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Guide for Authors

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City of Hope National Medical Center and Beckman Research Institute

City of Hope is an innovative biomedical research, treatment and educational institution, which is dedicated to the prevention and cure of cancer and other life-threatening diseases. The institution was originally established in May 1913 as a volunteer-driven organization, the Los Angeles Sanatorium. It was destined to become a national movement, with its mission of helping the afflicted "to find a new hope, a new healthy body and a new useful life." Beckman Research Institute is one of the nation's premier centers for pioneering biomedical research. Founded in 1951, it was endowed by the Arnold and Mabel Beckman Foundation in 1983 and renamed the Beckman Research Institute.

Editorial

Tribute to Promethean thinker — *in memory of Susumu Ohno*

Sergei N. Rodin, *Editor**
Arthur D. Riggs, *Co-Editor-in-Chief*

Susumu Ohno occupies a special place even among the most inventive and original thinkers of the 20th century biology. On February 1st 2008, he would be 80 years old; it is precisely on this day that Dr. Ohno's colleagues and friends gathered to pay tribute to his genius and numerous achievements. Dr. Ohno's career is virtually inseparable from the Beckman Research Institute of the City of Hope (Duarte, California); thus, it seems fitting that the tribute was realized as the special Beckman symposium entitled *Emergence of the genetic code, genomes and epigenomes* (*in memory of Susumu Ohno*).

In this time of ever-more-overspecialized researchers, Susumu Ohno was a remarkable exception. The range of his professional curiosity knew no limits. It has been said about Mikhail Lomonosov, the de facto father of modern Russian science, "… he [Lomonosov] was a founder of our first University …to be more precise, he *WAS* our first University" (Alexander Pushkin). Dr. Ohno was just like that, a true renaissance (or age of enlightenment) man, a man of encyclopedic interests and knowledge.

This said, he was also a man of focus, rigour and parsimony. Susumu Ohno was partial to the idea, put forward by the contemporary philosophers and historians of natural sciences (see, for example, Prigogine, I., Stengers, I., 1984. Order Out of Chaos. Bantam Books, New York, 349 pp) that the most powerful discoveries are the "set-back" ones, *i.e.* the discoveries that effectively ban, or inhibit, the concepts that appeared to be well-established, unshakeable, and "self-evident" before. For example, in physics, any conceptual revolution (or paradigm shift) has always been directly linked to a new ban / inhibition of some sort (*e.g.*, the second law of thermodynamics, inability to transmit information faster than the speed of light,

Dr. Susumu Ohno

uncertainty principle, *etc*.). In context of scientific research such bans are in fact very productive, for they "cut off" the nonsensical potential research venues, thus allowing us to concentrate our time, energy and resources along the "allowed" research directions. Susumu Ohno felt very strongly that theoretical biology, being much "younger" than theoretical physics, has not yet developed such a well-defined, powerful and logical "immune system", able to rapidly and painlessly separate research wheat from research chaff.

Susumu Ohno, D.V.M., Ph.D., came to United States from Japan as a postdoctoral fellow, and soon emerged as a scientist with international reputation and influence. The scope of his research interests was vast. His first project (while still in graduate school) was related to immunology, and he never lost interest in this subject, culminating in his work on the evolution of the immune system. At the City of Hope, his early work on using cytology techniques led to increased interest in chromosomes and, subsequently, to the first major milestone of his illustrious career --- discovery of X chromosome inactivation (1959). This discovery was a harbinger of the exciting (and currently flourishing) research area of epigenetics and epigenomics.

Reflection on chromosome and genome size evolution led, in 1971, to the publication of Susumu Ohno's seminal monograph (and arguably his greatest achievement, at least from the possibly biased viewpoint of this evolutionary geneticist) entitled *Evolution by Gene Duplication*. Thirty-six (and counting) years later, in our "post-modern" epoch of successfully realized human (and other species) genome projects, it is virtually impossible to find a research paper on the evolution of multigene systems and whole genomes that does *not* reference the above classic.

Another fundamental aspect of evolution that fascinated Dr. Ohno (fitting his interest in aforementioned "set-back" discoveries) was the "hindsight evolution" principle --- the natural selection is strictly a tactical, "here-and-now", force. As such, it never works in advance, never knows of any long-term strategy and has no foresight to meet the future demands. In this regard, the numerous origin(s)-associated paradoxes of the "chicken-or-egg" variety particularly intrigued him. The absurdity of the "evolutionary foresight" was always obvious to Susumu Ohno, whatever biological system of increasing complexity and uncertain origins would capture his mind and imagination at the time – from the tRNA cloverleaf as the genetic code adaptor to the enzyme that detoxifies atropine in the wild rabbit of Spain (*Oryctolagus cuniculus*) to the incredible complexity of the ribosome to the self-nonself discrimination in the immune system.

In Greek mythology vernacular, which Dr. Ohno liked to use, evolution works as Epimethius (who has hindsight only) rather than his brother Prometheus (endowed with foresight). However, if we consider evolution of knowledge, it appears that Susumu Ohno *himself* belonged to that rare group of thinkers whose Promethean ideas not only fail to fade out but, on the contrary, tend to shine even brighter with progression of time, as if he did indeed possess the foreknowledge of the future challenges of science. The X chromosome inactivation, the leading role of gene duplications in evolution of novelties and the intriguing repetitive pattern in gene sequences likely preserved since primordial RNA life are but three examples of Dr. Ohno's incredible foresight that immediately occur to any theoretical geneticist. There are more.

During the last 10-15 years of his career, having made significant contributions to such diverse fields as genetic predisposition to cancer, self-nonself discrimination by the immune system, *etc.*, Susumu Ohno's primary interests shifted towards understanding the origins of the genetic code and translation machinery. As a part of that research, he found many singular internal periodicity patterns in various genetic sequences, and somehow even managed to translate them into the musical notation. In his opinion, the beauty of these basic repeating motifs might have reflected the primary modules of emerging bilingual (built of nucleic acids and proteins) life. Thanks to the genius and whimsy of Susumu Ohno, today we can enjoy this ancient beauty not only by observing it in sequences but even by actually *listening* to it!

During the morning session of the Beckman symposium, three of the presenters, namely Ernest Beutler (Scripps Institute, La Jolla, USA), Bruce Cattanach (Mammalian Genetics Unit, MRC, Harwell, UK) and Melvin Cohn (Salk Institute, La Jolla, USA) reflected, among other things, on the aforementioned "foresight" of Dr. Ohno's many and diverse contributions.

Dr. Beutler's talk centered on the phenomenon of G6PD inactivation, in historical context. G6PD inactivation is a phenomenon of major public health importance, and thus its molecular and, eventually, epidemiological understanding was absolutely crucial. And it was Dr. Ohno's X-inactivation work that both contributed to, and benefited from, that understanding. Now we, of course, know the trait is X-linked, and only one X-chromosome remains active. We owe that knowledge to the early-60s Beutler-Ohno collaboration.

Dr. Cattanach's talk unfolded a fascinating story of how we arrived from the early hints and puzzles to the modern, comprehensive (but still evolving) understanding of the mechanisms and consequences of imprinting. One of the milestones along this journey was the seminal 1962 work by Ohno and Cattanach

in which they demonstrated a cytological proof of X-inactivation hypothesis in mouse.

In his presentation, Dr. Cohn concentrated on the conceptual and mechanistic logic of the immune system, and how it must have been necessarily shaped by the evolutionary pressures and considerations. More quantitatively-minded members of the audience especially enjoyed his description of the immune response simulator, a sophisticated computational modeling software package developed and maintained by Dr. Cohn's group.

In the afternoon session of the symposium, the discussion was continued by Paul Schimmel (Scripps Institute, La Jolla, USA), Sergei Rodin (Beckman Research Institute, City of Hope, Duarte, USA), Takashi Gojobori (Center for Information Biology National Institute of Genetics, Mishima, Japan) and Kenneth Wolfe (Trinity College, Dublin, Ireland).

In his talk, Dr. Schimmel explored an intriguing connection between, on one hand, origins of the genetic code, it's possible precursor in the acceptor stem of tRNA and translation machinery and, on the other hand, certain human diseases --- the somewhat unexpected link being provided by the aminoacyl tRNA synthetases. The latter, in addition to their protein synthesis responsibilities, are also procytocines, thus being of potential importance in angiogenesis.

Dr. Rodin continued the aminoacyl tRNA synthetases theme, touching upon the genetic code, two classes of synthetases and primordial complementarity-based link between the acceptor and anticodon domains of tRNA molecule. Vestiges of this link can still be detected in extant synthetases and tRNAs. Dr. Rodin combined the above into a theory of genetic code origin, with roots in the mid-90s Rodin-Ohno's hypotheses of the dual complementarity and the concerted origin of two synthetase classes from sense-antisense strands of same ancient gene.

Dr. Gojobori reflected on both his personal memories of Dr. Ohno, and on modern interpretation of some of the Dr. Ohno's evolutionary ideas in light of the massive amounts of genomic sequences available for analysis today. Specifically, he concentrated on the genomic evolution of neural genes, presenting massive composite phylogenetic trees thereof. This was of a special interest to the phylogenetic analysis / bioinformatics researchers in the audience.

Last but most certainly not least, Dr. Wolfe talked about eukaryotic genomic evolution and polyploidy. Until recently, the latter, including full genome duplications, was considered to be an important factor in plant evolution, but recent evidence suggests (in a full accord with Ohno's ideas) that it might be a relatively common phenomenon throughout eukaryotic kingdom in its entirety. Dr. Wolfe ended the afternoon session on a somewhat lighter tone, noting that some first and foreign-language editions of Evolution by Gene Duplication nowadays fetch more than 500 Euro on eBay.

In summary, a beautiful day and gathering in memory of Susumu Ohno, enjoyed by all. We would like to extend our special acknowledgements and gratitude to the Beckman Research Institute of the City of Hope, for sponsoring and organizing the event; to the speakers, who graciously found time in their busy schedules to join us in this celebration; and to Midori Ohno, for providing many pictures and materials.

Appendix:

Emergence of the genetic code, genomes and epigenomes

--- in memory of Susumu Ohno ---

(*Beckman Research Institute of the City of Hope, Duarte, CA, USA; February 1, 2008*)

Morning session

Arthur D. Riggs: 10 min

Ernest Beutler (*Scripps Institute, La Jolla, USA*)
G6PD Deficiency

Bruce Cattanach (*Mammalian Genetics Unit, MRC, Harwell, UK*)
Events leading to the discovery of imprinting

Melvin Cohn (*Salk Institute, La Jolla, USA*)
The logic of immune behavior

Paul Schimmel (*Scripps Institute, La Jolla, USA*)
Genetic Code Development and Connection to Disease

Sergei Rodin (*BRI COH, Duarte, USA*)
One ancestor for two codes

Takashi Gojobori (*Center for Information Biology National Institute of Genetics, Mishima, Japan*)
Genomic evolution of neural genes in light of Ohno's view of biological order and disorder

Kenneth Wolfe (*Trinity College, Dublin, Ireland*)
The impact of polyploidy on eukaryotic genome evolution

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Policy Forum

Long live the health care system in Japan

Yasuo Idezuki

Japanese Association of Medical Sciences, Bunkyo-ku, Tokyo, Japan; Japan Surgical Association, Chiyoda-ku, Tokyo, Japan.

Summary **In Japan, a cost-containment policy has been implemented with health care system reform. Increasing medical expenses are inevitable due to aging of the population, health transitions, and expanded and advanced medical services. Policies such as the introduction of regressive reimbursement for inpatient services as part of social insurance, the reduction in the number of hospital beds, fewer medical examinations, and curtailed usage of medical tests have jeopardized the accessibility and quality of medical services, especially for the elderly. Compared to other developed countries, both the proportion of the government's payment of health expenses and the proportion of health expenses in proportion to GDP are lower in Japan. To confront the challenges of an aging society in terms of finances and the health care system, policymakers from the Ministry of Health should take a comprehensive approach instead of a mere cost-containment policy.**

Keywords: Health care system, Japan, Cost-containment policy

In Japan, medical service fees were reduced 2.7% in 2002, when health care system reform was implemented. Although it was excused as "a burden equally borne by citizens, doctors, and the government" by Prime Minister Koizumi, the reform actually focused on the cost-containment and went against citizens and doctors: the proportion of total medical expenses borne by the national government and businesses decreased while that borne by individuals and local governments increased (Figure 1). To examine the rationality of such a cost-containment policy, two aspects should be determined: factors leading to increasing medical expenses and Japan's health care system in comparison to the rest of the globe.

Generally, medical expenses have been rising in developed countries due to increasing population, aging of the population, use of advanced medical technology, and health transitions. As is clear, progress in medical science and technology leads to prolonged life expediency and health transitions. There has been a dramatic shift in the distribution of mortality and the disease burden from infectious diseases to chronic lifestyle-related and debilitating diseases such as cancer, diabetes, and cerebral and cardiovascular diseases. Expensive advanced medical technologies such as MRI, PET, ultrasonography, computerized axial tomography, and endoscopy have been increasingly used in diagnosis and treatment. Medicine has become available for diseases that were not previously treatable and conditions that had not been viewed as "diseases." Like other developed countries, Japan is also already an aging society. According to a national census in 2005, the proportion of elderly over age 65 in the total Japanese population increased to as high as 19.5%. The elderly are more likely to use a number of medical services. The synergy of those factors definitely contributed to rising medical expenses.

Citing the necessity of reform, policymakers from the Ministry of Health stated that the large number of hospital beds and long hospitalizations, frequent

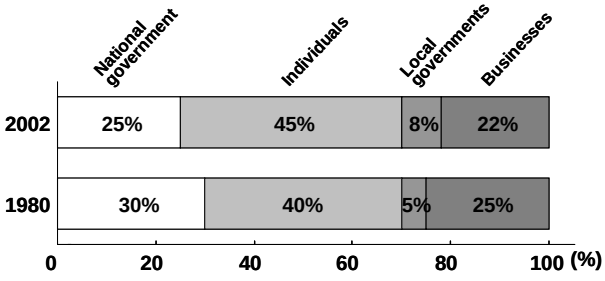

Figure 1. Proportion of medical expenses paid by the national government, individuals, local governments, and businesses in Japan (Source: Ministry of Health, Japan).

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medical examinations, and expanded usage of medical tests led to poor efficiency of the health care system in Japan. However, these issues require a comprehensive approach rather than a simply cost-containment policy.

The large number of hospital beds and long hospitalizations are attributed to "socialized hospitalization," which refers to the fact most patients prefer the hospital to their homes due to the poor rehabilitation environment at home. Policymakers should take social circumstances including a profound tendency towards nuclear rather than extended families, small living spaces, and household income into consideration. The current policies, such as the introduction of regressive reimbursement for inpatient services as part of social insurance and the reduction in the number of hospital beds, have increased the burden of patients as well as their families.

There are both positive and negative aspects to frequent medical examinations. Under the national health care system, patients can receive health care services they need from hospitals and doctors everywhere at their convenience. Although this may lead to relatively substantial use of health care services to some extent, such a system no doubt ensures universal accessibility and the achievement of health outcomes. To reduce the frequency of medical examinations as part of the reform, policymakers from the Ministry of Health have proceeded to consider the exclusion of a small portion of medical fees from reimbursements provided by social insurance. Such a cost-containment measure may potentially result in patients with a mild disease developing a more serious form of the disease and result in even higher medical expenses. What the policymakers should consider is implementation of a long-term health education program on common diseases and lifestyle-related diseases through schools, communities, and the media in order to improve citizens' behavior with regard to effective use of health care services.

During the reform, policymakers strictly restrained the categories and frequency of use of medical tests. This could limit the use of medical tests but it also hinders diagnosis and the quality of health care services since doctors' judgment on the use of medical tests is based on each patient's case rather than a regimented standard. A systematic study on this issue should be conducted in order to provide evidence for policymakers.

Additionally, relatively high drug expenses remain an important contributor to increasing medical expenses, which seems to be intentionally overlooked by policymakers. Compared to other developed countries, drug expenses in Japan have accounted for as much as 31% of total medical expenses (Figure 2), at the root of which is the high price of drugs as part of reimbursements provided by the national insurance system. For example, a capsule of Rythmodan, a drug used to treat arrhythmia, costs 14.3 yen in the UK and 66.8 yen in the US but is as expensive as 90.5 yen in Japan; similarly, 100 mL of Omnipaque, a contrast medium for arteriography, costs 5,244 yen in France and 12,854 yen in the US and yet commands a high price of 14,709 yen in Japan. With the high price of drugs fixed by the Ministry of Health, the ordinary profit rate of the pharmaceutical industry was as much as 22.1% even despite Japan's being in a recession. A rational approach to cost-containment would be to adjust drug prices and facilitate drug distribution rather than to reduce medical fees.

Influenced by exaggerated pieces in the media, such as recent reports that total medical expenses in Japan have already exceeded 30 trillion yen, most Japanese tend to misunderstand the health care system as well as the country's medical expenditures and yet rising medical expenses remain a heavy financial burden for the country. Policymakers asserted that medical expenses, and especially those of the elderly, had increased considerably. According to the Ministry of

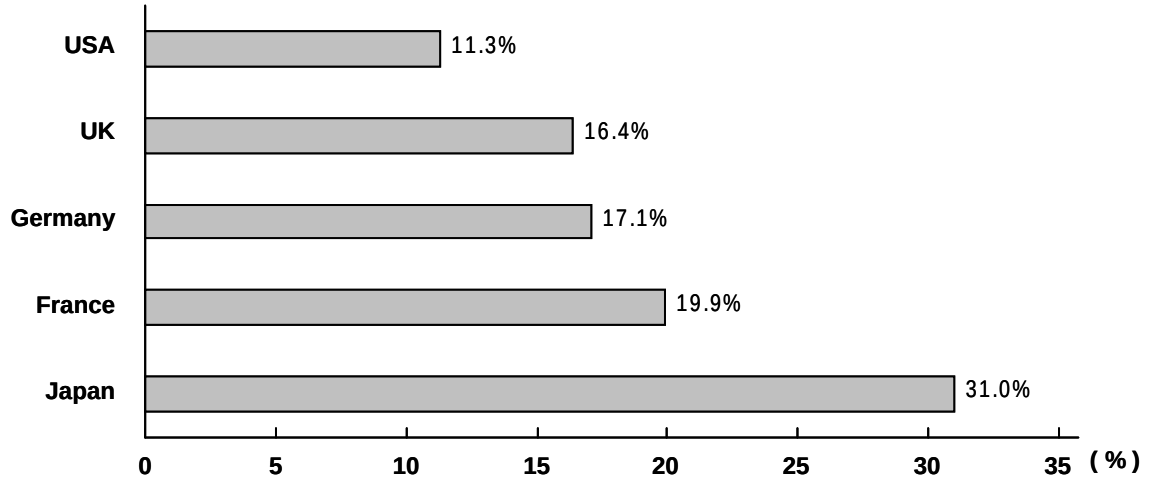

Figure 2. Proportion of drug expenses out of total medical expenses in developed countries (Source: Central Social Insurance Medical Council, Japan).

