficiency of nucleoside analog inhibitors of RT has also been shown to be highly dependent on T/P base composition (*16*). These "indirect effects" resulting from perturbation of RT-T/P points of contact remote from the polymerization and RNase H sites can have important consequences for HIV-1 RT fidelity (*17*), but the biochemical mechanisms are poorly understood. Crystallographic studies of HIV-1 RT bound to either DNA/DNA (*18,19*) or RNA/DNA (*20*) reveal van der Waals and hydrogen bonded contacts to the T/P stem upstream of the polymerization site by residues of the palm and thumb subdomains of the p66 subunit of HIV-1 RT. Mutagenesis of these residues can result in significant modifications to the polymerase, strand transfer and RNase H activities (*21-23*), suggesting that specific interactions between amino acid side chains of HIV-1 RT and the T/P can regulate different enzymatic functions of RT.

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We have taken a complementary approach to protein mutagenesis studies by varying single T/P basepairs predicted to be in close contact with the p66 palm and thumb subdomains of HIV-1 RT and measuring the resulting effects on dNTP incorporation and RNase H cleavage kinetics. We used an RNA template corresponding to nucleotides 962 to 996 of the *env* gene from HIV-1 clone WMJ1 (*24*) as a model sequence to examine these effects. Base substitutions were made at the -2 and -6 positions in the T/P by synthesis of the appropriate oligoribonucleotide templates and complementary DNA primers, and the corresponding effects on HIV-1 RT activity was measured using steady and pre-steady state kinetic models. Previous work using M13 DNA templates showed that the sequence composition at these positions exerted a significant effect on the termination of cDNA synthesis by HIV-1 RT (*7*), suggesting a potential role in modulating polymerase fidelity.

Materials and Methods

Oligonucleotides

RNA templates were synthesized by Dharmacon Research (Lafayette, CO, USA). RNA oligomers were deprotected according to the directions of the manufacturer and were used without further purification. DNA primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Deoxynucleoside triphosphates were purchased from Roche (Pleasanton, CA, USA). DNA oligomers were purified by 20% polyacrylamide gel electrophoresis (PAGE) and isolated using an ELUTRAP (Schleicher & Schuell BioSciences, Keene, NH, USA). DNA oligomers were desalted on Centri Spin 10 gel filtration columns (Princeton Separations, Adelphia, NJ, USA). RNA and DNA concentrations were determined spectrophotometrically using the absorbance at 260 nm according to the method of Borer (*25*). DNA primers (6 μM) were 5'-end-labeled with 10 μCi [γ-^{32}P] ATP (ICN Biomedicals, Irvine, CA, USA) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) using a standard procedure. Template/primer complexes were formed by mixing RNA with a 1.5 fold excess of the corresponding DNA primer in 50 mM Tris-HCl/NaCl (pH 7.5) for 3 min at 90ºC followed by cooling over 90 min in a heat block.

Purification of HIV-1 RT

HIV-1 RT was expressed from plasmid pHIV-RT (His) Prot transformed into *E.coli* BL21 (DE3) pLysS cells (Novagen, Madison, WI, USA) and purified essentially as described (*14*) with several minor changes. The cell-free extract obtained from a 6 L culture was applied to a pre-equilibrated Ni-NTA superflow affinity column (Qiagen, Valencia, CA, USA) in metal affinity equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.0) at a flow rate of 0.6 mL/min using an FPLC (LCC-500 Plus, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The column was washed with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% glycerol, pH 7.0) at a flow rate of 0.3 mL/min until the A_{280} was reduced to < 0.08 a.u. HIV-1 RT was eluted from the column using a gradient (10-250 mM imidazole in buffer A) at a flow rate of 0.75 mL/min. Fractions were analyzed for protein content using the Bradford reagent (Biorad, Hercules, CA, USA) and by SDS-PAGE with Coomassie staining. Fractions containing HIV-1 RT p66/p51 heterodimer were combined and concentrated using Centricon plus-20 tubes (Millipore Corporation, Bedford, MA, USA) prior to loading onto a gel filtration column (HiPrep 26/60 Sephacryl S-300; Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 50 mM Tris-HCl, pH 8.0 at a flow rate of 0.8 mL/min. Aliquots determined to contain HIV-1 RT were pooled and concentrated, and enzyme concentrations were determined spectrophotometrically as previously described (*14*). The fraction of catalytically active polymerase molecules was assessed by active site titration as described below.

Steady state kinetics

HIV-1 RT (56.5 nM) was added to T/P complexes (150 nM) containing 0.1 μCi 5'γ-^{32}P end-labeled primers in 4 μL 50 mM Tris-HCl/NaCl (pH 7.5). Reactions were initiated by adding 4 μL of dATP (0.005, 0.01, 0.02, 0.05, 0.1, and 0.2 μM) or dGTP (0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mM) containing 10 mM Mg^{2+} at 37°C. Polymerization was quenched within the linear phase (30 sec and 3 min respectively for reactions with dATP and dGTP) with 16 μL of 90% formamide, 50 mM EDTA (pH 8.0), 0.1% xylene cyanol, and 0.1% bromophenol blue. Primer extension products were separated using 20% PAGE and the data were quantified by PhosphorImager analysis using the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA, USA). Reaction velocity was plotted against dNTP concentration and steady state kinetic constants were determined by fitting the data to the Michaelis-Menten equation using Prism (Graph Pad Software, Inc., San Diego, CA, USA). Steady state parameters are compiled in Table 1.

Pre-steady state kinetics

Pre-steady state and active site titration experiments were carried out using a KinTek RQF3 rapid quench flow instrument (KinTek Corporation, Austin, TX, USA). Solution A, consisting of HIV-1 RT (74 nM) and T/P (30 nM) in 50 mM Tris-HCl/NaCl (pH 7.5); and Solution B, consisting of various concentrations

Table 1. Steady-state kinetic parameters

Template	Incorporation of dATP				Incorporation of dGTP			
	V_{max} (nM s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{2app} (s^{-1} μM^{-1})	V_{max} (nM s^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{2app} ($\times 10^5$) (s^{-1} μM^{-1})
Control (WMJ1)	1.80 ± 0.05	0.0318 ± 0.0007	0.108 ± 0.006	0.30	0.33 ± 0.04	0.0059 ± 0.0007	200 ± 56	3.0
-6U/A	0.42 ± 0.01	0.0074 ± 0.0002	0.025 ± 0.002	0.29	0.082 ± 0.004	0.0015 ± 0.00007	190 ± 25	0.78
-6A/T	3.68 ± 0.06	0.0651 ± 0.0011	0.015 ± 0.001	4.3	0.625 ± 0.0098	0.0110 ± 0.0002	52 ± 3.2	21
-6G/C	0.75 ± 0.03	0.013 ± 0.0005	0.022 ± 0.003	0.59	0.256 ± 0.002	0.00452 ± 0.00004	50 ± 1.7	9.1
-2U/A	1.16 ± 0.01	0.0205 ± 0.0004	0.062 ± 0.002	0.33	0.19 ± 0.04	0.0034 ± 0.0006	300 ± 140	1
-2G/C	0.75 ± 0.01	0.013 ± 0.0002	0.026 ± 0.002	0.50	0.146 ± 0.002	0.00257 ± 0.00004	25 ± 1.7	10
-2C/G	0.68 ± 0.03	0.012 ± 0.001	0.031 ± 0.004	0.39				

of dATP (0.005-1 μM) in 10 mM $MgCl_2$, were loaded into separate 18 μL loops of the RQF3 quench flow apparatus. Enzyme catalysis was initiated by rapidly mixing solutions A and B together followed by quenching with 100 mM EDTA at reaction times ranging from 10 ms to 60 sec at 37°C. Primer extensions were analyzed by 20% PAGE and the intensities of the gel bands were quantified by PhosphorImager analysis. Data were then plotted using the appropriate rate equations as previously described (*26*). Time course data for fixed nucleotide concentrations were fitted to Equation 1 by nonlinear regression analysis. In this expression, A is the amplitude of the burst phase, k_{obs} is the rate constant of the burst phase at the given [dATP], and k_{ss} is the steady state rate constant. Values of k_{obs} were graphed as a function of [dATP] using Equation 2 to obtain k_{pol}, the maximum rate constant of the pre-steady state burst phase and K_d^{dATP}, the dissociation constant of the nucleotide triphosphate from the HIV-1 RT•T/P complex.

Equation 1.

$$[product] = A\,(1\text{-}e^{k_{obs}t}) + k_{ss}t$$

Equation 2.

$$k_{obs} = \frac{k_{pol}\,[dATP]}{k_d^{dNTP} + [dATP]}$$

Active site titration

Active site titrations were carried out using varying concentrations of T/P under conditions of fixed HIV-1 RT concentration and saturating dNTP (*26*). Solution A, consisting of HIV-1 RT (37 nM) and T/P concentrations ranging from 12.5-200 nM in 50 mM Tris-HCl/NaCl (pH 7.5) were rapidly mixed with solution B (100 μM dATP and 10 mM $MgCl_2$) and reacted over times ranging from 5 msec to 30 sec in the RQF3 apparatus at 37°C. Primer extensions were analyzed and quantified as described above. Burst amplitudes were obtained from plots of product formation as a function of time for individual T/P concentrations. The dependence of the burst amplitudes as a function of [T/P] was then fitted to Equation 3 by nonlinear regression analysis. K_D is the dissociation constant for the HIV-1 RT•T/P complex and [E] is the concentration of active HIV-1 RT molecules.

Equation 3.

$$A = 0.5(K_D + [E] + [T/P]) - \sqrt{0.25(K_D + [E] + [T/P])^2 - [E][T/P]}$$

RNase H cleavage assay

100 nM T/P containing 0.1 μCi [γ-^{32}P]5'-labeled RNA template was incubated with 37 nM of HIV-1 RT in 50 mM Tris-HCl/NaCl (pH 7.5) at 37°C. Reactions were initiated by the addition of 5 mM $MgCl_2$. Aliquots (10 μL) were removed at time points between 0.25-20 min and quenched as described for steady state analyses. Fragmentation was analyzed by electrophoresis and quantified as described above. The rate of RNA cleavage was determined by fitting the data to a single-exponential decay equation.

Heteroduplex melting analyses

UV melting isotherms were measured on an Ultraspec 3000 *pro* equipped with a Peltier thermoelectric unit. Data were acquired using SWIFT-Tm software (Amersham Pharmacia Biotech). Equimolar concentrations of RNA template and DNA primer over a range of 0.5-3.0 mM were heated to 95°C for 10 min and then slowly annealed to 35°C in 50 mM Tris-HCl/NaCl, pH 7.5 prior to initiating melting at a rate of 0.5°C/min. Melting was monitored by absorbance at 260 nm over a temperature range of 35-70°C. Thermodynamic parameters were derived from plots of T_m^{-1} *vs.* ln [T/P]. ΔH was determined by fitting to the van't Hoff equation (Equation 4) where T_m is the melting temperature, [T/P] is the heteroduplex concentration, and R is the universal gas constant (1.987 cal•K^{-1} mol^{-1}).

Equation 4.

$$\frac{1}{T_m} = \frac{R}{\Delta H}\ln\frac{[T/P]}{4} + \frac{\Delta S}{\Delta H}$$

Results

Steady state kinetic analysis

Steady-state kinetic parameters were determined for the incorporation of dATP and dGTP opposite three contiguous uracil residues (U970-972) of an RNA template derived from the *env* gene of HIV-1 strain WMJ1. The effects of single base pair changes in the T/P stem on polymerase kinetics were determined. The WMJ1 control T/P and uniquely-substituted variants used in this study are shown in Figure 1. Steady state data for WMJ1, -2 and -6 substituted T/Ps are compiled in Table 1. The apparent second order rate constants ($k_{2app} = k_{cat}/K_M$) were used to evaluate the influence of upstream sequences on the relative efficiency of dATP or dGTP incorporation.

The observed k_{2app} value for the incorporation of dATP on the control WMJ1 template was 0.3 $s^{-1}\mu M^{-1}$. All possible single basepair substitutions at the -2 position did not greatly affect this value. Replacement of the A/T basepair at this position with U/A or C/G yielded nearly identical numbers, while replacement with G/C resulted in a less than 2-fold change. However substitution of the WMJ1 C/G pair at the -6 position with A/T resulted in a 14-fold increase in k_{2app} for dATP incorporation. Replacement with U/A at the -6 position had no effect on the incorporation efficiency of dATP, while replacement with G/C resulted in a 2-fold increase.

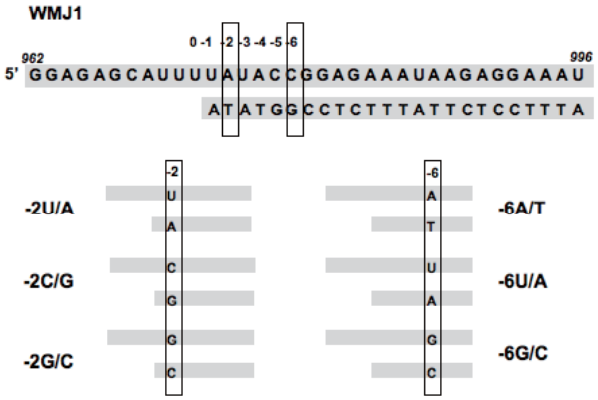

Figure 1. Template/primer (T/P) substrates used to measure the kinetic effects of sequence variation on HIV-1 RT catalyzed dNTP incorporation and RNase H activity. The control sequence corresponds to nucleotides 962-996 of the *env* gene of HIV strain WMJ1. Single basepair substitutions were made at either the -2 or -6 nucleotide positions, denoted by rectangles.