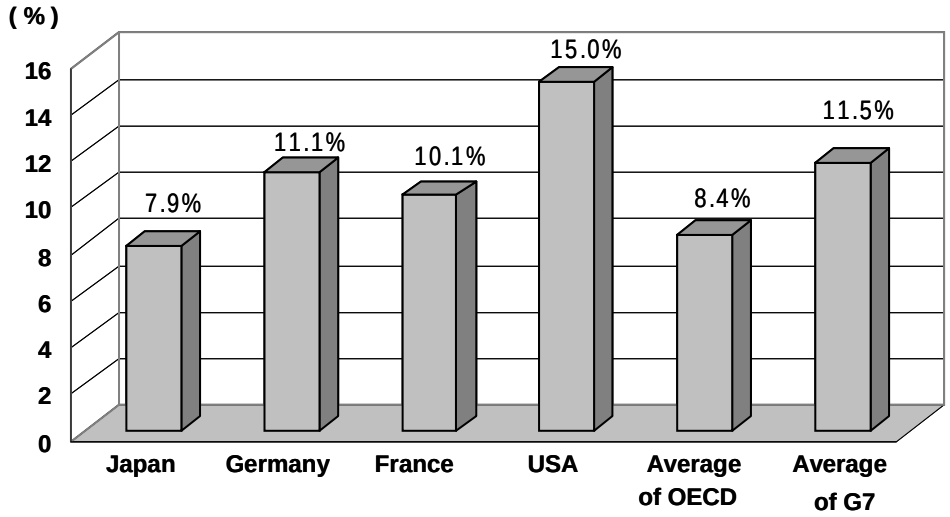

Figure 3. Medical expenses in proportion to GDP in developed countries (Source: OECD Health data from 2005).

Health, medical expenses totaled about 31.5 trillion yen, accounting for 7.9% of GDP. In fact, however, this proportion is the lowest of the developed countries (Figure 3). According to survey data from 2002, individuals paid as much as 45% of medical expenses while the national government only paid 25%. The national financial budget for health care is only approximately one-tenth of that of the US. Given the consequences of an aging society and the status of other developing countries, investment in health care needs not to be limited but to be augmented in Japan.

The cost-containment policy threatens the health care system in Japan, which was once world-renowned for its high quality and best overall health system. Increased payments by individuals have worsened accessibility to health care services particularly for the lower income and the elderly. Moreover, the reduction in medical service fees [paid to doctors by national insurance] (2.7% in 2002, 1.0% in 2004, and 3.16% in 2006) has hampered the management and the financial status of hospitals and clinics. Approximately 90% of government hospitals, 80% of national and public hospitals, and 25% of private hospitals have suffered deficits in recent years. With the sale of independent hospitals, the streamlining of national and public hospitals, and the bankruptcy of some small and medium-sized private hospitals, the health care system has approached the verge of collapse, especially in terms of obstetricians, pediatricians, and emergency services at the community level. Both individuals and doctors were sacrificed to achieve the current cost-containment policy. Ironically, the cost-containment policy of the Ministry of Health has been a godsend to pharmaceutical firms thanks to relatively high drug profits, while hospitals and clinics have suffered. Drug profits should be adjusted in relation to medical service fees.

In conclusion, a priority on the agenda of policymakers should be the quality of health care services and health attainment of all citizens rather than curtailing medical expenditures. Right now, both policymakers and Japanese citizens have to reconsider reform of the health case system: a rigorous cost-containment policy carries a strong presumption against the long life of the health care system in Japan, as shown by earlier lessons from the United Kingdom.

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Review

Des-γ-carboxyprothrombin: Clinical effectiveness and biochemical importance

Yoshinori Inagaki[1], Wei Tang[1, 2,*], Huanli Xu[1, 2], Fengshan Wang[2], Munehiro Nakata[3], Yasuhiko Sugawara[1], Norihiro Kokudo[1]

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[3] *Department of Applied Biochemistry, Tokai University, Kanagawa, Japan.*

Summary **Des-γ-carboxyprothrombin (DCP) is an abnormal prothrombin with a decreased number of γ-carboxylated glutamic acid residues in the Gla domain. DCP is also known to be an effective serological tumor marker for hepatocellular carcinoma (HCC), and highly sensitive methods of detecting serum DCP have enabled the detection of early and small-sized HCC in clinical settings. Several immunohistochemical studies have suggested that excessive production of DCP in HCC tissues may relate to worse tumor behavior such as the presence of vascular invasion and intrahepatic metastasis of HCC cells. Clinical availability of DCP, therefore, might be a more significant factor in the diagnosis of tumor behavior in HCC patients. Recently, some studies have suggested that DCP may play an important role in cancer progression *via* induction of cancer cell proliferation and angiogenesis around HCC tissues. Thus, DCP is expected to be effectively used not only as a tumor marker but also as a target of drug discovery.**

Keywords: Des-γ-carboxyprothrombin, Hepatocellular carcinoma, Tumor marker, Cancer cell proliferation, Angiogenesis

1. Introduction

Cancer cells are known to express various substances that are not expressed or expressed in small amounts, if at all, in normal cells. These cancer-related substances have been used as tumor markers in clinical settings. Various types of tumor markers have been discovered thus far and each tumor marker helps clinicians to diagnose a specific cancer disease in the early stage, to predict the cancer's behavior, and to determine a therapeutic strategy, thereby improving the survival of cancer patients (*1-3*).

Des-γ-carboxyprothrombin (DCP), also known as protein-induced vitamin K absence or antagonist II (PIVKA-II), is known to be an effective tumor marker for hepatocellular carcinoma (HCC) (*4-6*). Recent studies on DCP have revealed not only the effectiveness of this substance as a diagnostic marker but also its significant role in cancer progression. The present article reviews characteristics and clinical effectiveness of DCP as a diagnostic marker and describes further progression in the field of DCP investigation.

2. Mechanism of DCP Production

2.1. *Structural characteristics of DCP*

Prothrombin, a coagulation factor, is synthesized in a vitamin K-dependent manner in liver tissues. DCP is an abnormal prothrombin that lacks the ability to interact with other coagulation factors. The difference between normal prothrombin and DCP is the component of amino acid residues. The prothrombin molecule has some functional domain structures, and there are

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10 γ-carboxylated glutamic acid (Gla) residues in the N-terminal domain called the Gla domain (*7*). These Gla residues are originally glutamic acid (Glu) residues in the prothrombin precursor and are completely synthesized by vitamin K-dependent enzymatic reaction of γ-glutamyl carboxylase as post-translational modification (Figure 1) (*8*). When this reaction is insufficient under some conditions, such as a vitamin K deficiency, DCP with Glu residues in the Gla domain that do not undergo γ-carboxylation is expressed and secreted into extracellular regions (*9*). Thus, DCP comes in the form of many types of molecules with different numbers of Gla residues (*10*).

2.2. *Mechanism of DCP production*

The exact cause of DCP production in HCC tissues is not yet understood. One possibility is that the activity of γ-glutamyl carboxylase declines in HCC tissues. Shah *et al*. performed an analysis using rat models carrying a Morris hepatoma tumor to clarify the significance of γ-glutamyl carboxylase activity (*11*). Their study revealed that γ-glutamyl carboxylase activity was markedly lower in DCP-positive HCC tissues than in DCP-negative HCC tissues, while, abnormal γ-glutamyl carboxylase activity was not detected in normal liver tissues. Similar results were also obtained *via* an *in vitro* experiment using DCP-producing and non DCP-producing rat hepatoma cell lines (*12*). These studies indicated that an elevated DCP level may be a consequence of decreased γ-glutamyl carboxylase activity but not of abnormal prothrombin expression.

Another possibility is that the availability of vitamin K declines as a result of abnormal vitamin K metabolism. Okuda *et al*. performed a study using an HCC cell line to clarify the effect of vitamin K on the production of DCP and revealed that the HCC cell line produced DCP in a time- and cell number-dependent manner in the absence of vitamin K but not in the presence of vitamin K (*13*). Sakon *et al*. measured serum levels of DCP in patients with or without vitamin K administration and showed that the serum level of DCP declined as a result of administration of vitamin K while the levels of some vitamin K derivatives were significantly elevated in HCC patients (*14*), suggesting that elevated DCP may not be caused by vitamin K insufficiency but by abnormal vitamin K metabolism in HCC cells. In addition, Huisse *et al*. reported that a decreased vitamin K level and not decreased γ-glutamyl carboxylase expression was the most important factor for the production of DCP (*15*).

Furthermore, the overexpression of prothrombin precursor is also suggested to be one of causes of DCP production. Ono *et al*. indicated that the level of prothrombin precursor in HCC tissues was significantly elevated in patients with an elevated serum DCP level while there was no significant difference in the levels

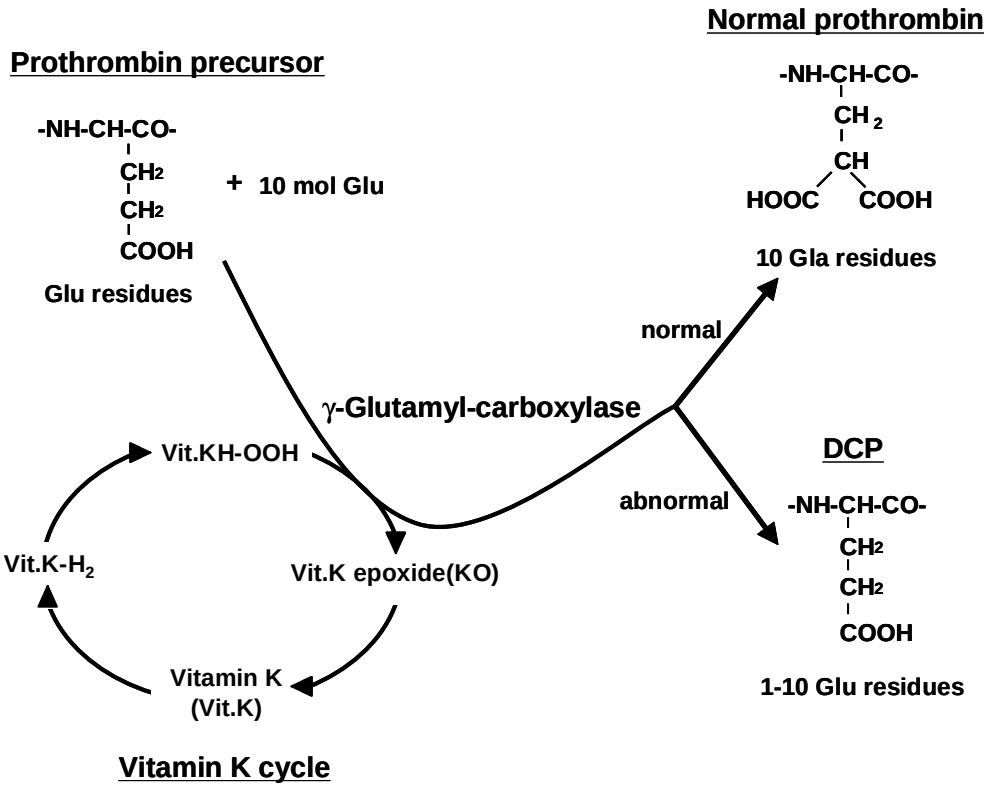

Figure 1. Production of normal prothrombin and DCP. Under normal conditions, γ-glutamyl-carboxylase completely alters 10 glutamic acid (Glu) residues in the Gla domain of a prothrombin precursor into 10 γ-carboxylated glutamic acid (Gla) residues. Under abnormal conditions, this reaction is insufficient, resulting in the production of an abnormal prothrombin, DCP, with a decreased number of Gla residues.

of endogenous vitamin K between HCC and non-HCC tissues (*16*). Yamagata *et al*. also suggested that the activity of γ-glutamyl carboxylase per unit amount of endogenous prothrombin precursor decreased in HCC tissues when compared to non-HCC liver tissues (*17*). These findings suggest that excessive production of prothrombin precursors may be a cause of DCP overexpression.

Production of prothrombin is affected by the expression and activity of various factors, so the mechanism of DCP production is also complicated. No single abnormal condition or phenomenon may cause DCP production but a combination of these conditions or phenomena may. In addition, these abnormalities are suggested to vary depending on the characteristics of HCC cell lines and cancer behavior in HCC patients. Further studies should be performed to clarify the mechanism of DCP production and evaluate its relationship to cancer behavior.

3. Clinical Effectiveness of DCP

3.1. *Development of tools for DCP detection*

As described above, DCP has been used as an effective tumor marker of HCC in clinical settings. In 1984, Liebman *et al*. first reported that the levels of DCP in sera of HCC patients were significantly elevated and that this elevation was related to recurrence of HCC after surgery (*18*). This study was performed by means of competitive radioimmunoassay with a polyclonal antibody to detect the level of DCP. Next, Fujiyama *et al*. determined plasma DCP levels using enzyme immunoassay (EIA) and reported that the levels of plasma DCP were frequently elevated in 63% of HCC patients with a cut-off level of 0.1 AU/mL; levels returned to normal after surgery in some patients while they rose again in cases of recurrence of the disease (*19,20*). These studies suggested that DCP could be used as a sensitive marker for HCC diagnosis and that the determination of DCP levels by EIA with monoclonal antibody is a powerful method of diagnosing HCC behavior easily and rapidly. However, the sensitivity of this method had to be increased to screen patients with a small-sized HCC.

An EIA kit with a higher sensitivity (Eitest PIVKA-II, Eisai, Tokyo, Japan) and an electrochemiluminescence kit (Picolumi PIVKA-II, Eisai) have been developed and are currently used in clinical settings. Their sensitivity is 10 times higher than that of the previous EIA kit thanks to modification of the EIA method or use of a new electrochemical method (*21,22*). Many investigations have been performed using these kits to clarify the usefulness of DCP as a diagnostic marker for HCC. Using this more sensitive EIA, Mita *et al*. analyzed serum DCP levels in 91 patients with HCC and 57 with cirrhosis and indicated that 56 of 91 HCC patients had positive DCP levels with a cut-off level of 40 mAU/mL while only 3 of 57 cirrhosis patients had positive DCP levels (*23*). Sensitivity and specificity of the test were estimated to be 62% and 95%, respectively, suggesting that determination of DCP levels by the sensitive EIA is a useful method for the early diagnosis of HCC. In addition, Nomura *et al*. and Sassa *et al*. indicated that the sensitive EIA has the potential to detect a small-sized HCC (*24,25*). These data accumulated using methods of measuring DCP levels with greater sensitivity have enabled clinicians to screen more patients with HCC, and DCP is now effectively used as a tumor marker for early detection of HCC in patients.

Anti-DCP monoclonal antibody has also been investigated and developed by some researchers (*26-28*). One anti-DCP monoclonal antibody called MU-3, which is now usually used to detect serum DCP levels in clinical settings, can recognize a specific part of the Gla domain as an epitope (*29*). Reaction of MU-3 is negatively dependent on the number of Gla residues in the DCP molecule. DCP variants produced in HCC patients chiefly have fewer than 4 Gla residues at the positions of amino acid residues 16, 25, 26, and 29 and are thereby strongly recognized by MU-3 (*10*). In contrast, DCP variants produced in patients with benign liver disease had more than 5 Gla residues. The kinds of DCP molecules which were detected in HCC patients were severely restricted, and MU-3 is a capable tool for screening HCC patients from patients with benign liver disease with a high level of specificity. Thus, using a sensitive test kit with MU-3 to measuring the serum level of DCP has significant clinical applicability.

3.2. *Clinicopathological significance of serum DCP levels*

Many studies on the relationship between the serum DCP level and various clinicopathological features of HCC have suggested that elevation of DCP reflects worse tumor behavior and prognosis for HCC patients (*30*). Imamura *et al*. indicated that patients' prognosis was significantly worse in patients with DCP-positive HCC than with DCP-negative HCC (*31*). Hamamura *et al*. reported that the positivity for DCP was frequently detected in patients with hepatitis B, a large tumor size, and enhanced tumor growth (*32*). Suehiro *et al*. also indicated that the serum level of DCP was related to the degree of proliferation of HCC tissues (*33*). Furthermore, several studies showed that elevated serum DCP is significantly related to portal vein invasion and/or intrahepatic metastasis as may be an independent prognostic factor. Sakon M *et al*. showed that a macroscopically massive carcinoma, intrahepatic metastasis, and portal vein tumor thrombus were detected at high frequencies in DCP-positive patients (*34*). Koike *et al*. performed a prospective study to clarify the significance of DCP in the prediction of portal vein invasion and revealed that portal vein invasion occurred at a significantly higher rate in patients who were positive

for DCP than those negative for DCP, and they suggested that positivity for DCP is the strongest predictive factor for portal vein invasion (*35*). Tang *et al*. also analyzed the clinicopathological significance of the serum level of DCP and indicated that a positive serum DCP level was significantly related to the presence of vascular invasion, intrahepatic metastasis, tumor size, TNM stage, and tumor recurrence (*36*). In addition, patients with positive serum DCP levels had a worse prognosis than those without such levels (*36*). Miyaaki *et al*. also indicated that DCP-positive HCC patients had higher frequencies of infiltrative growth, vascular invasion, and intrahepatic metastasis than DCP-negative HCC patients (*37*). These findings suggest that DCP is significant related to worse tumor behavior. Currently, the serum DCP level can be used not only as a diagnostic marker to screen HCC patients but also as an indicator of therapeutic effect and predictor of tumor recurrence and patient prognosis.

Other than DCP, there are two other serum markers for HCC diagnosis, α-fetoprotein (AFP) and the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3) (*35*,*38-40*). Although elevated DCP in sera of HCC patients is suggested to have no relation to elevated AFP and AFP-L3, a simultaneous test with a combination of these tumor markers is more effective for sensitive diagnosis of HCC (*41*,*42*). Some studies showed that elevation of DCP detected in patients with low AFP levels was frequently indicative of a large tumor size (*32*,*35*). Nakamura *et al*. suggested that DCP is more effective for diagnosis of large tumors than small tumors while AFP is effective for diagnosis of small tumors (*39*). Thus, simultaneous measurement of DCP and AFP or AFP-L3 levels has been proposed as a way to screen patients with HCC and particularly as a way to detect a small-sized HCC with a high level of sensitivity.

3.3. *DCP expression in liver tissue and its relation to an elevated serum DCP level*

Since DCP in circulation is originally produced by HCC tissues, one can reasonably conclude that the level of DCP expression in tissues determines the serum DCP level. Actually, the level of DCP expression in HCC tissues correlates with the serum DCP level and is related to the biological malignant potential of HCC and the prognosis of small HCC (*43*). However, serum DCP in HCC patients may not originate solely from HCC tissues. Tang *et al*. showed that the overexpression of DCP is detected not only in HCC tissues but also in the surrounding non-HCC liver tissues and that patients presenting with an overexpression of DCP in the surrounding non-HCC tissues had significantly elevated serum DCP levels (*36*). In addition, Yuan *et al*. performed an electrochemiluminescence immunoassay to determine quantities of serum and tissue DCP and reported that levels of DCP expression in non-HCC tissues correlated with those in HCC tissues and serum DCP levels (*44*). These findings suggest that an elevated serum DCP level may originate not only in HCC tissues but in surrounding non-HCC tissues. Moreover, Tang W *et al*. performed a further study to clarify the clinical effectiveness of tissue and serum DCP levels (*45*). In analysis of patient survival, a patient group with both overexpressed tissue DCP and elevated serum DCP levels displayed the worst outcome when compared to other patient groups with negative tissue DCP expression and a low serum DCP level and with either overexpressed tissue DCP or an elevated serum DCP level. Furthermore, multivariate analysis identified DCP expression in total liver tissue as a significant prognostic factor along with intrahepatic metastasis. The current study showed that DCP expression in either HCC or surrounding liver tissue may be a powerful tool for diagnosing HCC behavior along with the serum DCP level (Figure 2). Moreover, the overexpression of DCP not only in HCC tissue but also in surrounding non-cancerous liver tissue must be considered in order to investigate the biological mechanism of an elevated DCP level in HCC patients

4. Biochemical Significance of DCP and Further Progression of Investigation

Although numerous studies have contributed to the recognition that DCP has a clinical significance as a useful diagnostic marker for HCC, reasons why DCP is overexpressed in HCC tissues and why this overexpression is related to enhancement of tumor growth and malignancy of HCC have not been clarified thus far. However, several basic studies on the function of DCP in HCC cells may offer important clues to resolving these questions.

4.1. *DCP's role in the proliferation of HCC cells*

The structure of DCP contains two kringle domains that are similar to those of hepatocyte growth factor (HGF) (*7*,*46*). The kringle domains are necessary for HGF to bind its receptor, called Met, and to induce cell proliferation. DCP is hypothesized to have the ability to bind with Met and cause cells, and particularly HCC cells, to proliferate. In 2005, Suzuki *et al*. investigated the biological function of DCP in HCC cell proliferation (*47*). According to their report, levels of DNA synthesis in HCC cell lines were significantly enhanced by addition of purified DCP; this enhancement was more marked in non-DCP-producing cell lines than in DCP-producing cell lines, indicating that DCP can induce the proliferation of HCC cells. Furthermore, the researchers also analyzed the mechanism of this phenomenon and revealed that DCP bound with Met and stimulated the Met-JAK-STAT pathway as the signaling pathway for the induction of HCC cell proliferation. These results suggest that DCP can induce the proliferation of HCC cells by functioning like HGF. DCP may relate to worse

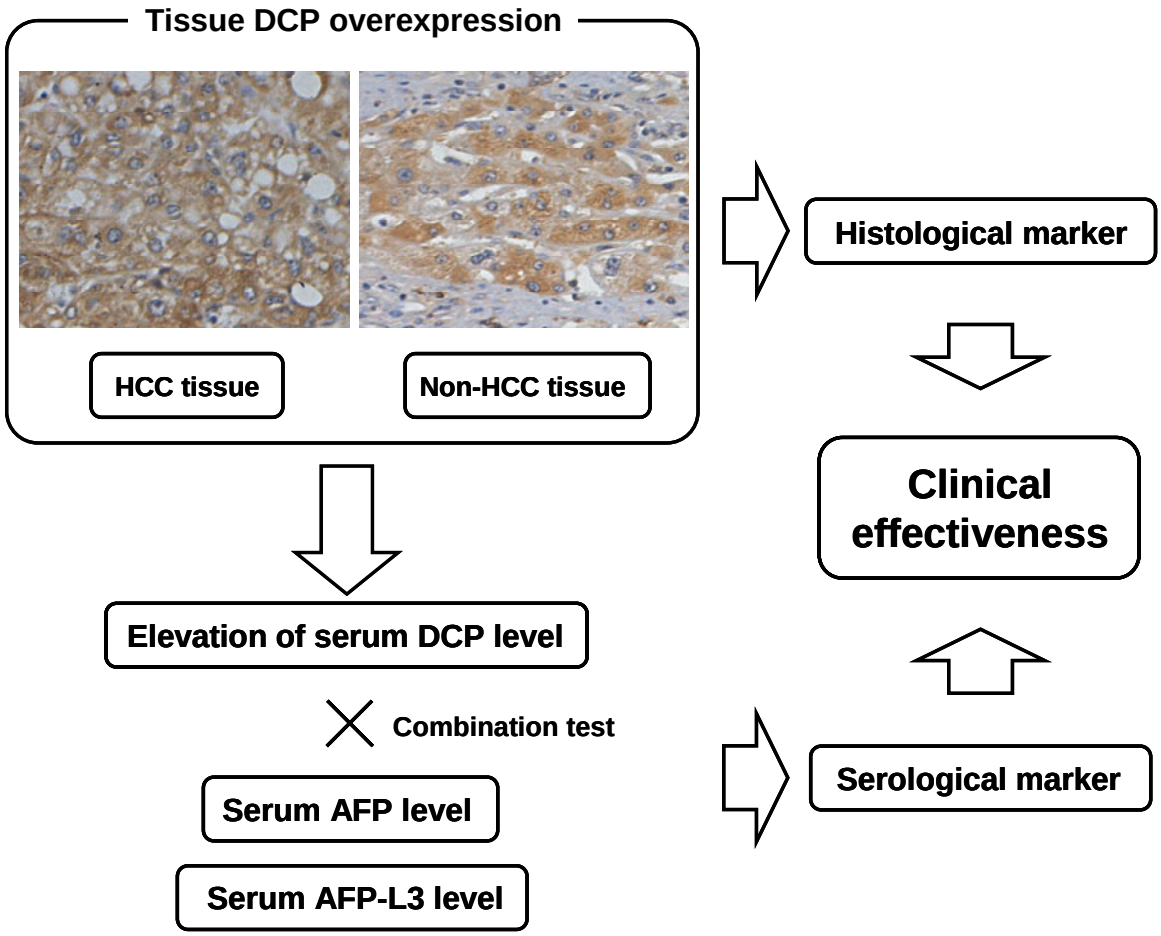

Figure 2. Clinical effectiveness of histochemical expression and serum level of DCP. Histochemical expression of DCP in both HCC and surrounding non-cancerous liver tissues is significantly related to worse tumor behavior. At the same time, the serum DCP level is also related to tumor malignancy; a combined serological test for DCP and AFP or AFP-L3 is particularly effective at diagnosing HCC in patients. Furthermore, the combination of histological and serological analyses may have novel clinical significance.

tumor behavior by enhancing the proliferation of HCC cells.

4.2. *DCP's role in angiogenesis around HCC tissue*

Another biological effect of DCP in malignancy of HCC may be that DCP has the ability to enhance angiogenesis around HCC tissues. Fujikawa *et al*. analyzed the biological function of DCP in angiogenesis by using human umbilical vein endothelial cells (HUVEC) (*48*). They indicated that DCP could stimulate the DNA synthesis and migrative activity of HUVEC but normal prothrombin could not. They reported that, as part of the activation of HUVEC, DCP could bind to kinase insert domain receptor (KDR), which is known to be a vascular endothelial growth factor receptor (VEGFR-2), and stimulate the KDR-PLC-γ-MAPK signaling pathway followed by the acceleration of DNA synthesis and cell migration (*48*). This fact suggests that DCP secreted from HCC cells can induce angiogenesis in surrounding tissues and cause a worse prognosis in HCC patients. Angiogenesis is a particularly important phenomenon for the continuous growth of tumor tissues and causes worse tumor behavior, such as dedifferentiation and hypervascularity (*49*); high tumor microvessel density is suggested to cause the recurrence of HCC (*50*). These studies suggest the significant role of DCP in cancer progression *via* the induction of angiogenesis and may helpful for understanding the mechanism by which DCP aggravates HCC behavior.

4.3. *Further progression of DCP investigation*

DCP has several important roles in HCC progression and may explain why cancer behavior and patient prognosis worsen in patients with DCP-positive HCC than in those with DCP-negative HCC. DCP is not just an abnormal prothrombin but may also be a potential cancer-enhancing protein (Figure 3). This fact suggests that DCP may be used not only as a diagnostic marker of HCC but also as a therapeutic target for HCC. As described above, DCP is related to some cancer-associated events such as self-proliferation and angiogenesis. Thus, if these events can be inhibited by some DCP inhibitor, progression of DCP-positive HCC may be suppressed. However, constructing novel DCP inhibitors will not be simple since the nature of DCP, such as its three-dimensional conformation, is not fully understood.

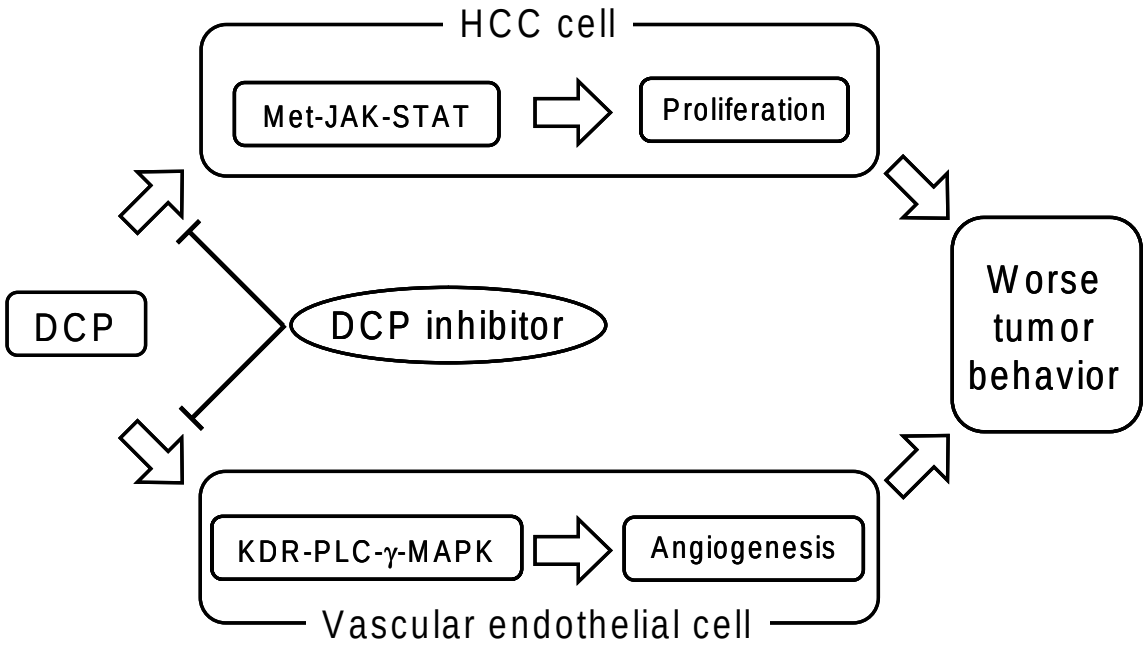

Figure 3. Proposed physiological functions of DCP and expected progression. Autocrine/paracrine secretion of DCP influences the self-proliferation of HCC cells through the Met-JAK-STAT signaling pathway. In addition, paracrine secretion of DCP influences the proliferation and migrative activity of vascular endothelial cells through the KDR-PLC-γ-MAPK signaling pathway. Secreted DCP can function as a growth factor, and hence the inhibition of DCP may contribute to the suppression of cancer aggressiveness.

Further investigation should be continued to develop the novel effectiveness of DCP.

5. Conclusions

In the current medical treatment for cancer, early diagnosis and appropriate therapeutics are the most important factors for the improved survival rate of cancer patients. This review has focused on the clinical effectiveness of DCP in the diagnosis and treatment of HCC. DCP is now established as an effective tumor marker for HCC and highly sensitive methods of detecting DCP contribute to the early diagnosis of HCC. In addition, recent findings indicating the novel biological functions of DCP in HCC progression may provide clues to novel therapeutics for HCC.

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Brief Report

Knowledge and practice of poultry handling and living environments of rural residents in China

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Summary **In China, most human cases of avian influenza affected rural residents and were reported to involve contact or intake of sick poultry, suggesting risks relevant to being a rural resident increase the possibility of exposure to the fatal virus. In this study, we investigated the living environments in rural areas and rural residents' knowledge, attitudes, and practices regarding poultry handling and avian influenza in Shandong Province, Anhui Province and the Inner Mongolia Autonomous Region. We thus hope to provide evidence on the risk of exposure to sick poultry and on the effects of health education for rural residents.**

Keywords: Rural resident, Avian influenza, Living environment, Health education

Introduction

According to situation assessment by the World Health Organization (WHO), all of the prerequisites for the start of a human influenza pandemic have been met save one: the establishment of efficient human-to-human transmission (*1*). The possibility that the virus will mutate into a new form communicable among humans increases as the geographical range of the infection expands and the number of people both near the disease and who have contracted the disease increases. Since 2003, outbreaks of highly pathogenic avian influenza A (H5N1) virus with sporadic transmission from birds to human worldwide have heightened concern regarding a potential pandemic. In China, like other Asian countries such as Indonesia, Thailand, and Cambodia, most reported patients and victims were rural residents and had contact with poultry, suggesting risks relevant to being a rural resident increase the possibility of exposure to the fatal virus. Many studies have confirmed that H5N1 infection has been associated with exposure to infected poultry (*2-5*). Until now, however, no systematic study sought to understand the knowledge and practices of rural residents regarding poultry handling and precautions against infection, the implementation of local surveillance systems, and the effects of health education and interventions such as vaccination of poultry. All of this information is urgently needed for policymakers to prepare for a potential pandemic. The objective of this study was to assess living environments in rural areas and rural residents' knowledge, attitudes, and practices regarding poultry handling and avian influenza.

Methods

This is a cross-sectional study conducted from September 2007 to January 2008 in Shandong Province, Anhui Province, and the Inner Mongolia Autonomous Region with an eye toward geographical diversity and feasibility. The latter two areas had experienced avian influenza and cases of human infection before. The target population was rural residents ages 18 and over who were selected by multi-stage sampling. First, 1 district was randomly selected from each area. Three counties were selected from each district, and then 1 village was selected from each county. In the 9 selected villages, all residents were interviewed, providing a total of 1,379 participants (Table 1), using a semi-structured questionnaire designed to collect information on their demographic characteristics, living environment, poultry handling practices, frequency of trips to the poultry market, attitudes regarding a potential pandemic, and knowledge of

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Table 1. Demographic characteristics of participants

Demographic characteristics		*n* = 1,379	(%)
Age		47.3	(± 17.6)
Location	Shandong	399	(28.9)
	Anhui	491	(35.6)
	Inner Mongolia	489	(35.5)
Gender	Male	662	(48.0)
	Female	717	(52.0)
Marital status	Single	180	(13.1)
	Married	1,199	(86.9)
Education	Middle school and below	1,258	(91.2)
	Above middle school	121	(8.8)
Occupation	Farmer	1,027	(74.5)
	Other	352	(25.5)
Annual income of household (RMB)	< 5,000	224	(16.2)
	5,000 - 9,999	486	(35.2)
	10,000 - 14,999	227	(16.5)
	15,000 - 19,999	187	(13.6)
	20,000 - 24,999	65	(4.7)
	≥ 25,000	190	(13.8)
Participant in community-based medical insurance scheme	Yes	1,322	(95.9)
	No	57	(4.1)
Poultry feeder	Yes	312	(22.6)
	No	1,067	(77.4)

Table 2. Living environments

Living environments		*n* = 1,379	(%)
Contact with wild birds	Often	426	(30.9)
	Occasionally	388	(28.1)
	Never	565	(50.0)
Type of yard	Yard encircled by wall	714	(51.8)
	Yard semi-closed	182	(13.2)
	Yard open	450	(32.6)
	Other	33	(2.4)
Frequency house cleaned	Every week	677	(49.1)
	Every month	289	(21.0)
	Every 6 months	248	(18.0)
	Every year	162	(11.7)
	Not cleaned	3	(0.2)
Waste dumping in designated place	Yes	586	(42.5)
	No	793	(57.5)
Water supply	Yes	1,242	(90.1)
	No	137	(9.9)
Type of toilet	Flushing toilet	189	(13.7)
	Pit toilet	1,190	(86.3)
Separate chopping board	Yes	443	(32.1)
	No	936	(67.9)
Poultry kept in household	Yes	714	(51.8)
	No	665	(48.2)

avian influenza and disease prevention. Additionally, health officers from primary healthcare settings and the local Health Agency were interviewed to ascertain their understanding of policies and interventions including surveillance, vaccination of poultry, and economic compensation during the outbreak.

Results and Discussion

The living environments of the rural residents surveyed are summarized in Table 2. Half of the participants have contact with wild birds ("often" and "occasionally"). Nearly 50% have a semi-closed or open yard that poultry can pass in and out of. Interviews revealed that some participants brought a dead wild bird home and prepared it for food when they found it outside. Over 51% keep poultry in their households. Some of those respondents had special chicken coops and others did not; the latter instead had poultry that were bred in a common room, an extra room, or the toilet. In most households with poultry and even those with special coops, poultry dung was often seen in the yard, living room, toilet, and kitchen. Based on the interviews, 30 of 714 total participants reported the death of poultry in the previous two weeks. When their poultry died, most participants buried or burned them while others took inappropriate measures such as throwing them out with the trash or eating them. In terms of sanitary conditions, 57.5% of participants dumped waste near the house instead of at a designated place. Of the households surveyed, 9.9% did not have a water supply. As many as 86.3% of respondents use a pit toilet instead of a flushing toilet. Of respondents, 67.9% prepared raw poultry and cooked food on the same chopping board. According to self-reports by participants, the vaccination rate of poultry bred in their households was 100%. The average frequency of trips to the poultry market was once every 19 days.

Regarding rural residents' knowledge of avian influenza and disease prevention, 15 questions were designed based on education guidelines for human infection prevention (*6*), and 1 point was allotted for each correct answer. The score therefore ranged from 0 to 15. In this study, the minimum, mean, and maximum scores were 0, 4.5, and 11, respectively.

Residents from Shandong Province were found to have better knowledge related to avian influenza and disease prevention than residents of the other 2 areas; interviews with local health officers indicated that this can be attributed to the effects of health education activities. Moreover, respondents with less education, farmers, and those with annual income of less than 5,000 RMB (approximately 75,000 Japanese Yen) tended to have worse scores. Among factors significantly associated with participants' correct answer rate were a separate chopping board for food preparation, influenza treatment, vaccination, interest in related information, eating habits, and attitudes regarding a potential pandemic. The main sources of related knowledge and information were TV and the Internet. Most participants were relatively interested in and concerned about a potential pandemic.

This study identified the frequent and inevitable contact between rural residents and poultry in living environments of rural residents. Sanitary conditions and especially waste disposal, toilets, and water supply must be improved. Some unsafe and inappropriate practices of poultry handling such as food preparation and handling of dead poultry still remain. The level of rural residents' knowledge of avian influenza and disease prevention in general was relatively low and was associated with regional differences, health behavior, and attitude, suggesting that health education for rural residents must be improved in the near future.