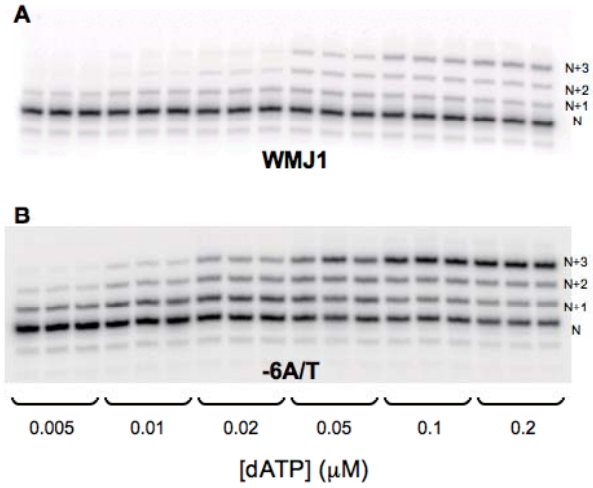

Figure 2. Primer extension data for dATP incorporation opposite U by HIV-1 RT on WMJ1 control and -6A/T substituted T/Ps. Enzyme and T/P concentrations were 50 and 150 nM, respectively. (A) Data acquired using WMJ1 (-6C/G), and (B) -6A/T substituted templates.

The relatively large increase in incorporation efficiency as a result of -6A/T substitution was readily observable from the primary gel electrophoresis data. Figures 2A and B present results for polymerization reactions with dATP conducted under identical conditions for control and -6A/T substituted T/Ps, respectively. Extension of primers of length N on WMJ1 control templates to yield products of length N+3 were observed between 20 and 50 nM dATP. Substitution with -6A/T lowered the concentration of dATP required for extension to between 5 and 10 nM. An increase in the efficiency of dGTP incorporation opposite U relative to control was similarly observed upon -6A/T substitution. This single base change resulted in a 7-fold increase in k_{2app} for U/G mispair formation. Product corresponding to formation of three successive U/G mispairs could not be observed in primer extension reactions using control template for dGTP concentrations up to 0.8 mM, but was readily detected in the -6A/T substituted T/P at 0.4 mM (data not shown). Smaller increases (~3-fold) in mispair formation were observed upon substitution of a G/C basepair at either the -2 or -6 positions. Substitution with U/A at the -2 or -6 position in the control template decreased the efficiency of U/G mispair formation by 4- and 3-fold, respectively.

The influence of both K_M and k_{cat} on the dNTP incorporation efficiency can be ascertained from Table 1. For example, the 14-fold increase in k_{2app} for U/A basepair formation resulting from A/T substitution at the -6 position reflects a 7-fold decrease in K_M and a 2-fold increase in k_{cat}. This same substitution influences the mispairing reaction with dGTP by lowering K_M 4-fold and similarly increasing k_{cat} by 2-fold. Although k_{cat} values obtained from the Michaelis-Menten approximation applied to polymerase reactions are normally considered to reflect rate limiting product release, a mechanistic interpretation of K_M is usually not possible. In order to obtain more detailed kinetic information on how the T/P sequence composition influences the various steps of the polymerization reaction, pre-steady state analyses were carried out using chemical quench flow methods (*26*) for WMJ1 and the -6A/T substituted T/Ps.

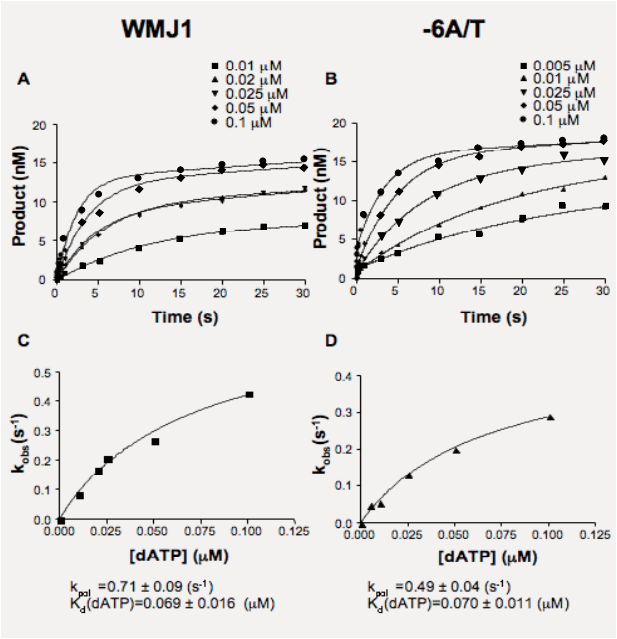

Figure 3. Pre-steady state kinetic analysis of U/A basepair formation. Product formation as a function of time was measured by the rapid quench flow technique using a KinTek RQF3 apparatus. Data were fitted to the single exponential of Equation 1 as described in Materials and Methods to obtain the curves shown in (A) for WMJ1 and (B) for -6A/T substituted T/Ps at the indicated dATP concentrations. Rate constants obtained in (A) and (B) were then graphed as a function of dATP concentration using Equation 2 in order to obtain values for k_{pol} and K_d^{dATP} for WMJ1 (C) and -6A/T (D) T/Ps.

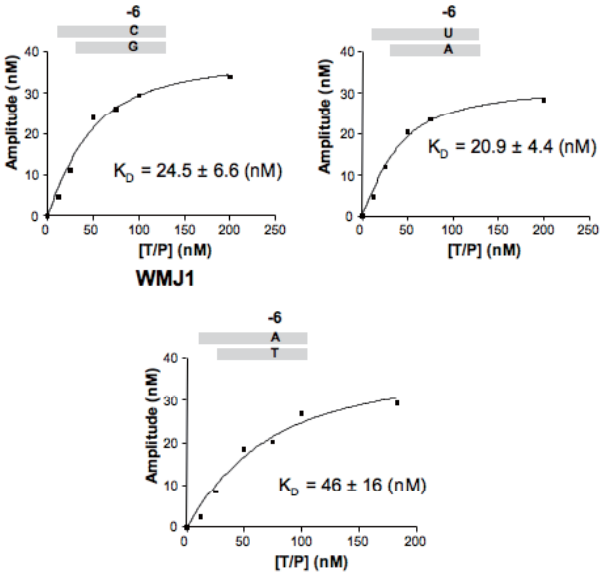

Figure 4. Determination of K_D for binding of HIV-1 RT to (A) WMJ1, (B) -6U/A, and (C) -6A/T T/Ps. Plots of product formation as a function of time for T/P concentrations ranging from 12.5-200 nM were initially obtained using conditions of saturating dATP (50 μM) and 30 nM HIV-1 RT. Plots of burst amplitudes as a function of T/P concentration were then constructed by fitting to Equation 3 to obtain K_D values.