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Brief Report

A rapid identification of *Radix inulae* and its active component alantolactone in the Tibetan medicine Manuxitang

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Summary **This study sought to establish a more reliable method of identifying the "monarch" or principal drug *Radix inulae* and its active component alantolactone (AL) in the Tibetan medicine Manuxitang. *Radix inulae* and AL in Manuxitang were effectively identified by thin layer chromatography (TLC). AL was quantitatively determined using gas chromatography in the range of 0.1-1.0 μg/mL (r = 0.9998). The precision was 1.20% (n = 6) with an average RSD of 1.74%. Recovery was in the range of 93.5-98.5% with RSD value of 1.85%. The methods established were simple, accurate, and specific and could be used for quality control of Manuxitang.**

Keywords: Manuxitang, *Radix inulae*, Alantolactone, Chromatography, Tibetan medicine

1. Introduction

Tibetan medicine, an important part of traditional Chinese medicine (TCM), is known as a source of sustainable and affordable healing preparations that are effective without lasting negative side effects. More than 3,000 different medicinal materials of traditional Tibetan medicine, including many precious and costly medicinal herbs such as aweto, snow lotus of Tianshan, saffron, and Solanum muricatum, grow on the Qinghai-Tibet plateau known as the "Roof of the World".

Traditional medicines are usually prepared by decocting multiple materials, which causes difficulty in identifying their active components and achieving quality control. Qualitative evaluation of a TCM decoction is often challenging since the active compounds in TCM may originally be from single herbs and may also result from the decocting process. There is an urgent need for systematic scientific standards to objectively evaluate their safety and efficacy and to strictly control their quality based on scientific research and evidence (*1*,*2*).

One of the most popular Tibetan medicines is known by the Tibetan name Manuxitang (玛奴西汤) or the Chinese name Siwei Zangmuxiang Tangsan (四味藏木香汤散) and has been widely used in clinical practice for more than 1,000 years. This medicine has proven to have considerable effects on diaphoresis and relief of "exterior syndrome" (signs and symptoms accompanying cold and flu) and has been widely used for the onset of pestis and influenza, chills, headache, joint pain, rheumatoid arthritis, and fever (*3*). Manuxitang consists of four traditional herbs, namely *Radix inulae* (藏木香; Zangmuxiang), *Tinosporae sinensis* Caulis (宽筋藤; Kuanjinteng), *Rubus niveus* Thunb (悬钩木; Xuangoumu), and *Zingiber officinale* Posc. (干姜; Ganjiang). Manuxitang has already been listed in Volume 1 of the "People's Republic of China medical department drugs standard-Tibetan medicine" in 2005 (*4*). However, identification and quantitative determination were not included in the standards for Manuxitang.

In a traditional medicine, the principal component is known as the "monarch". *Radix inulae*, the "monarch" of Manuxitang, is the dried roots of *Inula helenium*

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L. or *Inula racemosa Hook f*. (Family Compositae). This medicinal material is widely used in Mongolia, Xinjiang, Tibet, and Qinghai and is frequently used in TCM for abdominal distension and pain, acute enteritis, and bacillary dysentery (*5,6*). The major active component in *Radix inulae* has been determined to be alantolactone (AL) (*7,8*). This study attempted to establish a rapid method of identifying the "monarch" or principal drug *Radix inulae* and its active component AL in Manuxitang.

2. **Materials and Methods**

2.1. *Herbs and chemicals*

Manuxitang was provided by Qinghai Tibetan Medicine Hospital (batch code: 060101, 060102, 060103). *Radix inulae*, the dried root of *Inula helenium L*. (Composite), was provided by Qianghai Tibetan Medicine Research Institute and identified by the Institute for Drug Control. AL was purchased from China Materia Medica Biological Product Inspection Institute, Beijing, China.

2.2. *Thin layer chromatography (TLC)*

2.2.1. *Sample preparation*

Two g of Manuxitang were extracted with 25 mL of trichloromethane by ultrasonication for 30 min. The extract was filtered and then concentrated to 2 mL and the filtrates obtained were used for sample application (*9*). *Radix inulae*-positive control solutions were prepared from the *Radix inulae* herb using the same extraction methods as for Manuxitang. A *Radix inulae*-negative control was prepared in accordance with the preparation of a Manuxitang formulation without *Radix inulae* following the extraction as described above. AL was dissolved into 2 mL of trichloromethane to prepare an AL control solution.

2.2.2. *TLC for Radix inulae and AL*

About 5 μL of Manuxitang sample solution, AL control solution, and *Radix inulae*-negative and positive control solutions were spotted on a Silica Gel G plate (Qingdao Haiyang Chemical Plant, Qingdao, China) containing 0.5% sodium carboxymethyl cellulose by means of a semi-automatic sample applier. After development with a solvent system of petroleum ether (60-90°C)/ethyl acetate (15:3, v/v), the plates were withdrawn from the chambers and dried at room temperature. Color developing was performed by spraying plates with 5% vanillin sulfate solution and then heating them to 105°C until the spots were clear. Thin layer chromatography scanning (TLCS) fingerprint profiles were obtained by both UV absorption at 365 nm and fluorescence detection. Photographic TLC images were also acquired using ReproStar 3.

2.3. *Gas chromatography*

2.3.1. *Preparation of sample solutions*

Two g of Manuxitang were crushed to obtain a fine powder and then mixed well. After screening, the powder was accurately weighed, dissolved with 30 mL ethyl acetate, and then ultrasonicated for 30 min. The mixture was filtered through a 0.45 μm membrane filter and the filtrate obtained was used as the sample solution. The negative control solution (without *Radix inulae*) was prepared using the same method.

2.3.2. *Gas chromatography conditions*

A Finnigan Trace GC Ultra gas chromatographic system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with an SUPELCOWAXTM10 column (30 m × 0.25 mm × 0.25 μm), a flame ionization detector, and a GCD-500G high-purity hydrogen generator were used for gas chromatography. The oven temperatures were controlled as follows: the temperature was initially 50°C and then raised to 250°C at a rate of 20°C/min, kept for 6 min, and then raised again to 260°C at a rate of 5°C/min and kept for 5 min. The temperature of the injector block was set at 260°C. Samples (1.0 μL) were injected in split injection mode (10:1). Helium was used as the carrier gas at a flow rate of 1.0 mL/min.

2.3.3. *Method validation*

2.3.3.1. *Calibration curves*. A stock standard solution of AL (1 mg/mL) was prepared with ethyl acetate. Working standard solutions ranging from 0.1-1.0 mg/mL of AL were prepared from various aliquots of stock standard solution and then diluted to 1 mL with ethyl acetate to give a final appropriate analyte concentration. Six-point calibration curves were acquired by plotting the peak area with respect to the concentration of the calibration standards.

2.3.3.2. *Precision.* Same-day precision was estimated by six replicate injections of standard solutions at the concentrations of 0.1, 0.50, and 0.9 mg/mL on the same day. A relative standard deviation (RSD) within 5% was the criterion for acceptability of data.

2.3.3.3. *Recovery*. Recovery studies were conducted by spiking one batch of Manuxitang (batch code: 060101) with 1.00 mg/mL of recovery standard solution. Two mL of standard solution were added to separate aliquots of the Manuxitang powder. The spiked samples were then extracted, processed, and quantified in accordance with the established method ($n = 6$).

3. Results and Discussion

On TLC analyses, *Radix inulae* and AL were both successfully separated by development on the plate (Figure 1). The Manuxitang sample solution (lane 2) produced a purple spot, which developed at the same position as that produced by control AL (lane 4), and

some spots with colors differing from those produced by the *Radix inulae*-positive control solution (lane 3). R_f values of *Radix inulae* and AL were 0.18 and 0.54, respectively. These two spots were not observed with the *Radix inulae*-negative control solution (lane 1).

When Manuxitang was analyzed by gas chromatography under the optimal conditions as described in the Materials and Methods, AL was successfully separated and detected at a retention time of 16.8 min (Figure 2). The peaks in Manuxitang were identified by retention times and the contents were calculated by the areas of the peaks. The AL content in three batches of Manuxitang was 4.33, 4.21, and 4.30 mg/mL, respectively.

For evaluation of the quantitative applicability of the method, the following parameters were analyzed under optimum conditions. (i) The linear relationship between the concentration of the active compounds (X) and the corresponding peak areas (Y) was investigated. The regression equation for AL was: $Y = 9.0 \times 10^7 X + 2.0 \times 10^6$ in the range of 0.1-1.0 μg/mL ($r = 0.9998$). (ii) The limit of detection (LOD), a concentration that generates a signal-to-noise ratio of 3, was determined to be 10 μg/mL for AL. (iii) Precision was measured by performing same-day experiments of six replicate injections of standard solutions at three different concentrations. The precision (RSD) for AL was 1.20% with an average RSD of 1.74%, indicating good precision at low, medium, and high concentrations of the compound. (iv) Recovery was in the range 93.5-98.5% for AL, with RSD values of 1.85%, which met the criterion for acceptability of accuracy (95-105%) at the analyte concentration.

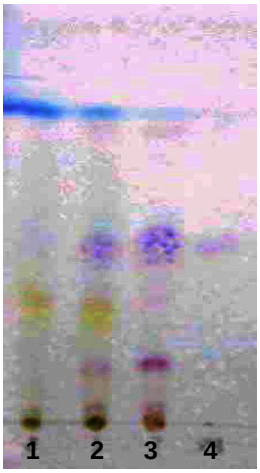

Figure 1. A typical TLC profile for *Radix inulae* and AL. Mobile phase: petroleum ether (60-90°C)/ethyl acetate (15:3, v/v). Lane 1, negtive control; lane 2, Manuxitang sample; lane 3, *Radix inulae*-positive control; lane 4, AL-positive control.

The last few years have seen a tremendous increase in regulatory activities regarding the herbal industry, and demand for analytical methods that can help to ensure safety and quality has been growing at an accelerated pace (*10-12*). Due to the complexity of components in Chinese herbs and especially in their preparation, however, these methods suffer from limitations such as being material-intensive and time-consuming. The present study established a relatively simple, convenient, and effective method of determining AL, a major active component in *Radix inulae* (*7,8*), and used it for the quality control of a Manuxitang decoction.

TLC is often the method of choice for routine compositional analysis and quality screening of medicinal plants when many samples have to be compared, when flexibility is important, and when rapid qualitative and semi-quantitative data are needed with a

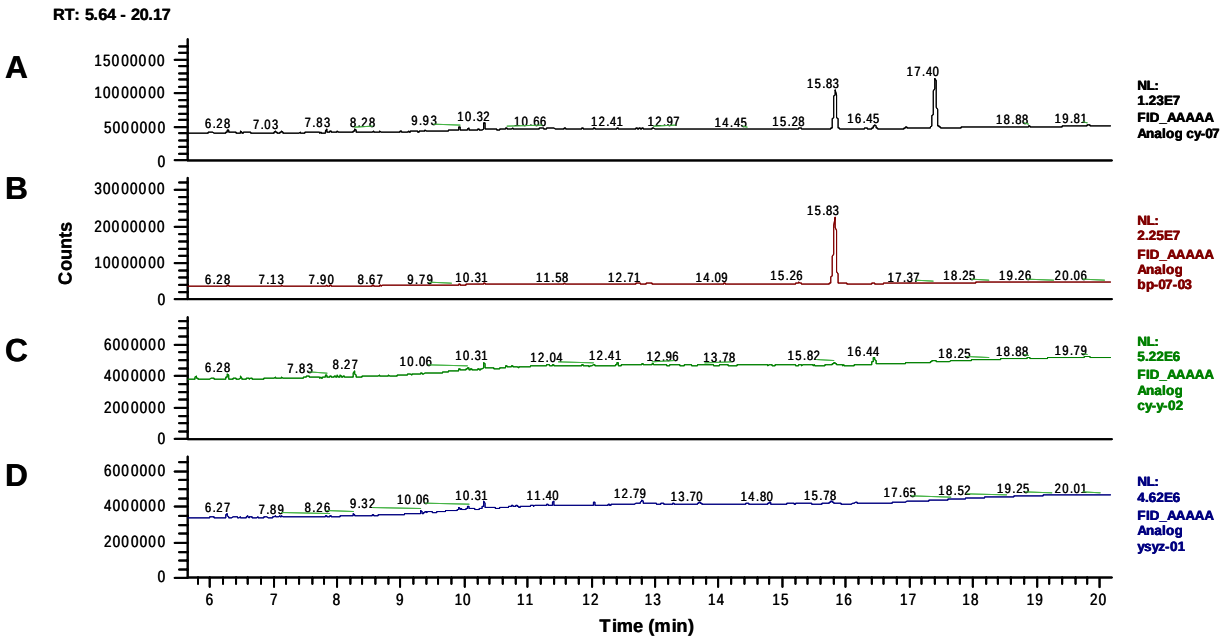

Figure 2. Gas chromatograph analyses of AL in Manuxitang. A, Manuxitang sample; B, AL control; C, negative control; D, solvent only.

low cost per sample. The present data showed that the identification of *Radix inulae* and its active component AL in a Manuxitang decoction could be achieved by TLC, although TCL analysis was semi-quantitative. In contrast, gas chromatography analysis is a simple, sensitive, and reproducible method of determining AL in *Radix inulae* and Manuxitang. Additionally, the proposed method may also be suitable for the quality control of TCM containing *Radix inulae*.

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Original Article

Assessment of hepatitis B vaccine-induced seroprotection among children 5-10 years old in Ulaanbaatar, Mongolia

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Summary **Hepatitis B virus infection is a serious public health problem. Mongolia is one of the countries with the highest rates of hepatitis B virus infection in the world. The routine immunization with the hepatitis B vaccine began nationwide in 1991. The purpose of this study was to determine the persistence of seroprotection (anti-HBs ≥ 10 mIU/mL) in children 5-10 years old that were immunized with the hepatitis B vaccine as infants. In total, 438 children were selected from six health facilities in Ulaanbaatar through a multistage random sampling method. Vaccination information was confirmed by checking the vaccination records kept in the health facilities. A blood sample was obtained from each child for anti-HBs, HBsAg and anti-HBc. Of 438 children, five (1.1%) were HBsAg positive and 58 (13.2%) were anti-HBc positive. Sixty infected children were excluded and a total of 378 (86.3%) sera were evaluated. The seroprotective antibodies were detected in only one-fourth of the children at the age of ten. Titres of anti-HBs decreased significantly with age (Linear regression $p = 0.01$). This decrease is primarily due to the rapid decrease in children living in ger areas ($p < 0.001$) compared to children living in apartment areas ($p = 0.152$). On the other hand, children living with higher socio-economic status had more exposure to blood-borne pathogens, probably due to inappropriate health-seeking behaviors.**

Keywords: Hepatitis B vaccine, Seroprotection, Mongolia

Introduction

Hepatitis B virus (HBV) infection is a serious public health problem. According to the World Health Organization (WHO), two billion people worldwide have been infected with HBV at some time in their lives (*1*,*2*). Of these, about 350 million remain chronically infected and become carriers of the virus (*1*,*2*). Every year there are over four million acute clinical cases of HBV and one million die from chronic active hepatitis, cirrhosis or primary liver cancer (*2*). Hepatitis B is the only type of chronic viral hepatitis that can be prevented by a vaccine. In 1991, the WHO called for all children to receive the hepatitis B vaccine, and 136 countries added this vaccine to their routine immunization program by the end of 2001 (*2*,*3*).

Mongolia included the hepatitis B vaccine in their national immunization program in 1991. Two doses of the vaccine were given at the ages of 0 and 2 months until 1996, then the third dose was added, being given at an age of 8 months. In general, the hepatitis B vaccine is recommended as a three-dose series given at the ages of 0, 1 and 6 months (*3*). The first dose should be given within 24 hours after delivery, and a total of three doses with an interval of at least four weeks, but not more than two months between the first and second doses, are recommended.

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In Western Pacific Regional countries, where hepatitis B vaccination has been included in national immunization programs routinely, seroprevalence of HBV infection has been reduced by over 85% (from 9.8% to 0.7% in Taiwan, from 11% to 0.7% in Fiji, from 10% to 0% in Singapore, from 16.3% to 3.0% in Vanuatu), whereas in Mongolia, it was estimated to decline by only 50% (from 14-39% in 1989 to 6-7% in 1998-1999) (*3,4*). The incidence of icteric viral hepatitis in Mongolia has also been reduced by more than two times in general, and the HBV carrier rate has been decreased by 3.3 times in the vaccine-age population since the introduction of hepatitis B vaccination (*5*). However, HBV infection was still prevalent at a relatively high rate of 10% in apparantly healthy individuals in 2002 (*6*). Furthermore, prevalence of antibody to hepatitis B surface antigen (anti-HBs) induced by the vaccine was only 70.2% among 2-year-old children in rural areas compared to 94.2% among children in urban area (*7*) because of incomplete vaccination and freezing of the vaccine during transportation (*8*). Although more than 95% of all children received the hepatitis B vaccine in Mongolia, children in rural areas were less likely to complete the recommended doses than children in urban settings, probably due to seasonal difficulties in locating nomadic households (*7*).

Although the hepatitis B vaccine is highly effective, the duration of protection and indications for its booster dose need to be further investigated (*9-11*). A study in Taiwan reported that fifteen years after routine immunization with the hepatitis B vaccine, a large proportion of the children exhibited waning immunity (*12*). The real threat may emerge in the future when the immunized children become older and begin engaging in sexual activity with generations that still have a high HBV carrier rate. Currently, a booster dose of hepatitis B vaccine after the three-dose routine immunization is not recommended because carriers of the virus are rarely found among children who were immunized 10-15 years previously with hepatitis B vaccine (*13-17*). However, some reports have suggested that boosters are necessary because of the progressive decline of anti-HBs over time and the potential risk for development of HBV infection (*12,18,19*). Therefore, efficacy of the vaccine, long-term immunity and necessity of a vaccine booster are important issues in endemic areas.

To the best our knowledge, there has been no study assessing the duration of seroprotection induced by the hepatitis B vaccine in Mongolia. In this paper, we examined the persistence of seroprotection in 5-10 year old children who were immunized with the hepatitis B vaccine as infants and explored the risk factors affecting the efficacy of the hepatitis B vaccine.

Methods

This study was conducted from January to February 2006 in Ulaanbaatar, Mongolia. Through cluster sampling, we selected three of the nine districts in Ulaanbaatar: Songinokhairkhan, Chingeltei and Baganuur. Areas within the districts can be classified into two distinct categories: apartment areas and ger areas. Two health facilities, one from an apartment area and the other from a ger area, were chosen at random from each selected district. Living conditions of the Mongolian population depend primarily on the type of dwelling. In Ulaanbaatar, *gers* (traditional Mongolian dwellings consisting of tent-like wooden structures covered with felt, without inside hygienic facilities) and *houses* (structures with one or more rooms, some with piped water and/or inside hygienic facilities) co-exist on dirt roads in ger areas. In contrast, *apartments* (structures that are provided with piped water, heating systems, and hygienic facilities) are located in areas that have paved roads and often have local shopping centres (*20*). Children aged 5 to 10 years who had previously received two or three doses of hepatitis B vaccine were selected from 6 health facilities by reviewing vaccination records. The study population in each health facility comprised six age groups with approximately 72 children in each age group, 36 boys and 36 girls: 5 years, 6 years, 7 years, 8 years, 9 years, and 10 years of age.

The dates of administration, number of doses received, and serial numbers of hepatitis B vaccines were confirmed by checking the vaccination records kept in the health facilities. Detailed information was obtained from the children's mothers by a semi-structured questionnaire that included child's age, sex, date of birth, residence, personal history of blood exposure (*e.g.* previous blood transfusion, operation, intravenous injection and dental manipulation), family history of liver diseases, experience of home injection and toothbrush sharing, and socio-economic status of the child's parents.

A 5 mL blood sample was obtained from each child to test for anti-HBs, hepatitis B surface antigen (HBsAg) and antibody to hepatitis B core antigen (anti-HBc). All blood samples were delivered to the laboratory in Ulaanbaatar on the same day that the blood was drawn. Serum samples were divided into two labeled, sterile Ependorff tubes to avoid repeated freezing and thawing, then were maintained in long-term storage under deep-freeze conditions until processed. Anti-HBs was quantatively estimated on a mini VIDAS immunofluorometric autoanalyzer manufactured by bioMerieux, France. Levels of anti-HBs were expressed in milli-international units per millilitre (mIU/mL). The range of reliability of the mini VIDAS autoanalyzer is between 5 mIU/mL and 500 mlU/mL. Values of anti-HBs < 10 mIU/mL were considered un-protective, and values ≥ 10 mlU/mL were considered protective. The presence of HBsAg, and anti-HBc were determined with ACON immunochromatographic tests (Acon Laborartories, USA).

Statistical analysis was performed using SPSS 12.0.1

for Windows. Linear regression was used to analyze continuous values of anti-HBs titer. The chi-square test was used to test the strength of the relationship between categorical variables, and a *p*-value below 0.05 was considered statistically significant.

This study was approved by the Ethical Committee of the University of Tokyo, Japan, and Ministry of Health, Mongolia. The purpose of the study was carefully explained to children's mothers and written informed consent to participate in the study was obtained before we started the study. The parents were informed of their child's serological results and hepatitis B vaccine booster inoculations were offered to those with un-protective titres.

Results

A total of 438 children and their 438 mothers participated in this study. Children's age, gender, and place of residence were distributed equally (Table 1). We found that 72.1% of mothers and 59.9% of fathers of study children living in an apartment area had more than 10 years of education, significantly higher than the parents living in ger areas: 28.8% and 16.2%, respectively ($p < 0.001$; Table 2). Similarily, parents living in apartment areas were more likely to be employed and had higher incomes than those living in ger areas. These results were statistically significant ($p < 0.001$; Table 2).

Of 438 children, 5 (1.1%) were HBsAg positive, indicating current infection or carrier status, and all 5 were male, school-age children (Table 3). Three of the 5 children were also anti-HBc positive. The presence of anti-HBc without HBsAg is indicative of past infection. The overall anti-HBc positive rate was 13.2% (58/438), with 7.9% (12/152) of preschool-age children and 16.1% (46/286) of school-age children testing positive for anti-HBc. There was a significant difference in anti-HBc seroprevalence between children of preschool age and school age ($p < 0.05$; Table 3). Surprisingly, all 60 infected children were documented to have received at least 2 doses of the hepatitis B vaccine.

We excluded the 60 children with evidence of HBV infection from the analysis of vaccine-induced seroprotection. A total of 378 (86.3%) sera were evaluated and titres of anti-HBs decreased significantly with age (Linear regression $p = 0.01$; Table 4). Protective titres (≥ 10 mIU/mL) of anti-HBs were detected in 184 (48.7%) children. There was a signifincant difference in the percentage of children with protective anti-HBs between age groups: 63.0% for 5-year-olds, 61.2% for 6-year-olds, 58.9% for 7-year-olds, 45.0% for 8-year-olds, 35.5% for 9-year-olds, and 25.0% for 10-year-olds ($P < 0.001$; Table 4). This decrease is primarily due to the rapid decrease in children living in ger areas ($p < 0.001$) compared to children living in apartment areas ($p = 0.152$).

Two children with incomplete vaccination records were excluded from the analysis of seroprotection rate due to vaccination. Of the remaining 376 children without HBV infection, 23.1% received two doses of the vaccine and 76.9% received three doses (Table 5).

Table 1. Study population characteristics ($n = 438$)

		n	%
Gender			
	Male	222	50.7
	Female	216	49.3
Age in years	5	79	18.0
	6	73	16.7
	7	75	17.1
	8	70	16.0
	9	70	16.0
	10	71	16.2
District	Songinokhairkhan	146	33.3
	Chingeltei	147	33.6
	Baganuur	145	33.1
Residential area	Apartment	220	50.2
	Ger	218	49.8
Activity of child	At home	23	5.3
	At kindergarten	105	24.0
	At school	310	70.8

Table 2. Household socioeconomic characteristics

		Apartment ($n = 220$)		Ger area ($n = 218$)		*p*-value*
		n	(%)	*n*	(%)	
Parental educational years						
Mother:	≥ 10 years	158	72.1	62	28.8	< 0.001
	< 10 years	61	27.9	153	71.2	
Father:	≥ 10 years	121	59.9	30	16.2	< 0.001
	< 10 years	81	40.1	155	83.8	
Parental occupation						
Mother:	working	161	73.5	122	56.7	< 0.001
	not working	58	26.5	93	43.3	
Father:	working	185	91.6	142	75.9	< 0.001
	not working	17	8.4	45	24.1	
Monthly family income (togrog[a])						
	≤ 70,000	30	14.1	104	49.3	< 0.001
	70,001 - 100,000	53	24.9	79	37.4	
	100,001 - 250,000	90	42.3	24	11.4	
	> 250,000	40	18.8	4	1.9	

* By chi-square test; [a] 1000 togrog is equivalent to approximately US$1.

Table 3. Prevalence of HBsAg and anti-HBc

	Total *n* (%)	HBsAg *n* (%)	Anti-HBc *n* (%)
Total	438 (100)	5 (1.1)	58 (13.2)
Gender:			
Male	222 (50.7)	5 (2.3)	25 (11.3)
Female	216 (49.3)	0 (0)	33 (15.3)
Age groups:			
Preschool-age (5-6)	152 (34.7)	0 (0)	12 (7.9)*
School-age (7-10)	286 (65.3)	5 (1.7)	46 (16.1)
Number of received vaccine[a]:			
2 doses	99 (22.7)	1 (1.0)	12 (12.1)
3 doses	377 (77.3)	4 (1.2)	46 (13.6)
Residential area			
Apartment	220 (50.2)	3 (1.4)	28 (12.7)
Ger	218 (49.8)	2 (0.9)	30 (13.8)

* $p < 0.05$ by chi-square test; [a] 2 children were excluded due to incomplete vaccination records.

Table 4. Distribution of anti-HBs titers in children living in apartment and ger area by age ($n = 378$)

Age in years	Anti-HBs titer	Anti-HBs		Anti-HBs (+)	
	Median (IQR 25% - 75%)	(–)[a] *n* (%)	(+)[b] *n* (%)	Apartment area *n* (%)	Ger area *n* (%)
Total	8 (3.0 - 37.0)	194/378 (51.3)	184/378 (48.7)	103/191(53.9)	81/187 (43.3)
5	15 (4.5 - 44.5)	27 (37.0)	46 (63.0)	26 (72.2)	20 (54.1)
6	14 (3.0 - 55.0)	26 (38.8)	41 (61.2)	18 (56.2)	23 (65.7)
7	18 (6.0 - 42.7)	23 (41.1)	33 (58.9)	16 (57.1)	17 (60.7)
8	7 (2.2 - 34.5)	33 (55.0)	27 (45.0)	15 (48.4)	12 (41.4)
9	4 (1.7 - 27.0)	40 (64.5)	22 (35.5)	14 (43.7)	8 (26.7)
10	4 (1.2 - 10.2)	45 (75.0)	15 (25.0)	14 (43.7)	1 (3.6)
p-value	0.01†	<0.001*	<0.001*	NS	<0.001*

IQR: interquartile range; † By linear regression analysis; * By chi-square test; [a] Anti-HBs<10 mIU/mL; [b] Anti-HBs ≥ 10 mIU/mL.

Table 5. Distribution of anti-HBs among children depend on the number of received doses of hepatitis B vaccine

		Total ($n = 376$[a])		*p*-value*
		2 doses *n* (%)	3 doses *n* (%)	
Total		87 (23.1%)	289 (76.9%)	
Age in years:				
	5	1 (1.4)	71 (98.6)	< 0.001
	6	0 (0)	67 (100)	
	7	1 (1.8)	55 (98.2)	
	8	10 (16.7)	50 (83.3)	
	9	36 (58.1)	26 (41.9)	
	10	39 (66.1)	20 (33.9)	
Anti-HBs:				
Total	(–)[b]	66 (75.9)	126 (43.6)	< 0.001
	(+)[c]	21 (24.1)	163 (56.4)	
9 year-olds	(–)	28 (77.8)	12 (46.2)	0.01
	(+)	8 (22.2)	14 (53.8)	
10 year-olds	(–)	32 (82.1)	12 (60.0)	< 0.05
	(+)	7 (17.9)	8 (40.0)	
Apartment area	(–)	26 (65.0)	61 (40.7)	< 0.01
	(+)	14 (35.0)	89 (59.3)	
Ger area	(–)	40 (85.1)	65 (46.8)	< 0.001
	(+)	7 (14.9)	74 (53.2)	

* By chi-square test; [a] 2 children were excluded because of incomplete vaccination records; [b] Anti-HBs < 10 mIU/mL; [c] Anti-HBs ≥ 10 mIU/mL.

There was a significant difference in seroprotection rate (anti-HBs) between children that received two doses (24.1%) and three doses (56.4%) of the vaccine ($p < 0.001$; Table 5). More than half of the children aged nine and ten received only two doses of the vaccine, due to the two-dose policy in effect at the time they were receiving their vaccinations. The protective anti-HBs were detected in a significantly higher proportion of 9-year-olds that received three doses (53.8%) of the vaccine compared to 9-year-olds that received two doses (22.2%; $p = 0.01$; Table 5), and a similar difference was found in 10 year-olds: 17.9% for two doses and 40% for three doses ($p < 0.05$; Table 5). In addition, this difference in protective anti-HBs due to the number of received doses was the same, regardless of living in an apartment area ($p < 0.01$) or in a ger area (< 0.001; Table 5).

Of 58 children with evidence of past infection (anti-HBc positive), 23 (39.7%) had protective titres of anti-HBs, and 35 (60.3%) had non-protective titres of anti-HBs (Table 6). The prevalence of potential carriers (positive for anti-HBc, but without protective anti-HBS) was 8% (35/438), and of the suspected 35 carriers, 23 (65.7%) children lived in an apartment area (Table 6). Based on interviews of 434 children, 261 (60.1%) had at least one exposure to a risk factor for HBV infection in the past, such as having a blood transfusion (1.6%), operation (6.2%), intravenous injection (28.9%) or dental manipulation (44.4%; Table 6). In particular, children living in apartment areas were significantly more likely to be exposed to intravenious

Table 6. Comparison of positive cases of anti-HBc and the risk factors between residential areas

	Total *n* (%)	Apartment *n* (%)	Ger *n* (%)	*p*-value*
Past infected cases:	58/438 (13.2)	28 (48.3)	30 (51.7)	0.001
Anti-HBc (+) and anti-HBs (–)	35/58 (60.3)	23 (65.7)	12 (34.3)	< 0.01
Anti-HBc (+) and anti-HBs (+)	23/58 (39.7)	5 (21.7)	18 (78.3)	
Existence of past exposure:	261/434 (60.1)	151 (57.9)	110 (42.1)	< 0.001
Blood transfusion	7/438 (1.6)	3 (42.9)	4 (57.1)	NS
Operation	27/436 (6.2)	15 (55.6)	12 (44.6)	NS
Intravenous injection	126/436 (28.9)	76 (60.3)	50 (39.7)	< 0.01
Dental manipulation	193/435 (44.4)	114 (59.1)	79 (40.9)	0.001
Sharing toothbrush	50/435 (11.5)	12 (24.0)	38 (76.0)	< 0.001
With family history of liver diseases	180/438 (41.1)	101 (56.1)	79 (43.9)	< 0.05
Practiced injection at home	155/436 (35.6)	95 (61.3)	60 (38.7)	0.001

* By chi-square test.

injection ($p < 0.01$) or dental manipulation ($p = 0.001$) than children living in ger areas. In addition, 41.1% (180/438) of children had family members with a history of liver disease, with a higher proportion (56.1%) found in apartment children compared to ger children (43.9%; $p < 0.05$; Table 6). Also, 35.6% (155/436) lived in families which practiced self injection at home, with 61.3% living in apartment areas and 38.7% in ger areas ($p = 0.001$). There was significant difference ($p < 0.01$) in the history of liver disease between families which practiced injection at home (77/155; 49.7%) compared to families which had never practiced home injection (102; 36.3%).

Discussion

As far as we know, this is the first study designed to determine the long-term persistence of seroprotection induced by hepatitis B vaccination among children aged 5-10 who were immunized as infants in Mongolia. This study revealed that only 48.7% of children had seroprotective antibody levels in connection with hepatitis B vaccination administered 5-10 years earlier. This was higher than a previous study among the immunized 0-7 year olds (39.7%) in Ulaanbaatar in 1998-1999 (*21*). However, one of our important findings was that the seroprotective rate markedly declined with age so that as time after vaccination increases, the seroprotection rate decreases. The protective anti-HBs were absent in more than half of children at the age of 8, and only 25% of children had seroprotective antibodies at the age of 10. Moreover, the decline occurred earlier than in other countries. Among 10-years-olds who received three doses of the vaccine, 40% had seroprotective antibodies, which was lower than the findings of a study conducted in Taiwan (a hyperendemic country), where a 50% seroprotection rate was found among 13-15 year-old children (*11*).

A possible reason for the earlier decline of seroprotection was the number of doses received by the children. We found that children vaccinated with three doses had higher titres of seroprotective antibodies than children vaccinated with two doses. The importance of a third dose for protective anti-HBs was also confirmed by comparisons within the 9-year-old and 10-year-old age groups. The third dose of the vaccine in the infant immunization schedule produces a large and rapid rise in antibody titres and therefore, might be considered as a booster dose (*13,22*).

This study reported that children living in ger areas were more likely to lose seroprotection than children living in apartment areas. The result is similar to a study among two-year-old Mongolian children comparing urban (Ulaanbaatar) and seminomadic rural areas (*7*) reporting that rural children had lower seroprotection than urban children. Although our study was carried out only in Ulaanbaatar, infrastructures of ger areas are similar to seminomadic settings and the people are probably living in relatively low socioeconomic conditions, including poor nutrition. Our study reported that people living in ger areas had characteristics associated with low socioeconomic status such as low education level, employment rate and income. In the city, vaccines are distributed from the National Center for Communicable Diseases (NCCD) to each district once a month, to health centers located in both apartment and ger areas monthly, and are stored by the health centers in a refrigerator. Even though there were different formulations of the hepatitis B vaccine, such as Hepavax B, Hepavax Gene, and Engerix B, in use at the time when our study children were born, all children in this study were immunized with the same vaccine in a given month, regardless of living area. None of the children received the hepatitis B vaccine through mobile services. In addition, many studies have shown that high socioeconomic status positively affects health status, morbidity, and mortality (*23,24*). Thus, we suppose that low socioeconomic status and poor nutrition might affect the persistence of anti-HBs induced by the vaccine.

Although hepatitis B is usually minimally symptomatic in early childhood, the carrier state (the infectious reservoir for hepatitis B) is likely to occur if the infection is acquired at a young age. Our finding of only 1.1% HBsAg seroprevalence shows the substantial improvement in the reduction of HBV infection from

the previous prevalence rate of 2.6% among 0-7 aged immunized children in 1998-1999 (*21*) and 10% among the healthy adult population in 2002 (*6*). This would be attributable to two major interventions in the Mongolian health program that began in 1991: the introduction of the hepatitis B vaccine to the national immunzation program and an end to the practice of reusing needles for injection. Currently, all such needles are sterile, individually packaged and disposable (*25*). Also, our finding that none of the 5-year-olds had HBsAg indicates a step towards achieving the Western Pacific Regional goal of reducing HBsAg seroprevalence to less than 1% in 5-year-old children immunized with the hepatitis B vaccine (*3,4*).

The prevalence of current infection or the carrier state (HBsAg) decreased, however, past infection (anti-HBc) prevalence remained high. A previous study conducted in Mongolia between 1998 and 1999 revealed that the positive anti-HBc rate was 2.6% among 2-year-olds (*7*) while this study found anti-HBc positive rates of 7.9% in preschool-age children and 16.1% in school-age children. Although these data were derived from different study subjects, the risk of HBV infection appears to increase with age. Moreover, WHO defines children who have positive anti-HBc and negative anti-HBs as low-level HBV carriers with undetectable HBsAg (*26*). In this study, the prevalence of anti-HBc without anti-HBs was 8%, which was higher than the prevalence reported from a similar study in Taiwan (1.2%) (*11*). The above findings suggest that Mongolian children were exposed more often to HBV. Children in apartment areas, with comparatively better socioeconomic status, had a higher proportion of anti-HBc without anti-HBs, indicating that they had more exposure to risk factors for blood-borne pathogens than children in ger areas. We speculate that it might be related to frequent dental manipulation, intravenous and unsafe injections, and exposure to infected family members.

In developing countries, most injections are unnecessarily given for nonspecific symptoms such as colds, fatigue, dizziness, diarrhea, abdominal pain, and fever, although oral alternative medicines could be used (*24,27*). This also occurres in Mongolia. The practice of home injection in our study included 35% of the families, which was similar to the findings of a previous study (*27*) where 32% of the Mongolian general population living near 20 health facilities occasionally administered injections themselves to relatives at home. Used injection devices were disposed with the home garbage, leading to an increased risk of spreading blood-borne pathogens through unsafe injections and medical waste. Our finding of a higher prevalence of home injections in apartment families might also cause a higher prevalence of liver diseases. A current study in Mongolia reported that hepatitis B accounted for more than one-third of several types of hepatitis (*28*). Thus, family members with a positive history of liver disease can be a source of HBV infection to children.

We also revealed that a higher proportion of children in ger areas were sharing toothbrushes with family members, classmates and playmates. This may also be a mode of transmission of the virus through contact with mucous membranes or open skin breaks, since HBV can survive for at least one week on inanimate surfaces (*29*).

Conclusion

This study has shown that seroprotection induced by hepatitis B vaccination decreases earlier in Mongolian children compared to other hyperendemic countries and that a third dose is important for preserving the efficacy of the vaccine. Children living in poorer areas are more likely to lose the seroprotection, probably due to low socioeconomic status and poor nutrition. However, children living in areas with higher socioeconomic status had more exposure to blood-borne pathogens because of inappropriate health-seeking behaviors such as home injections, frequent dental manipulations and intravenous injections. This study recommends emphasizing the use of disposable medical supplies and appropriate disposal systems, the sterilization of reusable supplies and equipment, and strict adherence to and enforcement of safe and standard medical practices. In addition, community-based education highlighting various modes of transmission and prevention of HBV and the risk factors for blood-borne pathogens is needed for the general population in Mongolia. Finally, further prospective studies are needed to determine the duration of protection, and the necessity and timing of booster doses.