Pre-steady state kinetics

Figures 3A, B show the pre-steady state kinetic data for product formation using the WMJ1 and -6A/T substituted T/Ps. Time course plots of product formation as a function of dATP concentration were obtained by fitting to Equation 1. The concentration dependence of the burst amplitudes (k_{obs}) was then fitted to Equation 2 to obtain K_d, the equilibrium dissociation constant for binding of dATP to the HIV-1 RT•T/P complex, and k_{pol}, the rate of phosphodiester bond formation. These results are shown in Figures 3C, D for the control and -6A/T T/Ps, respectively. The values of K_d obtained for control and -6A/T T/Ps were identical, indicating that the increases in incorporation efficiency resulting from -6A/T substitution were not due to increased polymerase binding of dATP. The values for k_{pol} were 0.71 ± 0.09 s^{-1} for control and 0.49 ± 0.04 s^{-1} for -6A/T T/Ps. The similarity of these values suggested that the rate of phosphodiester bond formation was not markedly influenced by T/P base composition at the -6 position.

Active site titrations were carried out by varying the concentrations of T/P (12.5-200 nM) for a fixed amount of HIV-1 RT at saturating dATP in order to establish the value of K_D (k_{off}/k_{on}) for initial enzyme binding to the RNA/DNA heteroduplexes. K_D values were determined for control, -6A/T and -6U/A substituted T/Ps. The latter T/P yielded the same k_{2app} value for U/A basepair formation as the control (Table 1), and hence served as an additional probe for examining the influence of K_D on incorporation efficiency. Burst amplitudes of product formation for different concentrations of T/P were initially determined (data not shown). These data were then fitted to Equation 3 in order to obtain values for K_D as shown in Figures 4A-C. Values for K_D were the same within experimental error for the control (-6C/G) and -6U/A T/Ps (~20 nM). However the value for the -6A/T substituted T/P was significantly larger, ~50 nM. Since the burst amplitudes directly reflect the concentration of active polymerase, comparison of these values with the protein concentration determined from spectrophotometric or Bradford assays provides an estimate of the percent of functional enzyme. These calculations typically indicated 50-60% polymerase activity.

RNase H cleavage

The RNase H activity associated with HIV-RT plays a critical role in the removal of the RNA template during 1st strand DNA synthesis (*27*). The effect of T/P sequence context on the RNase H activity was examined by measuring the rate of disappearance of 5'-end labeled RNA template under conditions of catalytic RT in the absence of dNTPs. The time course for hydrolysis of variously substituted T/Ps is shown in Figure 5. Rate constants for RNA cleavage were determined by fitting to a single exponential and are shown in the inset. The substitution of -6A/T was

observed to slow the rate of RNA cleavage by ~3-fold relative to the control sequence, whereas all other substitutions at either the -2 or -6 positions did not affect the relative cleavage rate.

T/P thermodynamic stability

In order to determine whether a relationship might exist between T/P sequence composition, thermodynamic stability, and polymerase incorporation efficiency, enthalpies (ΔH) associated with melting were determined for WMJ1, -6A/T and -6U/A templates. Van't Hoff plots obtained from optical melting data are shown in Figure 6. The WMJ1 control T/P with a C/G basepair at the -6 position was found to be the most stable, characterized by ΔH = -129 kcal/mol. Single substitutions with -6A/T or -6U/A basepairs lowered this value to -99 and -86 kcal/mol respectively. In spite of the 43 kcal/mol difference observed for -6U/A and control T/Ps, the apparent second order rate constants for dATP incorporation were identical (Table 1).

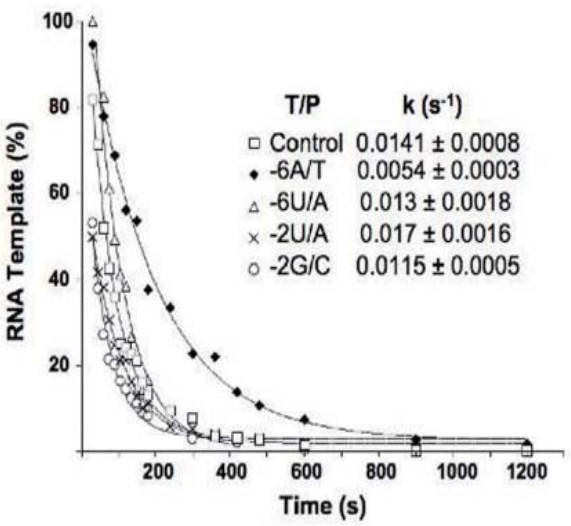

Figure 5. The influence of T/P base substitutions on the HIV-1 RT associated RNase H activity. The rate of disappearance of 5'-end labeled RNA within the heteroduplex T/P was determined by fitting integrated gel-electrophoresis data to a single exponential. Rate constants (average of 3 determinations) are shown in the inset.

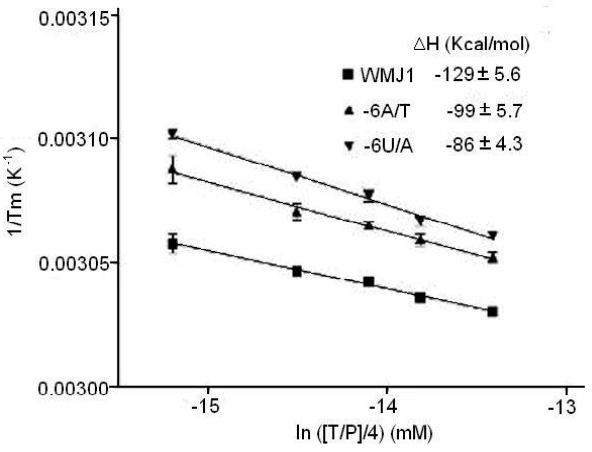

Figure 6. Enthalpies of melting for WMJ1 (-6C/G), -6A/T and -6U/A T/Ps. Values for ΔH were determined from plots of T_m^{-1} *vs.* ln ([T/P]/4) using the van't Hoff equation (Equation 4).

Discussion

In this work we have attempted to provide a mechanistic rationale for how certain sequence contexts may modify the activity of polymerases such as HIV-1 RT and influence fidelity. We have shown that a specific base substitution within an *env*-derived T/P sequence can exert a nearly 10-fold effect on polymerase incorporation efficiency even when situated remotely from the dNTP incorporation site. In contrast, 6 other T/P base substitutions examined exerted either small (≤ 3 fold) or negligible effects on the polymerase and RNase H activities. Base substitutions within the T/P likely regulate nucleotide incorporation efficiency by modulating non-covalent interactions with HIV-1 RT during one or more of the reaction steps. A simplified polymerase reaction mechanism which omits the conformational changes required for activated complex formation (*28,29*) is shown in Scheme 1. Several plausible models for how sequence context might regulate RT activity were considered within the context of this mechanistic scheme, known crystallographic information and the results of our steady and pre-steady state kinetic data.