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Original Article

The Health Management Information System of Pakistan under devolution: Health managers' perceptions

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Summary **Devolution implies that use of data for decision making starts at the level of data generation. However under a newly decentralized system, managers may face different hurdles in utilizing the preexisting Health Management Information System (HMIS). This qualitative research explores the perceptions of health managers regarding HMIS under the devolution reforms enacted in 2001 in Pakistan. The study was carried out by interviewing 26 managers at various levels in seven selected districts in all provinces. There was general dissatisfaction and confusion over roles and responsibility: respondents reported that the overall atmosphere was characterized by the reluctance of provincial managers to release data under their authority, the absence of prerequisite human resources, and conflicts of interests between political and administrative leadership. The devolution didn't bring immediate good effects for the HMIS. Treated as a least priority area, staff was distributed from provincial HMIS cells, causing overburdening of remaining staff and jeopardizing data analysis. Reporting regularity from the districts was also compromised secondary to political interference and loss of provincial control. The present HMIS is in need of redesigning so that it may keep pace with the devolved system. The HMIS reforms are needed to improve information systems at the district level, capacity building of district managers, political commitment, and administrative ownership of the system and to earmark and make available resource and promote evidence-based decision making. Change in the public administration culture towards encouraging initiative taking at lower levels, introduction of performance incentives, inculcating work ethics, encouraging local accountability, and good governance are all essential.**

Keywords: HMIS, Health managers, Devolution, Pakistan

Introduction

Information plays a vital role in effective management of any system. The Health Management Information System (HMIS) provides specific information support to the decision makers at various levels of the health system to assist evidence-based decision making.

Under the devolution initiative, Pakistan's Ministry of Health (MOH) has recommended strengthening of health information systems for informed decision making in planning, management, monitoring, and supervision of health services for improved service delivery in the districts (*1*). However, attempts to strengthen information systems have generally proved unfruitful and at times counter-productive (*2*). One reason is that is stakeholders' perceptions have been ignored (*3*).

HMIS and Devolution – The experience in Pakistans' context: Before the 90s, Pakistan had several vertical programs with stand-alone information systems. The resulting fragmented data transmission made it difficult for managers to assess program effectiveness (*4*). In 1991-92, MOH transformed those vertical information systems into a comprehensive National Health Management Information System through a consultative process (*5*). The national feedback reports of the new HMIS pointed out that albeit gradual, there

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has been improvement in the scope and regularity of reporting, improving the quality of information and encouraging information use at various supervisory levels (*4,6,7*). In view of provincial managers' growing concerns about the HMIS, MOH held a series of workshops through which vertical information systems were found still to exist, together with a culture of non-evidence-based decision making as indicated by planning and management decisions which most of the time disregarded relevant information (*8,9*).

As opposed to typical centralized information systems which draw essentially upon data collection and morbidity and mortality profiles, the HMIS is understood primarily to enable field level managers to carry out information based decision making (*10*). This function of HMIS is plausible under a devolved system.

To address political, social, economic, demographic and epidemiological needs, Pakistan launched health care reforms in 2001 (*11*). Since promulgation of these devolution reforms, the new District Health System has faced crucial challenges stemming from a dearth of basic information such as data on population, health status, disease patterns, and coverage of essential health care, *etc*., which are fundamental requisites for planning and setting priorities, targets, and objectives for health and health care. This is coupled with an absence of adequate resources, logistics, organizational arrangements, and incentives to ensure prompt implementation of any program (*12*).

Though the literature suggests that devolution (or decentralization) is required for decision making at the lower management level and HMIS *per se* is meant to serve the needs of lower level managers, there may yet be various impediments in a decentralized system to the realization of evidence-based decision making. The devolution process as an organizational change may have a mismatch with the preexisting information system.

Decentralization implies that whoever collects the data, analyzes it: *i.e*. analysis and self-assessment are to be carried out at the level where data are collected and used for decision making at that level and not collected merely for upward reporting (*13*).

With decentralization, local decision-makers gain new responsibilities for planning and resource allocation and hence require additional skills (*14*). If skill development is ignored, the process of decentralization is likely to fail (*15*).

Decentralization has caused major problems for health and health care (*16*). Aas, for example, mentions several problems that might follow decentralization, such as lack of organizational control and co-ordination, deterioration of competency due to isolation, limited ability to release creativity, and conflict resulting from an unclear division of authority or simply the pursuit of personal ambition (*17*). All these factors have the same implications for information systems and information-based decision-making under decentralization.

Another dimension of decentralization is community involvement. A fundamental principle of the Alma-Ata Declaration was that individuals and communities need to be involved in the generation, dissemination, and use of health information for planning and implementation of health care (*18*). Research suggests that the effect of decentralization on local health system performance is more influenced sometimes positively, sometimes negatively, by the local political culture than by resources from central government (*19*). Hence use of information in decision-making may be encumbered by a political culture with its own tradition of decision making.

This study was conducted to explore the perceptions of health managers regarding HMIS, within their organizational setting and in the context of Pakistan's decentralization process. Little, if any, work has been done on this important aspect of HMIS in Pakistan, particularly under the new devolution initiative.

Materials and Methods

Since the purpose of the study was exploring (understanding, describing, and explaining rather than measuring) the perceptions of managers, a qualitative design was adopted. Patton notes that qualitative approaches emphasize the importance of getting close to the people and situations being studied (*20*).

In-depth, face to face, semi-structured interviews were conducted at the federal, the four provincial, the district, and the local health facility levels. Overall, twenty-six managers were interviewed. Data analysis was done at the level of statements, meanings, themes, and general descriptions of experiences (*21*).

Results

The context: perceptions about the devolved system

There is little awareness and more than sufficient confusion over the new system's status: to some respondents, the devolution was in a state of transition, others perceived it to be static at a certain point, leading nowhere, like ‘a hanging object’. Another respondent expressed his confusion by describing devolution as a ‘hodgepodge’. Others considered the need to bring devolution of power to the union council (sub-district) level.

Despite having the authority, districts lack the apparatus and skilled human resource to manage their affairs. Inertia was cited as another reason for the poor state of affairs; owing to centralization in the past, the staff is habituated and comfortable with working in a centralized system, while at the higher echelons of management at the provincial level, managers seems unwilling to release power from their grasp.

As stated by one respondent:

Apparently, I think, we are reluctant to yield power to district managers. [A provincial level health manager]

To some respondents, one of the prerequisites of appropriate implementation of devolution is the existence of uniform development in all areas, while in reality there is great disparity in development terms among districts, as some are at 'a higher level of development and others at a humble level'. Hence devolution was viewed as beneficial wherever there was skilled manpower, infrastructure, and intellect. But the real problem resides in underdeveloped areas which lack sources and resources.

The new district setup has caused deterioration of many systems... All authority is now in the hands of the District Coordination Officer (D.C.O.)[a]*. Nazims*[b] *are also being given authority. [A Nazim] has enormous authority but has no idea of what this means; having come from the business sector, [Nazims] will never let down their business. When requested to join hands in some noble cause (e.g. health care), they expect some personal benefit in return. [A facility level health manager]*

This self-interest also takes the form of bribes:

In the past, whatever task used to be accomplished by giving [bribing] around one hundred to hundred and fifty rupees is now done by giving a thousand or more… there used to be ten people around who took bribes but now there is a huge list of takers. [A district level health manager]

Respondents were quite concerned about political interference. It was pointed out that political influence after devolution has focused upon the Executive District Officer - Health (EDO).

HMIS and devolution: perceived benefits

Respondents were in agreement that both HMIS and devolution reforms may conceptually reinforce each other.

One respondent mentioned the merit of consolidation:

Now there are no multiple chains of communication to move upward and/or downward. The more there are steps of HMIS data transmission involved, greater the chances of errors. But now it is a single-step process. [A district level health manager]

Rationally HMIS was conceived to support district health management and become an essential tool in the devolved system by providing information support to the managers in their very surroundings, enabling them to tally the information with the real scenario and thereby to monitor the data generation process as well.

HMIS under devolution was perceived to work with more efficiency, as districts were given authority to make decisions on an '*as is-where is*' basis instead of reporting to the higher authorities and waiting for feedback, creating delays.

Referring to equity, the respondents perceived that under devolution there will be more chances for the district government to rearrange resources and focus on the respective diverse needs of each facility, since at the lower levels of district management, the needs and problems vary.

The HMIS under the devolution reforms was thought to bring more effectiveness to management as well. With the involvement of various stakeholders the resources used through a team work were perceived to entail more potential for effective management in terms of quick action on complaints, better control over absenteeism, regular assessment of staff performance, checks on pilferage, and scrutiny on the misuse of resources *etc.*

HMIS and devolution: perceived reality

Effect on organizational structure: In the pre-devolution period, the HMIS was controlled at divisional levels and later after the dissolution of divisions, at the provincial level. Subsequent to devolution, a gross reduction in HMIS cells at the provincial offices was observed. For example, in some areas, the regular post of Deputy Director HMIS was abolished during devolution reforms. A time came when there was not a single Deputy Director left to look after the HMIS section and it *'was done away with'* by assigning it as an additional responsibility to one of the many other Deputy Directors in the health department *'against the wishes of the individuals concerned'*. It was reported that certain provincial cells experienced a severe shortage of human resource due to retrenchment of staff, jeopardizing their reporting and data analysis work as a result. The HMIS organization was weakened at the provincial level by shifting power to the district level. Provincial offices had little awareness of the plans to strengthen the HMIS at the district level. To respondents, these reductions happened because *'the implementers at the local level perhaps did not know that information is the backbone of the health department'*.

Effect on communication: Respondents mentioned that communication declined after devolution at various levels of HMIS organization. Regular monthly HMIS meetings, held in the past, were discontinued in some provinces.

One respondent complained:

If facilities complain of absence of feedback, they are unaware that after devolution there is no one to analyze their reports and extend feedback, and at the

[a] District coordination officer: Bureaucratic representative at the district level government;

[b] Politically elected representative & head of house, at the district level of government.

district level, the situation is worse. There is only one officer, without any support staff. [A provincial level health manager]

Effect on decision-making: Prior to devolution, the District Health Officer-DHO had discretionary powers of budget utilization. Now that the Nazims are local government district heads and the public sector administration is headed by the DCO, the EDO-Health has no budget utilization powers. The system can run smoothly only if the DCO and Nazim are on good terms. The share of the health department due out of the district budget can only be allocated on the discretion of the DCO coupled with the consent of the Nazims.

As was mentioned by a respondent:

The DCO enjoys the overall discretion to incur expenditure in the areas deemed 'profitable' by him (health and education are exempted, as these do not generate cash). He has no background knowledge of health. [A district level health manager]

Another complained:

There hasn't been any criterion for budgeting. In spite of having populations similar in number, districts received funds that were poles apart. Certain facilities haven't received a single penny for even six months. These are the personal contacts, the level of awareness of the DCO, and the individual priorities that influence the budgetary allocation. [A district level health manager]

Reporting: In the previous centralized system, there were HMIS cells at provincial levels that received and gave feedback to the small number of districts within their jurisdictions. This was much more efficient for proper reporting. Now the districts are free to send reports or not as they wish, and the DHO blithely checks the timely submission of error-free reports, nothing else.

HMIS under devolution: the perceived hurdles

Capacity issues: The HMIS is teamwork dependent and requires a blend of many systems and skilled human resources, such as demographers, public health professionals, statisticians, epidemiologists, *etc*., in every district. However, in a developing country setting, the capacity to use and analyze data is scarce, due to the scarcity of human resources and finances.

Lack of accountability: Respondents commented that since the districts were currently free of central-level influence and authority, the districts might stop obliging the requests of national HMIS cells for submission of reports, which, according to some respondents, had already been observed in some districts.

Before devolution we were receiving reports on a regular basis but now the numbers of districts that are sending reports regularly has been drastically decreased. [A provincial level health manager]

Indifferent attitude of EDO-Health: In the new devolved system, the role of EDO-Health is very crucial but, according to respondents, the most worrisome issue is that for many EDOs, HMIS is not on the priority list. Respondents opined that it was because they were either not dynamic, or unaware, or they needed motivation.

Political interference: Respondents noted that, in the past, funds for facilities that failed to submit HMIS reports to the central level in due time were withheld. Recently that system is now being interfered by the Nazims, who to oblige their vote banks extend undue favors by issuing orders for release of salaries to individuals they wish to influence. This has resulted in an 80 to 90% decline in reporting regularity in certain districts after devolution. In addition, in the past, the EDO-Health practiced full authority in his sphere, but now administrative authority rests with DCO while the Nazim enjoys political authority, and this situation has influenced HMIS based decisions and activities on merit.

Privatization and control of NGOs: Some respondents felt that devolution was the beginning of privatization. One respondent shared his suspicions:

I think this is a trap advocated by NGOs. They have already bought some public health facilities and God knows whether they will share the data of those facilities with us or not [A provincial level health manager]

Ambiguity of power: Inherent in the concept of information-based decision making is the idea of power to make decisions for implementation and these two cannot be separated. So far in the devolution process, the issue of decision-making authority and control were reportedly unclear at both the provincial and the district government levels.

Respondents' suggestions for improving HMIS under devolution

Training of civil servants in the use of data: The District Management Group officers, having only a bureaucratic background but with a key role in decision making at the district level as DCOs, should also be trained and sensitized on HMIS and its significance in administration matters and decision making.

Interaction with health management teams: There is a need to appraise the DCOs and Nazims of the usefulness of HMIS in their ambits. It was suggested that HMIS capacity building and data use should begin from the facility and extend to the district level.

Creating a state of healthy competition between facilities: Some respondents argued that the performance of facilities could be compared on the basis of the HMIS, which might ultimately serve as a tool of healthy competition among health managers of various facilities. This competition might be extended to various constituencies.

Sharing budget allocation information: Few of the respondents opined that information sharing about finances would be more beneficial in terms of health management and information-based decision-making. The union council Nazim, the doctor, the vaccinator- all these people should be thoroughly conversant in the budget allocations of their union councils *etc.*

However small the share of the national budget being provided to health, even that does not trickle down to the masses because of misappropriations and bureaucratic delays: the solution is to link the HMIS to finances. The budget arrives and stays lying at the district office, and the BHU doctor is ignorant of his budget allocation. [A district level health manager]

Technical assistance by the provinces: There should be a task force at the provincial level which should come forward to assist district management in technical matters as such as HMIS training of human resources, as well as technical and management issues.

Dissemination of information at the community level: It was proposed that female representatives such as Lady Health Workers (LHW), as they are in direct contact with the community, should be involved in the dissemination of information generated through the HMIS at the community level. This will not only help in creating health issues awareness but will also inform people about the types and range of services available at facilities.

They (female representatives) should share the data at the grass roots level. They may reflect upon utilization of services and inform the masses as to what health services are available. They may also promote prevention by informing opinion leaders and the masses about common diseases prevalent in the community as reported by the HMIS and encouraging them to take preventive measures. [A facility level health manager]

Discussion

The initiative of devolution of power by the Government of Pakistan to district governments is facing numerous challenges interrelated with health systems and the HMIS.

Devolution did not bring immediate positive changes to the existing HMIS; rather it was associated with loss of certain achievements gained over time, such as established provincial HMIS cells and reporting regularity. Provincial HMIS offices have also faced certain setbacks. It is evident from the responses that no well-thought-out interim plan existed for the HMIS functioning or, had it been conceived, it was not implemented during replacement of the old system.

Jean Gladwin *et al.*, while investigating the effect of health services' decentralization on health information management in low-income countries, proposed that in order to improve information management under decentralization, existing management practices need to be related with the new tools of information management and to draw on existing experience and research in the introduction of HMIS (*22*).

The situation calls for HMIS reforms to improve the information systems at the district level by establishing a system to generate district-level data, capacity building of district managers to use this data, and political commitment and administrative ownership of the system to earmark and make resources available and promote evidence-based decision making. Last but not the least, giving performance-based incentives, inculcating work ethics, encouraging local accountability, and good governance are indispensable.

Enabling the field-level health managers to carry out information-based decision-making necessitates change in the public administration culture as well, where it might encourage staff at lower echelons to make decisions, create changes, and take initiatives to improve the health care system. Otherwise none of the attention paid to information will be fruitful (*23*).

As mentioned in the literature (*24*), the present HMIS is in need of redesign to enable it to keep pace with the devolved system. In the newly decentralized system, the existing information system may also need modifications to enable managers to use the information to develop their operational and financial plans and thus make reforms at the local level according to the needs of the community for effective and efficient service provision.

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Original Article

Secular trends towards delayed onsets of pathologies and prolonged longevities in Japanese patients with Werner syndrome

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Summary **Recent cases of increasingly elderly Werner Syndrome (WS) patients have paralleled increased lifespan in the general population, however, historical temporal lifespan variations in WS have not yet been ascertained. To assess temporal changes in life-span and progeroid comorbidity in WS, all Japanese WS patients documented from 1966-2004 were analyzed for age at onset of diabetes mellitus (DM), malignancy, and death. Of 1,019 WS analyzed, average age significantly increased for all variables studied over the study period. Average age of onset of malignancy and DM in all WS increased from 35.8 to 48.8 years and from 34.9 to 39.7 years, respectively ($p < 0.001$), while age at death increased from 38.2 to 52.8 years ($p < 0.001$), *vs.* 71.7 to 82.7 years ($p < 0.001$) in the general population. Lifespan increases in WS and the general population suggest a common environmental influence. Unlike the general population, no gender-specific difference in life-span occurred in WS, suggesting a gender-specific differential environmental effect on mutated WRN. Identification of factors responsible for age differences could facilitate improvement in survival and ageing phenotypes of WS patients and the general population.**

Keywords: Diabetes mellitus, Environment, Genetics, Longevity, Werner syndrome

Introduction

Werner syndrome (WS: MIM#27770) is caused by autosomally-recessive inheritance of a mutated RecQ3 DNA/RNA helicase gene (WRN) and is characterized by a variety of clinical manifestations which mimic features of advanced ageing (*1*). Patients with WS usually develop normally early on, but experience premature termination of the teenage growth spurt. This is commonly followed by hierarchical deterioration of a variety of connective tissue systems resulting in physical symptoms such as gray hair, alopecia, skin atrophy, skin sclerosis, skin hyper/hypo-pigmentation, vocal cord atrophy, osteoporosis, sarcopenia, bilateral cataracts, metastatic subcutaneous calcification, and atherosclerosis. Other systems adversely affected include the endocrine system, resulting in type II diabetes mellitus (DM), hypogonadism, and thyroid disorders; the metabolic system, resulting in hyperlipidaemia, hyperuricaemia and hyaluronuria, and malignancy (particularly sarcomas); and to lesser degrees the immune system, resulting in excessive auto-antibody production, defective cytokine responses and natural killer cell activity; and the nervous system, resulting in cognitive disorders and brain atrophy (*2*,*3*). Mutation of the WRN helicase, therefore, primarily affects mitotic rather than post-mitotic cell systems. Death due to malignancy or atherosclerosis-related conditions such as myocardial infarction typically occurs in the late 40's (*4*,*5*).

Since the first description of WS by the German family physician Otto Werner in 1904 (*6*), other WS case reports have accumulated worldwide (*7*). Interestingly, the majority (~75%) of WS patients are of Japanese descent (*8*), likely due to the relatively high frequency of consanguineous marriage in rural areas and an extremely high prevalence (1:100) of heterozygosity in the general Japanese population (*9*). Approximately 10 WS patients per year are documented in Japan, while only 3.3 patients

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per year are reported outside the country.

Recently, numbers of more elderly WS patients (over 60 years of age) have been trending up in Japan in parallel with increased longevity in the general Japanese population, indicating a possible common environmental link. To determine the extent of influence of environmental factors on the genotypic and phenotypic expression of WS, we reviewed all WS case reports published in Japan since 1966 and assessed whether the pattern of clinical manifestations had changed over time.

Materials and Methods

The first case of WS in Japan was reported in 1917 (*10*), the first death in 1966 (*11*), and the first association of WS with malignancy in 1968 (*12*). We analyzed the clinical manifestations of WS as described in all papers published between 1966 and 2004. WS publications were selected through a citation index (Igaku-Chuo-Zasshi) and bibliographies of each report were extensively examined for additional references. For comparison of Japanese WS patients with those outside Japan, searches were performed through PubMed. Care was taken to thoroughly identify patient family details, personal histories, authors, institutions and demographic characteristics to avoid the inclusion of duplicate patient data.

As most patients were diagnosed clinically with WS, diagnoses given by the original authors were carefully re-evaluated based on the presence of the following phenotypes: unusual body habitus, bilateral cataracts, skin sclerosis, painful corns, sarcopenia, metastatic subcutaneous calcification, skin ulcers, DM, and hyperlipidaemia (*8,13*). Of all cases analyzed, 77 patients reported after 1985 were confirmed as having WRN mutations after 1996, the year in which WRN was first identified (*14*). We selected age of diagnosis for clinically overt stage DM, age of diagnosis of the first malignancy, and age at death, as relatively reliable manifestations or outcomes of WS. Although early onset of cataracts is the hall mark of WS, we excluded this criterion from our study because accurate determination of when onset occurred could not be achieved due to differences in reporting criteria between case studies. Body weight, height and body mass index (BMI) as calculated by height and weight, were evaluated to determine environmental effects on metabolic syndrome, including DM (*15*). We further examined if longer life-span was linked with slower progeroid outcomes and if earlier onset of clinical symptoms was associated with a shorter life-span. Since most symptoms characteristic of WS usually overlap with those of natural ageing at an early stage of life, most patients, family members and even doctors, do not acknowledge presence of the disease before the age of 36.7 ± 10.1 years (*2*) even if additional family members are affected. Reliable records, particularly of early pathophysiological manifestations of WS, are therefore limited.

Data source

A total of 1,019 Japanese WS patients reported in 468 publications between 1966 and 2004 (> 99% of all documented patients during this time frame) were analyzed to confirm age differences in the onset of DM, malignancy, and age at death. Age of DM onset was collected from 430 patients; age of onset of the first malignancy from 345 patients; and age at death from 219 patients. To examine possible trends in BMI changes, body weight and height measurements were also determined. Data from the general population was obtained through the Annual J Health and Welfare Statistics Japan (Annual J Health and Welfare Statistics Japan, 2007 and website *http://www.mhlw.go.jp-toukei-youran-aramashi-ichiran.pdf*).

Statistical analysis

Using multiple regression analysis, we investigated temporal effects on the age of onset of DM, malignancy, and death in WS patients between 1966 and 2004, adjusting for patient sex as a confounding factor. The regression models were based on the following equation:

$$Y = b0 + b1(Year - 1985) + b2(Sex)$$

where Y represents the published patient age, Year represents the calendar year of the initial publications for each WS patient, and Sex is represented by zero (0) for males and one (1) for females. Estimation of regression coefficients are represented by b0, b1 and b2, where b0 corresponds to the intercept and is interpreted as the mean male age in 1985, the mid point of the study period; b1 expresses the age change per year, and b2 represents the effect of gender by the difference in age between males and females. Analyses of variance (ANOVA) were performed to confirm the fitness of the regression model. Statistical analyses were performed with the statistical package STATISTICA.

Results

A total of 1,019 cases documented in 468 Japanese articles published between 1966 and 2004 were included in the analysis. Using multiple regression models, temporal effects on the age of onset of DM, malignancy and death in WS patients were determined. Table 1 indicates regression coefficients with standard error, *p*-values and variance analyses. The ANOVA indicated that all models fit the data well.

In 1985 which was the midpoint of the study, the average age of onset of DM in WS patients was estimated to be 38.8 years for males and 3.07 years younger for females ($p = 0.002$) (Figure 1A). The

Table 1. Regression analyses for sex adjusted period effects on age of onset for DM, malignancy and age at death in the patients with Werner syndrome

		Estimated coefficient	Standard error	*p*-value
Diabetes mellitus (*N* = 430)				
Intercept		38.81	1.513	< 0.000001
Year-1985		0.128	0.048	0.00894
Sex		-3.074	0.970	0.00165
	ANOVA	F(2,427) = 8.4021	*p* = 0.00026	
Malignancy (*N* = 345)				
Intercept		43.37	0.836	< 0.000001
Year-1985		0.352	0.069	0.000001
Sex		-2.548	1.125	0.0243
	ANOVA	F(2,342) = 15.513	*p* = 0.00001	
Death (*N* = 219)				
Intercept		46.30	0.949	< 0.000001
Year-1985		0.384	0.082	0.000005
Sex		-1.705	1.394	0.223
	ANOVA	F(2,216) = 11.389	*p* = 0.00002	
Death in general Japanese population				
Intercept		74.21	0.090	< 0.000001
Year-1985		0.288	0.0056	< 0.000001
Sex		5.881	0.127	< 0.000001
	ANOVA	F(2,75) = 2391.7	*p* < 0.000001	

Sex: male = 0, female = 1

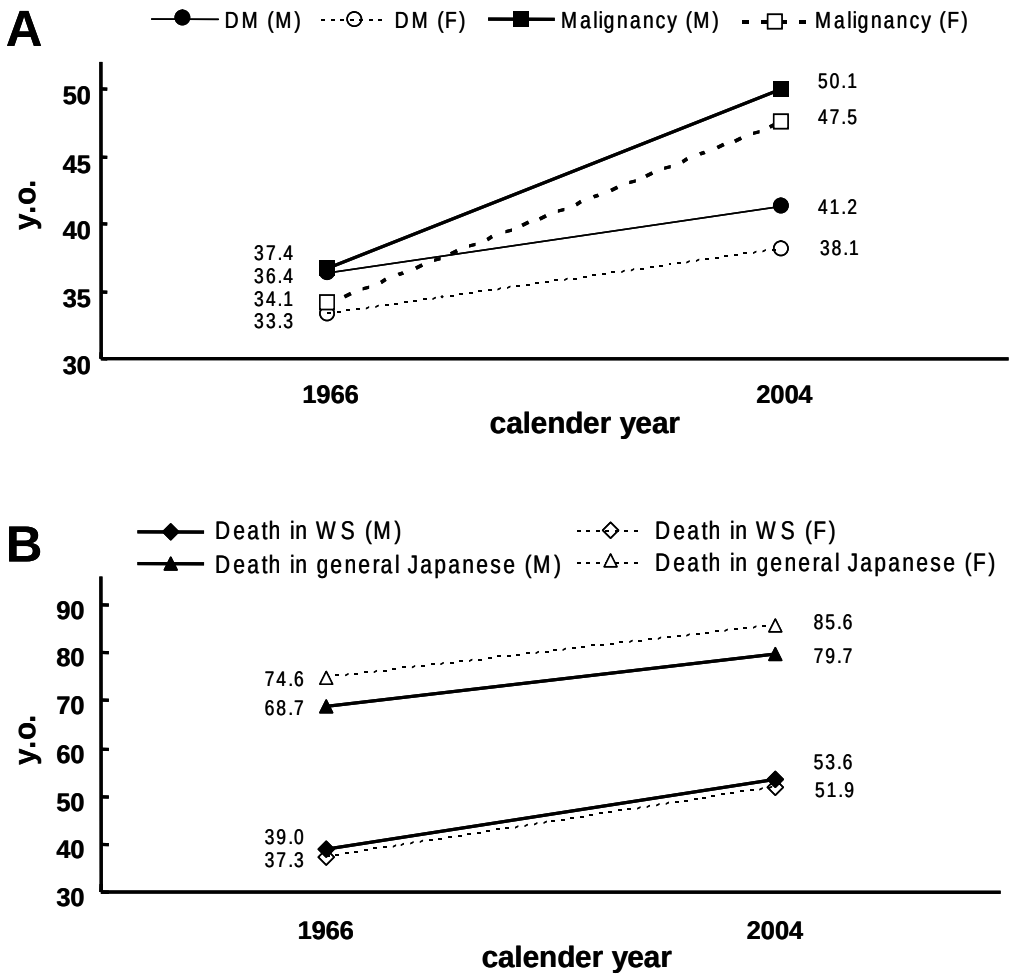

Figure 1. (A) Temporal trends of the age of onset of DM and malignancy, and (B) age at death in Japanese WS patients and the average life-span in the general population. Age of onset of DM and malignancy in male and female Japanese WS patients and age at death in WS patients and the general population are compared between 1966 and 2004.

incidence of DM in WS patients was ~70% for both sexes throughout the study period. A statistically significant increasing trend in the age of onset of DM in WS patients was observed in both males and females, with an estimated value of 0.128 years annually (p = 0.009). From the period of 1966 to 2004, therefore, the average age of DM onset in all WS patients increased by 4.8 years (from 34.9 years to 39.7 years).

Since BMI is an accurate indicator of DM in the general population, regression analyses of sex-adjusted temporal effects on body weight, height and BMI in WS patients were performed. Results in Table 2 show significant increases in body height ($p < 0.001$), weight ($p < 0.001$) and BMI ($p = 0.043$) in WS patients over time, in concert with the general Japanese population (Annual J Health and Welfare Statistics Japan, 2007 and website *http://www.mhlw.go.jp-toukei- youran-aramashi-ichiran.pdf*). Male WS patients, as with males in the general population, consistently surpassed female patients in body weight, height and BMI. However, BMI in WS patients in general is usually less than 18.1, and no significant difference in BMI was observed between patients with and without DM (data not shown). The estimated coefficients for DM were –0.61 (p = 0.405) for body weight, –0.20 (p = 0.782) for height, and –0.23 (p = 0.385) for BMI. The estimated coefficients of intercept, year and sex were almost identical between patients with and without DM.

With age of onset of the first malignancies, the regression curve patterns were similar to those of DM onset in regard to the annual age increase for both sexes throughout the analysis period, although the magnitude of the annual increase was larger (0.352 years annually, $p < 0.001$, Table 1) (Figure 1A). This amounts to a 13 year increase from the period of 1966 to 2004 (from 35.8 years to 48.8 years). Additionally, similar to DM, a gender difference in the age of onset of the first malignancies was noted, with a female onset age 2.548 years lower than that of the male average of 43.4 years at intercept (p = 0.024). The primary types of malignancies observed throughout the study period included a preponderance of rare tumors such as soft tissue sarcomas, malignant melanomas, thyroid carcinomas, meningiomas and hematologic disorders, as described (*4*).

A highly significant annual increase of 0.38 years in the age at death was seen in WS patients ($p < 0.001$), with average age at death of all patients increasing from 38.2 years to 52.8 years from 1966 to 2004. The increase was gender-independent, in sharp contrast to the general population, in which Japanese females live significantly longer than males (Annual J Health and Welfare Statistics Japan, 2007) (Figure 1B). From 1966 to 2004, the average life span of male WS patients increased from 39.0 years to 53.6 years, giving an average increase of the age at death of 14.6 years. In each year throughout the study period, the average life span of female WS patients was 1.705 years lower than that of males. For those with a molecular diagnosis of WRN, average age at death after 1985 was 50.7 years for males and 52.0 years for females, comparable to that reported by others (*16*). Fifty patients over the age of 50 with confirmed WRN mutations are still alive.

To compare results of WS patients with those of the general population, we examined age-adjusted temporal effects among the Japanese population during the same study period using National Statistics data (Annual J Health and Welfare Statistics Japan, 2007 and website *http://www.mhlw.go.jp-toukei-youran-aramashi-ichiran.pdf*). As seen in WS patients, a highly significant, though lower annual increase in the age at death in the general population was observed (0.288 years, $p < 0.001$, Table 1). From 1966 to 2004, average lifespan for both males and females in the general

Table 2. Regression analyses for sex adjusted period effects on body height, weight and BMI in the patients with Werner syndrome

		Estimated coefficient	Standard error	p-value
Height				
Intercept		152.01	0.479	< 0.000001
Year-1985		0.205	0.034	< 0.000001
Sex		-9.753	0.673	< 0.000001
	ANOVA	F(2,395) = 118.39	$p < 0.000001$	
Weight				
Intercept		40.97	0.483	< 0.000001
Year-1985		0.158	0.034	0.000044
Sex		-7.415	0.678	< 0.000001
	ANOVA	F(2,395) = 67.66	$p \leq 0.000001$	
BMI				
Intercept		17.63	0.176	< 0.000001
Year-1985		0.025	0.012	0.0426
Sex		-1.076	0.247	0.000017
	ANOVA	F(2,395) = 11.01	$p =$ 0.000022	

Sex: male = 0, female = 1

Table 3. Longevity of Werner syndrome patients

Reported year	Male			Female		
	Age at death	Cause of death	ID	Age at death	Cause of death	ID
1966-1985	70	AMI	WS61901			
1986-2004	79	malignancy	WS36101	69	still alive	unknown
	77	AMI	WS1801	63	AMI	WS9101
	69	AMI	WS1601	63	AMI	WS8101
	67	malignancy	WS1501	62	malignancy	WS15601
	67	malignancy	WS29901	61	malignancy	WS5701
	64	malignancy	WS1201	61	malignancy	WS1701
	63	AMI	unknown			
	63	malignancy	WS52301			
	63	malignancy	WS13201			
	62	malignancy	WS7801			
	62	malignancy	WS21701			
	62	malignancy	unkown			
	61	malignancy	WS23501			
	61	malignancy	WS23502			
	61	unkown	WS60901			
	61	unkown	WS60301			

AMI: acute myocardial infarction

population increased roughly ~11 years, from 71.7 years to 82.7 years.

Prior to 1985, no females and only one male WS patient aged over 60 were documented (Table 3) compared to 17 male and 6 female WS patients over 60 years after 1986. Strikingly, a 77-year-old male patient was diagnosed with WS by genetic analysis (mutation 4/4) (*2,17*) after 1986. Outside Japan, only one 62-year-old Irish male patient and one 63-year-old Dutch female patient were noted prior to 1985 (*18,19*), and only one 62-year-old Israeli female patient was reported after 1986 (*20*).

Two long-lived patients (WS1801, WS1501) were not diagnosed with WS prior to 35 years of age. In a few patients who had shorter life-spans of < 40 years, premature aging phenotypes typical of WS before age 10 were noted (data not shown). The percentage of WS patients suffering early death at < 40 years of age was 34.9% prior to 1985, and 13.3% after 1986 for both sexes. There was no significant difference between males and females in the frequency of early death.

Thus, although data is limited, there appears to be no correlation between delayed onset of WS-specific progeroid symptoms and a longer life-span or vice versa. The major causes of death in WS patients were malignancy (70%), atherosclerosis-related conditions such as myocardial infarction and cerebral infarction (20%), and infection (10%). The causes of death in WS patients were comparable in males and females prior to and after 1985 as described previously (*2*).

Discussion

There are several possible reasons why Japanese WS patients from both sexes similarly showed temporal-dependent delayed outcomes such as DM, malignancy and death. One explanation is that even in a genetically-defined disease such as WS, clinical phenotypes that mirror the natural ageing process in the general population may be strongly influenced by environmental/epigenetic factors in a similar fashion irrespective of sex. This is illustrated by the significant increase in longevity, body weight, height and BMI in WS patients from both sexes which was mirrored in the general population (Annual J Health and Welfare Statistics Japan, 2006 and website *http://www.mhlw.go.jp-toukei-youran-aramashi-ichiran.pdf*). With economic growth burgeoning in Japan from the mid 60's, developments in lifestyle, nutrition, hygiene and medical care improved population health as a whole, particularly in newborns and the elderly, resulting in a low percentage of neonatal death and increased longevity (Annual J Health and Welfare Statistics Japan, 2007). This rapid economic growth is a double-edged sword, however, as the prevalence of obesity, metabolic syndrome and malignancy among the general population also soared (*21*).

The annual increase in the age at death in WS patients (0.384 years) was statistically higher than that of the general population (0.288 years), based on a 95% confidence interval (Table 1). Environmental influences may therefore benefit WS patients more than the general population. This is supported by the observation that even though body weight, height and BMI in WS patients and in the general population increased significantly over time, BMI in WS patients was far below the normal range and may not be directly linked with the onset of DM as has been observed in the general population (Table 2). Recent environmental influences in Japan may therefore act in a beneficial manner for both WS patients and the general population prior to puberty, but may be detrimental, at least in relation to the onset of DM, after maturity.

Even though we do not have detailed data for the

age of onset of DM and malignancy in the general population, it is possible to estimate the trend over time based on population studies of the prevalence and incidence of DM (*22-25*) and malignancy (website *http://ganjoho.ncc.go.jp-public/statistics/backnumber/2000-en. html*). Population studies suggest that a recent trend in the general population of DM onset at younger ages is probably partly due to parallel increases in obesity among young people, which is in sharp contrast to WS patients.

The delay in progeroid outcomes in WS patients is likely not due to the simple technical advances in diagnosing DM and malignancy that have occurred over the last 40 years. If it had been, changes in the time of onset of both conditions would have been similar in both the general population as well as in WS patients, which was not the case. Additionally, most patients were diagnosed with DM before being diagnosed with WS (*2*). DM and malignancy are both hallmarks of WS and therefore monitoring for these conditions is more rigorous in WS patients than in the general population. It is likely, therefore, that detection of these conditions in WS patients would be enhanced, not delayed, as was seen.

Between 1966 and 2004, Japanese women were found to live ~7 years longer than men, a difference that was not maintained in WS patients. In 2004, male WS patients died at an average age of 53.6 years, compared to females at a similar age of 51.9 years. The lower female age at death may at least partly be due to the consistently lower age of onset of DM and malignancy seen in females compared to males. Thus, recent environmental factors may therefore favor male WS patients over female patients and the general population, which may suggest a gender-specific differential effect of the environment on mutated WRN. Another possibility is that female-specific genes (possibly hormone-related) are more negatively affected by the loss of WRN, although such evidence is lacking at present.

Although WS is an autosomal recessive disease that affects both sexes basically in a similar fashion, most clinical manifestations in WS are ageing-related phenotypes and as with general population ageing phenotypes especially longevity may favor female. Some phenotypic manifestations in WS could be regulated by both the loss of WRN condition which is environmentally dependent and also gender-related genes which are independent from the environmental effects. The gender-related genes may affect ageing on WS.

Finally, some of the ageing-associated phenotypes seen may relate directly to the loss of normal WRN function. Ageing is believed to induce genetic instability leading to cancer (*26*,*27*), therefore, the complete loss of WRN function may epigenetically and genetically impact other genes, promoters or proteins related to ageing-associated pathophysiology (*28*).