One mechanism which could account for the observed sequence context effects on polymerase activity involves basepair recognition by RT amino acid side chains via the major or minor groove of the T/P. However, structures deduced from crystallographic data of HIV-1 RT•T/P complexes reveal very few direct basepair/amino acid interactions. Structures obtained at ~3Å resolution for HIV-1 RT complexes with either RNA/DNA or DNA/DNA T/Ps indicate that the majority of amino acid side chains interact with the primer or template ribophosphate backbone (*18-20*). Specific binding interactions between the T/P and HIV-1 RT appear to be highly conserved since nearly identical points of contact have been described for complexes with different T/P sequences (*18-20*). These are shown

$$E+T/P_n \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E\text{-}T/P_n \overset{dNTP}{\underset{k_D}{\rightleftharpoons}} E\text{-}T/P_n\text{-}dNTP \overset{k_{pol}}{\underset{PPi}{\rightleftharpoons}} E\text{-}T/P_{n+1} \overset{k_{cat}}{\rightleftharpoons} E+T/P_{n+1}$$

Scheme 1. A simplified mechanism for primer elongation catalyzed by HIV-1 RT.

in Figure 7 for a sequence corresponding to the -6A/T substituted T/P. These structural considerations are not consistent with a mechanism involving a direct "readout" of the base composition.

Perturbations in van der Waals and hydrogen bonding interactions induced by local changes in helix geometry as a result of specific basepair substitutions may be relayed to the polymerase active site via conformational changes in the polypeptide backbone. In this mechanism, enhancement or inhibition of catalytic efficiency could occur as a result of a slight spatial rearrangement of the polymerase active site. This might be expected to result in variations in either the dNTP binding affinity (K_d) and/or in k_{pol}, the rate of phosphodiester bond formation (*30*). However, pre-steady state kinetics did not reveal any variations in these parameters as a result of -6A/T substitution. Sequence dependent variation in T/P thermodynamic stability could in principle influence the kinetics of dNTP incorporation by modifying interactions with the polymerase. However, no relationship was observed between melting enthalpies and apparent second order rate constants. Identical k_{2app} values for dATP incorporation were observed for T/Ps with widely divergent ΔH values (ΔΔH ~40 kcal/mol), suggesting that T/P thermodynamic stability does not influence the polymerase function of HIV-1 RT.

Alternatively, nucleotide sequence composition may modify polymerization kinetics by controlling the binding and dissociation of RT to the T/P. In order to determine whether base substitutions at the critical -6 position could modulate K_D (k_{off}/k_{on}) for initial polymerase binding (Scheme 1), active site titrations were carried out for the control WMJ1 (-6C/G), -6U/A and -6A/T T/Ps. The K_D values for the wild type and -6U/A T/Ps were nearly identical; however, a more than 2-fold increase was observed upon replacement with A/T at the -6 position. Increases in K_D result in a decrease in polymerase processivity, requiring multiple cycles of RT binding and dissociation in order to effect primer extension (*26*). Since only a fraction of HIV-1 RT molecules possess polymerase activity, increasing the T/P encounter frequency elevates the probability that productive reactions will occur. This results in enhanced incorporation efficiency. Product release by polymerases is typically the rate-limiting (slowest) step in the reaction sequence, and is directly reflected in the steady state k_{cat} values. Additional evidence that the -6A/T substitution influenced RT binding interactions was provided by the fact that the values of k_{cat} for both correct and incorrect basepair formation were increased to the same extent (2 fold) relative to the control WMJ1 sequence (Table 1). No other basepair substitution examined in this study was observed to increase k_{cat} relative to the control T/P. Thus perturbation of RT binding interactions both prior to and subsequent to the dNTP incorporation step by a specific basepair change can influence polymerization efficiency.

In order to further examine the effects of sequence composition on the enzymatic functions of RT, RNA hydrolysis by the RT associated RNase H activity was measured for different T/P substrates. RNase H cleavage of the template strand was monitored in the absence of dNTPs in order to uncouple RT catalyzed RNA hydrolysis from the polymerization reaction. Results of these experiments (Figure 5) revealed little variation in the extent of RNA hydrolysis among the tested substrates except for the -6A/T substituted T/P, which was cleaved at a 3-fold lower rate. Since the RNase H active site of RT is located 18-19 nucleotides from the primer terminus (*8,20,31,32*), it is unlikely that the -6A/T substitution directly inhibits the hydrolysis reaction. Reduction in RNase H activity is likely due to enhanced dissociation of RT from the T/P, a conclusion which is consistent with the increase in K_D determined from the pre-steady state experiments.

The earlier work of Abbotts *et al*. demonstrated enhanced termination probabilities for HIV-1 RT catalyzed primer extensions at sites 2 and 6 nt downstream from template adenines in an M13mp2 DNA template (*7*). These observations are partly in agreement with our results for -6 A/T substitutions on RNA templates, although adenine substitutions at the -2 position did not have as large an impact. The enhanced termination frequencies reported for the M13mp2 DNA template and our data obtained using RNA templates suggest that modulation of RT efficiency by a substitution at the -6 position may be operative for both RNA and DNA templates of unrelated sequence. Crystallographic studies suggest that the -6T/P local environment interacts in a unique manner with HIV-1 RT. Reverse transcriptase binding induces a bend of ~41º extending over the -5 to -9 positions, inducing an A to B form helical transition (*18,20,33*). The axis of curvature is most acute between the basepair step corresponding to positions -6 and -7, which forms the junction region between A and B forms. This may allow especially close approach of amino acid side chains of the αH and αK helices to the -6 basepair environment. Comparison of the amino acid side chains in contact with the -2 and -6 basepair regions reveals a significantly higher local concentration of basic and amide containing residues at the latter position. These differences are highlighted in the 3 dimensional representation shown in Figure 8, based on the crystal structure of HIV-1 RT bound to an RNA/DNA T/P (*20*). Many of these amino acids are involved in potential hydrogen bonds with the ribophosphate backbone of both the primer and template strands. Residues N255, Q258 and N265 reside in helix αH, while K353 and K374 are localized within β18 and helix αK, respectively. In contrast, the majority of polymerase contacts to the -2T/P basepair consist predominantly of acidic and neutral side chains (with the exception of

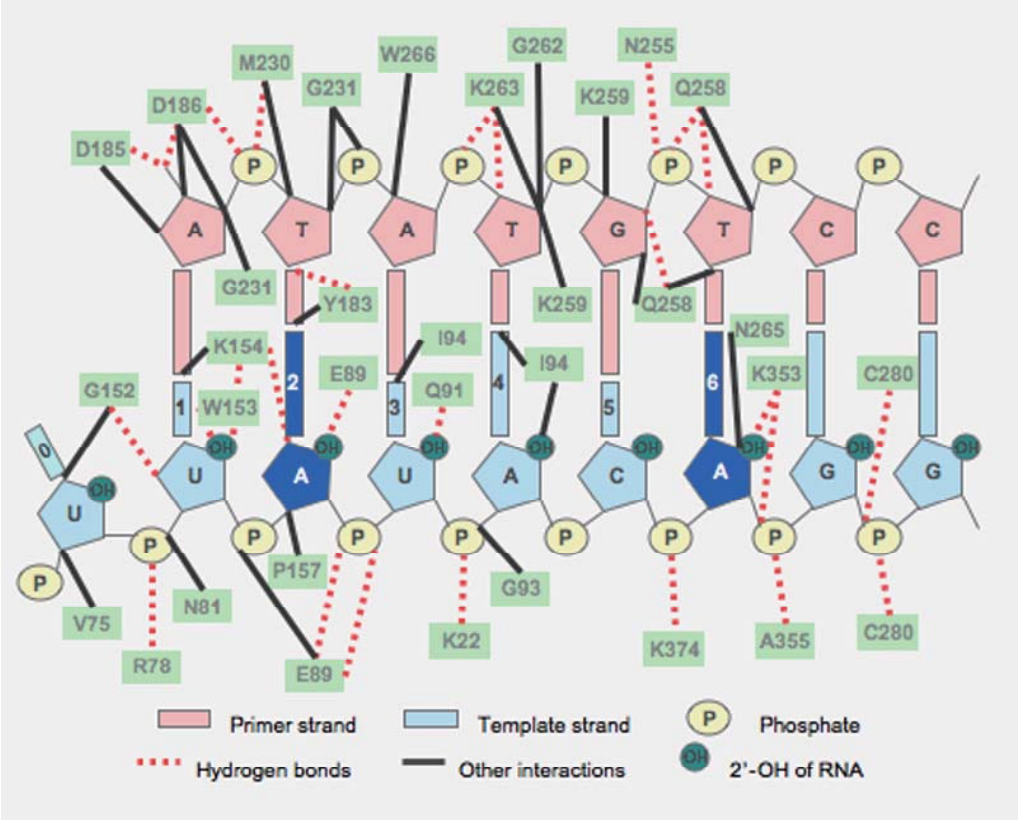

Figure 7. Schematic of non-covalent interactions observed between heteroduplex T/Ps and amino acid side chains of HIV-1 RT based on the crystal structure of Sarafianos *et al*. (reference 20).

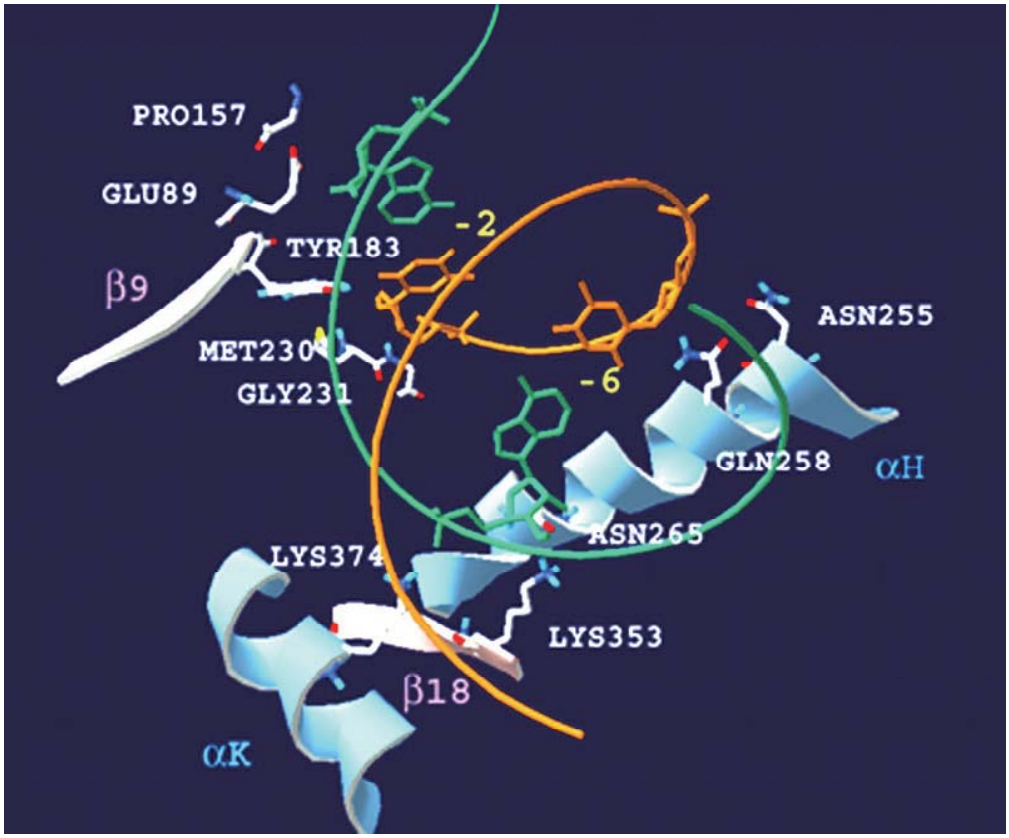

Figure 8. 3-D representation of the complex between HIV-1 RT and T/P substrate emphasizing the -2 and -6 basepair environments. The template RNA and DNA primer strands are shown in green and orange respectively. An increased density of basic and amide containing side chains can be observed at the -6 basepair. Image was constructed from protein data bank file IHYS using the Deep View Swiss – PDB viewer.

K154). We suggest that -6A/T substitution results in a local conformational change which leads to partial destabilization of some of these highly polar non-covalent interactions with the ribophosphate backbone, which in turn influences the binding and dissociation of HIV-1 RT. A modest decrease in hydrogen bonding distance of only ~0.5Å would result in a significant decrease in stabilization energy (*34*). The observed changes in k_{2app} values can be used to calculate the effects of T/P substitutions on HIV-1 RT mismatch frequency. The frequency of mismatch formation is defined by the reciprocal insertion frequency, $1/f_{ins}$, defined by Goodman and coworkers as the ratio $k_{2app}^{correct}/k_{2app}^{incorrect}$ (*35*). Using the values in Table 1, formation of a U/G mismatch at position 973 of the *env* gene of HIV strain WMJ1 would be observed once in 10,000 replication events. Substitution of A/T at the -6 position would lower this frequency to 1 in 20,000, resulting in a 2-fold increase in polymerase fidelity.

The effects of T/P basepair substitutions on the apparent second order rate constants were more variable for the mispairing reaction than for U/A basepair formation (Table 1). For example, -6U/A and -2U/A substitutions lowered k_{2app} for U/G mispairs by ~4 and 3-fold, respectively. In contrast, the -6G/C and -2G/C substitutions increased k_{2app} for this mispair by ~3-fold. These were the only base substitutions examined which would decrease RT fidelity. It is not clear why the apparent second order rate constants for the U/G mispairing reaction were more sensitive to T/P basepair substitutions. Perhaps in the absence of the greater kinetic and thermodynamic stabilization afforded by Watson-Crick base pairing, the energetically disfavored process of mismatch formation becomes more influenced by small perturbations in RT-T/P interactions resulting from sequence variation. However, even for the U/G mismatch, the largest effect on k_{2app} (7 fold change) was observed for the -6A/T substitution. It is not possible to assess the generality of the specific sequence context effect observed in this study, since only a small sample of sequence space can be feasibly probed using kinetic methods. However, we suggest that K_D modulation by specific base substitutions may constitute one mechanism whereby basepair composition can influence the fidelity of RT and other polymerases for certain sequence contexts.

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Role of protocol ultrasonography for detecting biliary stricture in adult living donor liver transplantation recipients

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SUMMARY **The role of protocol ultrasonography (US) in detecting biliary stricture (BS) after adult living donor liver transplantation (ALDLT) remains to be clarified. We reviewed 268 ALDLT cases with BS to assess the role of protocol US. The sensitivity and specificity of serum values of gamma-glutamyl transpeptidase (G-GTP) and alkaline phosphatase (ALP) as indicators of BS were evaluated and compared with the US findings. Diagnosis of BS was made by drip-infusion cholangiography or direct endoscopic retrograde cholangiography. Fifty patients (19%) developed BS; anastomotic stricture in 46 and non-anastomotic in 4. The incidence of BS was not affected by the method of bile duct reconstruction or the type of graft. Protocol US detected dilated bile ducts (≥ 3 mm) in 45 cases (90%) with BS. The mean diameter of bile ducts in the BS group was 5 ± 2 mm, which was greater than that of patients without BS (2 ± 1 mm, $P < 0.001$). When the bile duct diameter was over 3 mm, the sensitivity and specificity of protocol US for the diagnosis of BS was 96% and 91%, respectively, whereas those of G-GTP were 60% and 74% when the values were more than 5-fold greater than the upper normal limit, and those of ALP were 56% and 62% when the values were 3-fold greater than the upper normal limit. The bile duct diameter and the G-GTP and ALP values did not correlate. US might be a useful and efficient imaging method to follow-up patients after ALDLT.**

Key Words: Live donor, bile duct, stenosis

Introduction

Adult living donor liver transplantation (ALDLT) is now one of the most common effective treatments for end stage liver failure, because of a severe cadaver graft shortage. In ALDLT, thinner bile ducts and multiple holes make the bile duct anastomosis procedure more challenging than that in whole liver transplantation (*1*). Biliary stricture (BS) remains one of the most common postsurgical complications.

Early diagnosis of BS is critical for preventing septic cholangitis and graft failure in liver transplantation patients. A definite diagnosis based on clinical and laboratory features alone is often difficult, as these abnormal features also occur with other complications, such as acute or chronic rejection, exacerbation of hepatitis, non-biliary septicemia origin, and recurrence of primary biliary cirrhosis or sclerosing cholangitis.

Ultrasonography (US) is a non-invasive imaging modality that can be performed quickly at the bedside. It is now the most frequently used imaging procedure to monitor many post-operative complications in ALDLT. Its role in the diagnosis of BS, however, remains controversial.

In whole liver transplantation, Hussaini and colleagues (*2*) reported US as a valuable tool to diagnose BS with a sensitivity and specificity of 77% and 67%, respectively. Other studies (*3-6*), however, reported that the sensitivity was only 34% to 54%, and concluded that the role of US to detect BS is

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was limited. The role of protocol US in ALDLT has not yet been reported. A comparison of protocol US findings and the laboratory values of gamma-glutamyl transpeptidase (G-GTP) or alkaline phosphatase (ALP) has not been discussed in detail. In this study, we analyzed the ALDLT cases with BS in our database to assess the role of protocol US.

Patients and Methods

Patients

Clinical information was retrospectively obtained from hospital charts. From January 1996 to December 2005, 280 patients (156 men, 124 women; average age 47 years) underwent ALDLT at Tokyo University Hospital, Japan. The indications included hepatitis B and C-related cirrhosis (n = 130), cholestatic liver diseases (n = 77), fulminant hepatic failure (n = 24), biliary atresia (n = 16), metabolic disease (n = 9), alcoholic liver cirrhosis (n = 3), and others (n = 21). The most commonly used graft was right liver graft (n = 144) and left liver with or without the caudate lobe (n = 117), followed by the right lateral sector (n = 19). During this period of time, 12 patients failed to come in for long - term follow-up at our hospital and were therefore excluded from the study. In 268 cases of ALDLT, duct to duct anastomosis was performed in 193 and right liver graft was used in 157.

Protocol ultrasonography examination

Protocol Doppler US was performed once or twice per day for 2 weeks postoperatively and several times per week after the initial 2 weeks using a color Doppler US instrument (SSD 2000 or SSD 6500, Aloka Co. Ltd., Tokyo, Japan) in B, M, and Doppler modes. Protocol US was performed every 3 months in the outpatient clinic and when aspartate aminotransferase (AST), alanine aminotransferase (ALT), G-GTP, and ALP levels were above the upper normal limits, or when AST, ALT, G-GTP, or ALP values were abnormal and the intrahepatic biliary duct was dilated more than 3 mm. We examined all the bile ducts in the graft and chose the point where the intrahepatic bile duct was the most dilated and measured the diameter.

G-GTP, ALP, AST, and ALT were measured twice per day during the first 2 weeks postoperatively and once a day thereafter until discharge. In the outpatient clinic, these data were collected on a weekly basis during the first 3 months and every month in the succeeding period.

Diagnosis and treatment of biliary stricture

The final step of diagnosis consisted of three-dimensional reconstruction done by helical computed tomography on drip-infusion cholangiography or direct endoscopic retrograde cholangiography. Type of stricture, anastomotic or non-anastomotic, was determined using these examinations.

Balloon dilatation under endoscopic retrograde cholangiography or percutaneous transhepatic biliary drainage was first applied when feasible. If the stricture was not resolved by conservative methods, surgical revision was performed.

Statistical analysis

Data were expressed as percent and mean ± standard deviation or median and range. We compared categorical variables using the chi-square test and, when appropriate, the Mann-Whitney U test. A Spearman correlation test was used to analyze the relation between the bile duct diameter and the cholestatic liver enzyme values. A P value of less than 0.05 was considered significant. Statistical analysis was performed using SPSS software version 10.0.6 (SPSS Inc., Chicago, IL, USA).

Results

Incidence of biliary stricture

Fifty patients (19%) developed BS (20 women, 30 men); anastomotic stricture in 46 (17%) and non-anastomotic stricture in 4 (2%). The median time from ALDLT to the diagnosis of BS was 7.5 (range, 0.1-87) months and median follow-up of each patient from the first treatment to the last follow-up visit or death was 23.8 (range, 1-92) months. The different bile duct reconstruction types (P = 0.3) and graft types (right *vs.* left, P = 0.7) did not relate to the occurrence of BS.

G-GTP and ALP

In the patients with BS (BS group), 16 (34%) had clinical symptoms of cholestasis such as high fever, jaundice, and itching. On the other hand, the other 34 (68%) remained asymptomatic. G-GTP and ALP values exceeded the upper normal limit in 47 (94%) and 48 (96%) patients, respectively. The differences were statistically significant compared with the maximum values in the control group (Table 1). There was no correlation between bile duct diameter and G-GTP or ALP (P = 0.8 or 0.7). Sensitivity and specificity for multiple discriminatory thresholds of G-GTP were 60%

Table 1. G-GTP and ALP levels in BS group (before treatment) and control

Liver Enzymes	BS Group	Control	P
G-GTP (IU/l)	502 ± 368	302 ± 324	< 0.001
ALP (IU/l)	680 ± 336	384 ± 348	< 0.001

Abbreviations: G-GTP, gamma-glutamyl transpeptidase; ALP, alkaline phosphatase

Table 2. Sensitivity and specificity to diagnose biliary stricture for multiple discriminatory thresholds of G-GTP

Threshold	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)
ULNL	47	15	203	3	94	7
5-folded of ULNL	30	161	57	20	60	74
10-folded of ULNL	15	200	18	45	25	92

Abbreviations: ULNL, Upper limit of normal level; TP, true positive; TN, true negative; FP, false positive; FN, false negative; G-GTP, gamma-glutamyl transpeptidase

Table 3. Sensitivity and specificity to diagnose biliary stricture for multiple discriminatory thresholds of ALP

Threshold	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)
ULNL	48	7	211	2	96	3
5-folded of ULNL	28	136	82	22	56	62
10-folded of ULNL	10	192	26	40	20	88

Abbreviations: ULNL, Upper limit of normal level; TP, true positive; TN, true negative; FP, false positive; FN, false negative; ALP, alkaline phosphatase

Table 4. Sensitivity and specificity for multiple discriminatory thresholds of ultrasound to diagnose biliary stricture

Threshold	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)
≥ 3 mm	45	198	20	5	90	91
≥ 5 mm	24	216	2	26	48	99

Abbreviations: TP, true positive; TN, true negative; FP, false positive; FN, false negative

and 74% when the values were more than 5-fold greater than the upper normal limit (Table 2); those for ALP were 56% and 62% when the values were more than 3-fold greater than the upper normal limit (Table 3).

Ultrasonography in the detection of biliary stenosis

In our study, bile ducts were found to be dilated by US in 45 cases in the BS group (90%). The mean bile duct diameter in BS group was significantly greater than that of the control group (5 ± 2 mm and 2 ± 1 mm, $P < 0.001$). The sensitivity and specificity for discriminatory thresholds of US to diagnose BS are shown in Table 4. The cutoff value of the diameter of the bile duct was 3 mm with a sensitivity and specificity of 96% and 91%, respectively.

Follow-up after treatment

During the follow-up, 7 (14%) patients in the BS group died. One patient died because of pulmonary embolism, one had sepsis of biliary origin, three patients died due to recurrent malignancy, 1 due to multiple organ failure, and 1 secondary to pneumonia and peritonitis.

In the BS group, all patients with long-term survival eventually had satisfactory results and the dilated bile ducts returned to normal in 32 cases (64%) based on US at the last follow-up visit. The bile duct dilation remained unchanged in 22 cases (44%), based on US: 12 cases remained unresolved and the other 10 cases had recurrent bile duct dilatation and were treated more than 3 times. All these cases were confirmed to have recurrent or unresolved BS by helical computed tomography on drip-infusion cholangiography or endoscopic retrograde cholangiography and were treated with the same or other modalities.

Discussion

Duct to duct anastomosis for biliary reconstruction in ALDLT has made endoscopic access to the biliary tree for diagnostic and therapeutic management more feasible. The method also provides a more physiologic situation for preventing ascending cholangitis and delayed bowel movement (*7*).

The relation between the type of reconstruction and the incidence of BS is a matter of controversy. Gondolesi and colleagues (*8*) reported that patients with duct to duct reconstruction had a higher incidence of BS (32%) than patients with hepaticojejunostomy. Soejima and colleagues (*9*) reported no association between these two groups. According to our data, the overall prevalence of BS was 19% (20% in duct to duct and 15% in hepaticojejunostomy with no inter-group difference). In contrast to the findings of Soejima (*9*), we did not detect a tendency towards an increased incidence of BS using left liver graft in ALDLT (20%) in our series, although the incidence of BS was higher than in those with right liver graft (18%).

Biliary stricture occurred at the anastomotic site in 92% of the patients. Previous studies (*10,11*) have demonstrated that technical issues are the most important etiologic factors for anastomotic stricture. Ischemic and immunologic injury to the biliary epithelium is suggested to have a role in causing non-anastomotic stricture (*12-14*). The need for more than one bile duct anastomosis in ALDLT can aggravate these technical difficulties and cause anastomotic stricture. Shorter ischemia time and good quality liver from living donors might contribute to the lower rate of

non-anastomotic stricture in our series compared with the rate in deceased donor liver transplantation (*15*), which is 18% of patients with BS.

In the present study, 68% of the patients with BS were asymptomatic. The finding supports our approach, in which protocol US is used as the first line imaging examination during follow-up after ALDLT. Intrahepatic bile ducts are not visualized by US in the absence of biliary obstruction. In 90% of the cases with BS, US successfully detected dilated intrahepatic bile ducts. The presence of the classical intrahepatic "parallel channel sign" and the combined use of color Doppler US to identify the vessels from bile ducts might contribute to this favorable result (*16*).

One concern in the condition of chronic hepatic disease is that enlargement of the intrahepatic branches of the hepatic artery might also result in the intrahepatic parallel channel sign. To avoid such a fundamental misinterpretation, Doppler ultrasonography is an indispensable tool to distinguish bile ducts from blood vessels (*16*). In combined application of M-mode Doppler examination with the conventional B-mode ultrasonography, the cutoff value of 3 mm had high specificity and sensitivity for predicting the presence of BS in our study.

Our findings suggest that caution is required in interpreting the elevated G-GTP and ALP levels. In our study, G-GTP and ALP exceeded the upper normal limit in 47 cases (94%) and 48 cases (96%), respectively, when BS was diagnosed. The sensitivity and specificity of G-GTP and ALP for the detection of BS, however, were poor; thus, a confirmatory study using US is mandatory for the diagnosis of BS.

In conclusion, protocol US is useful for detecting BS at the earliest stage. A bile duct diameter greater than 3 mm detected by US suggests the presence of BS with high rates of sensitivity and specificity.

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