The present study suggests common as well as unique temporal environmental influences on hereditary disease and on the general population that affects ageing-associated phenotypic expression. The results also suggest that even in a genetically determined disease such as WS, the possibility of gene therapy-independent therapeutic intervention may exist. While this notion is highly speculative, the prospective cohort study by using the large number of mutation-proven Japanese patients may allow direct testing of these concepts in the future.

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Original Article

Inhibition of survivin expression to induce the apoptosis of hepatocarcinoma cells by adenovirus-mediated siRNA

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Summary **In order to provide more efficient transduction of plasmid siRNA into target cells, develop more susceptible transduction into cancer cell types, and more easily explore application in animal experiments, we examined development of an adenoviral vector-mediated siRNA expression system and inhibition of survivin gene expression to induce the growth and apoptosis of hepatocarcinoma cells. A system of adenoviral vector-mediated siRNA expression was constructed for the survivin gene. Survivin gene expression in HepG2 cells infected with recombinant adenovirus was detected by Western blot and RT-PCR, and apoptotic cells were investigated by FAC. Western blot analysis showed that the infection of adenovirus-mediated siRNA against survivin efficiently inhibited the expression of survivin in hepatocarcinoma cells with an inhibitory rate of 66.32%. Semi-quantitative RT-PCR showed that survivin gene mRNA transcription was reduced by nearly 72.34% with a peak at 72 h. The number of apoptotic cells increased. In conclusion, results demonstrated that this adenovirus-mediated siRNA system could serve as a useful tool for both basic research on the analysis of gene function and cancer therapy applications.**

Keywords: Adenovirus-mediated siRNA, Survivin, Hepatocarcinoma cells, Apoptosis

Introduction

The role of survivin, an inhibitor of apoptosis proteins (IAP), has gradually become apparent since 1997, when it was found to be specifically expressed in cancer cells (*1*). Using small interference RNA (siRNA) to inhibit survivin gene expression and induce the apoptosis of cancer cells is a new form of cancer gene therapy (*2-4*). However, most previous siRNA vectors were plasmids. The use of plasmid siRNA vectors was limited by its disadvantages such as limited transfection cell type, low and instable transfection efficiency, and difficult use in animal experiments. Virus vectors function *via* a recombinant virus that can infect cells. Since virus vectors can highly and stably infect various types of cells, they are more convenient for animal experiments. Therefore, virus vectors have recently enjoyed wide use, and much research describes siRNA vectors such as the adenovirus (Ad) and adeno-associated virus (AAV) (*5-9*). The present study, based on the siRNA construct (*4*), describes use of the adenoviral vector to construct an adenoviral vector-mediated siRNA (Ad-siRNA) vector of the survivin gene and discusses the recombinant virus's role in infecting HepG2 tumor cells, inhibition of survivin gene expression, and induction of apoptosis of tumor cells *in vitro*. This paper identifies the function of the Ad-siRNA vector system and establishes a foundation for animal experiments *in vivo* with AdsiRNA-survivin.

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Materials and Methods

siRNA sequences and plasmids

Survivin siRNA expression vectors were constructed as described previously by the authors (*4*). siRNA

sequences of survivin genes are as follows: Survivin-1 siRNA, 5'-TCGAG GAC CAC CGC ATC TCT ACA TTC TTCG GAA TGT AGA GAT GCG GTG GTC TTTT T-3' (sense) and 5'-CTAGA AAAA GAC CAC CGC ATC TCT ACA TTC CGAA GAA TGT AGA GAT GCG GTG GTC C-3' (antisense); Survivin-2 siRNA, 5'-TCGAG CTG AGA ACG AGC CAG ACT TG TTAGTACT CAA GTC TGG CTC GTT CTC AG TTTTT-3' (sense) and 5'-CTAGA AAAA CTG AGA ACG AGC CAG ACT TG AGTACTAA CAA GTC TGG CTC GTT CTC AGC-3'(antisense); Survivin-3 siRNA, 5'-TCGAG AAA GCA TTC GTC CGG TTG C GAGTACTG GCA ACC GGA CGA ATG TTT TTTTT-3' (sense) and 5'-CTAGA AAAA AAA GCA TTC GTC CGG TTG C CAGTACTC GCA ACC GGA CGA ATG CTT T C-3' (antisense). Transfection of plasmid DNA was carried out by lipofection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The plasmids pAdTrack, pAdEasy-1, and *E. coli* BJ5183 were provided by Dr. Tong-chuan He of the University of Chicago Medical Center.

Cell culture

AD293 and HepG2 cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a 5% CO_2 atmosphere. The cell growth state was observed periodically and cells were digested with 0.25% PentaZyme every 2-3 days to incubate generations.

Recombinant adenovirus

Plasmids of siRNA-pshRNA-survivin3 (constructed by the authors to efficiently inhibit survivin gene expression) and pTZU6+1 (as a control with no siRNA fragments inserted) were digested with Kpn I and Hind III and then separately cloned into the shuttle vector pAdTrack. For the generation of recombinant adenovirus, each plasmid was transfected into 293 cells by lipofection using Lipofectamine 2000 reagent (Invitrogen). The new constructs were named pAd-siRNA-survivin and pAd-control.

The plasmids pAd-siRNA-survivin and pAd-control were digested with Pme I to linearized them and then separately co-transfected with adenovirus frame vector pAdEasy-1 into *E. coli* BJ5183. Restriction analysis verified that the recombinant plasmids were pAd-Easy-siRNA-survivin and pAd-Easy-U6-control. The resulting plasmids were linearized with Pme I again and transfected into the adenovirus package 293 cell line for 7-13 days, virus formation was determined by GFP gene expression of the pAdTrack plasmid, and then the virus AdsiRNA-survivin and AdU6-control were harvested by alternately, freezing and thawing cells and then storing them at -70°C.

When AD293 cells were 80% confluent with fresh cells 24 h before infection, 5×10^5 cells were separately infected with 100 μL each of AdsiRNA-survivin and AdU6-control adenovirus original suspensions. The adenovirus original suspension was diluted to 1 mL with serum-free RPMI-1640 and replaced with fresh medium containing 10% FBS after 6 h. Virus formation was determined by GFP expression under fluorescence microscopy, and AD293 cells were harvested when virus was obviously formed or two-third of cells were floating. The cells were centrifuged at 800 rpm for 5 min, resuspended in 1 mL of PBS, and stored at -70°C.

Adenoviral vectors were purified by cesium chloride ultracentrifugation (*10*). Purified virus was dialyzed in phosphate-buffered saline (PBS) with 10% glycerol and stored at -70°C until use. Virus titer was determined according to the manufacturer's instructions (Stratagene).

Cell transfection

HepG2 cells were grown in RPMI-1640 with 10% FBS supplemented with 100 U/mL penicillin/streptomycin. The day before virus infection, HepG2 cells were plated in each well of 6-well plates. The following day, cells were incubated with recombinant virus (AdsiRNA-survivin and AdU6-control) at MOI of 10-20 at 37°C. After adsorption for 1-2 h, 2 mL of fresh growth medium was added and cells were placed in the incubator for an additional 2-3 days.

Immunoblot analysis

HepG2 cells were infected with AdsiRNA-survivin or AdU6-control virus suspension of the same titer (2.0×10^8 pfu/mL), harvested after 72 h, washed twice in PBS, and lysed in lysis buffer containing 1 mM PMSF (Merck, USA). The total protein was extracted and quantified with an ultraviolet spectrophotometer. Forty μg of the protein were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a NC membrane that was subsequently blocked for 2 h at room temperature. The membrane was first probed with rabbit anti-survivin (Santa Cruz, USA) (1:1,000 dilution) at 4°C overnight and then washed with PBST. The membrane was incubated with secondary antibody sheep anti-rabbit IgG-HRP (1:4,000 dilution) at room temperature for 1.5 h and visualized with an enhanced chemiluminescence (ECL) kit (Pierce, USA). The images were analyzed with the ImageMaster TotalLab System. The luminous intensity of the control protein served the reference to compare the relative proteins and ascertain weak intensity.

RT-PCR analysis

RT-PCR was performed to determine the relative quantity of survivin gene in hepatocarcinoma cells.

Cells were infected for 72 h, digested with 0.25% PentaZyme, and harvested. After cells were washed twice with PBS, total RNA was extracted according to instructions. There were two pairs of primers, the first of which was the GAPDH gene primer as the endogenous control: 5'-GGAAGGTGAAGGTCGGAGTC-3' and 5'-GACCACCTGGTGCTCAGTGT-3'; the second was the survivin gene primer: 5'-ATAGTCGACATGGGT GCCCCGACGTTG-3' and 5'-CTCGGATCCTCAAT CCATGGCAGCCAG-3'; procedures were performed according to the kit instructions. The quantity of added plates was expressed by density ratios to GAPDH. The degree of survivin mRNA silence was determined based on the quantity of plates.

TUNEL assays

Infected cells were grown for 72 h on coverslips, and TdT-mediated UTP end labeling (TUNEL) analysis was performed using the *in situ* Cell Death Detection kit (Roche Molecular Biochemicals). Apoptotic strand breaks were visualized by transmission epifluorescence microscopy.

Flow cytometric analysis (FCA) of cell apoptosis

HepG2 cells were seeded at a density of 1×10^6 cells/well into 6-well plates in which slides were previously laid, incubated for 24 h, and then separately infected with AdsiRNA-survivin (2.0×10^8 pfu/mL) and AdU6-control (2.0×10^8 pfu/mL) for approximately 72 h (according to the expression of fluorescence). The cells were harvested after absorbing all of the medium and the depositions were sufficiently mixed. The cells were kept in 75% glacial ethanol overnight and then analyzed by FCA.

Statistic analysis

The results of the tests were analyzed using SPSS10.0 software, and the difference between the two groups was compared using a student test. *P* values of less than 0.05 were considered to be significant.

Results

Construction of pAdsiRNA virus-vector

The pAdsiRNA vector was constructed on the basis of the constructs pshRNA-survivin3 plasmid and adenovirus vector pAdTrack (Figure 1). Results of restriction digest analysis of recombinant pAdsiRNA-survivin are shown in Figure 2, indicating that the U6+1 promoter and siRNA inverted repeat sequence fragment had been cloned into the vector pAdTrack. The linearized recombinant and pAdEasy-1 were transfected into *E. coli* BJ5183 to construct the recombinant pAdsiRNA-survivin. The pAdsiRNA-survivin was digested with Pac I, and the results of electrophoresis indicated that the recombinant yielded a 4.5 kb or 3.0 kb DNA fragment (Figure 3). The protocol to construct the plasmid pAdTrack-U6 was identical, but the digested vector pTZU6+1 had no siRNA fragment inserted, only including the U6+1 promoter.

The plasmids Ad-vector pAdsiRNA-survivin and pAdTrack-U6 were separately digested with Pac I and transfected into AD293 cells. The cells were harvested when virus spots and some floating cells were visible microscopically, and original virus suspensions were prepared. A large number of AD293 cells was infected with the original virus suspensions in order to provide a large amount of adenovirus. The virus was collected in accordance with GFP expression, and the process repeatedly yielded abundant recombinant virus *via* infected cells.

When the recombinant virus had infected AD293 cells for 36 h, almost 100% of cells displayed GFP expression under fluorescence microscopy. The titer of the recombinant virus was: AdsiRNA-survivin, 2.1

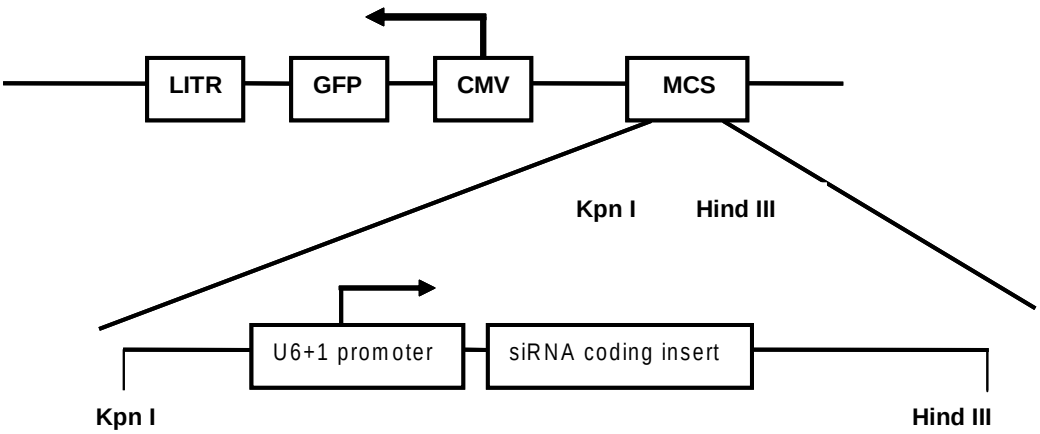

Figure 1. Schematic outline of recombinant adenovirus expression siRNA targeting survivin. The plasmid psiRNA-survivin was cut by Hind III and Kpn I and then inserted into plasmid pAdTrack to construct the vector pAdsiRNA-survivin.

× 10^8 pfu/mL; AdU6-control, 1.8 × 10^8 pfu/mL. After cesium chloride gradient centrifugation, the titer was: AdsiRNA-survivin, 2.4 × 10^9 pfu/mL; AdU6-control, 2.1 × 10^9 pfu/mL.

AdsiRNA efficiently inhibited survivin gene expression

Western blot analysis was performed to examine the levels of survivin expression after infection of the various vectors and to reveal the knockdown efficiency. The luminous intensity of survivin bands for cells infected with AdsiRNA-survivin was significantly weaker than that for cells that were not infected or were infected with AdU6-control, while the level of GAPDH expression as a control was not influenced (Figure 4). There was a significant difference ($P < 0.01$) in survivin expression between cells infected with AdsiRNA-survivin and those not infected, while no significant difference ($P > 0.05$) was found between uninfected cells and those infected with AdU6-control. These results indicate that the AdsiRNA construct efficiently inhibited survivin gene expression. Analysis with the ImageMaster TotalLab System showed that the intensities of survivin bands for AdsiRNA-survivin and AdU6-control were 33.68% and 99.20%, respectively ($P < 0.01$), in comparison to those for bands with no virus present.

As shown in Figure 5, semi-quantitative RT-PCR results for survivin and GADPH were similar to the results of Western blot analysis. The intensity of survivin amplification for cells infected with AdsiRNA-survivin was significantly weaker than that for uninfected cells or cells infected with AdU6-control ($P < 0.01$), while amplification of GAPDH as a control was not influenced. Analysis with the ImageMaster TotalLab System showed that the intensities of survivin mRNA for AdsiRNA-survivin and AdU6-control were 27.96% and 100%, respectively ($P < 0.01$), in comparison to the intensity with no virus present.

Apoptosis of cells infected by AdsiRNA

In order to analyze the cellular consequences of siRNA-mediated silencing of the survivin gene, TUNEL analyses were performed. As shown in Figure 6A and B, the uninfected HepG2 cells or cells infected with AdU6-control retained their normal shape and were closely packed; a small number of stained nuclei and lightly stained cytoplasms were observed. In contrast, many TUNEL-positive cells were observed among cells infected with AdsiRNA-survivin; HepG2 cell nuclei were stained brownish red and displayed karyopyknosis.

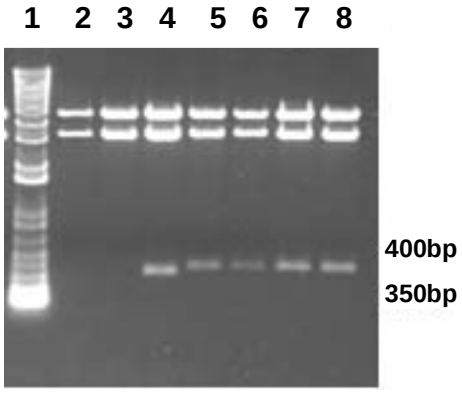

Figure 2. Restriction analysis of recombinant plasmids pAdTrack-survivin and pAdTrack-U6. Lane 1, Marker; lanes 2 and 3, pAdTrack + Kpn I + Hind III; lane 4, pAdTrack-U6 + Kpn I + Hind III; lanes 5-8, pAdTrack-survivin + Kpn I + Hind III.

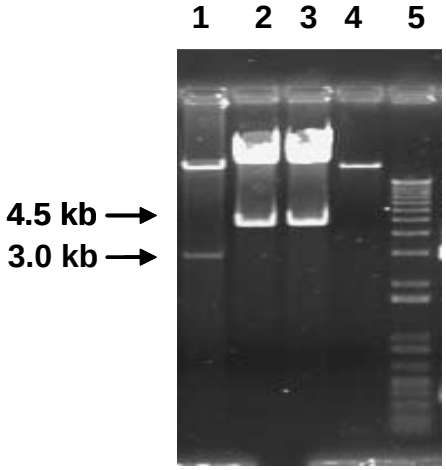

Figure 3. Restriction results for recombinant pAdsiRNA-survivin and pAd-U6-control. Lanes 1 and 2, pAdsiRNA-survivin + Pac I; lane 3, Ad-U6-control + Pac I; lane 4, pAdEasy-1 + Pac I, lane 5, Marker.

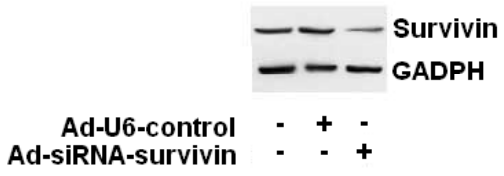

Figure 4. Western blot analysis of survivin expression in infected HepG2 cells.

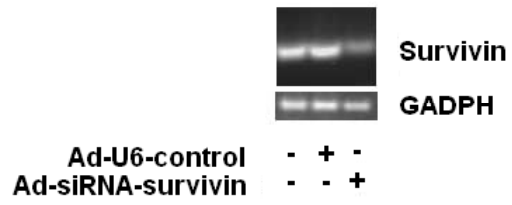

Figure 5. Semi-quantitative RT-PCR of survivin and GAPDH in infected HepG2 cells.

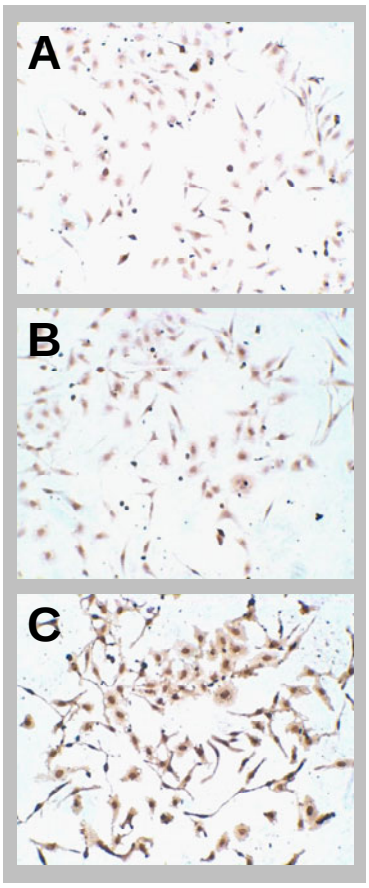

Figure 6. Detection of apoptotic cells in HepG2 as determined by TUNEL after infection of AdsiRNA. A, HepG2 cells; B, HepG2 cells + AdU6-control; C, HepG2 cells + AdsiRNA-survivin.

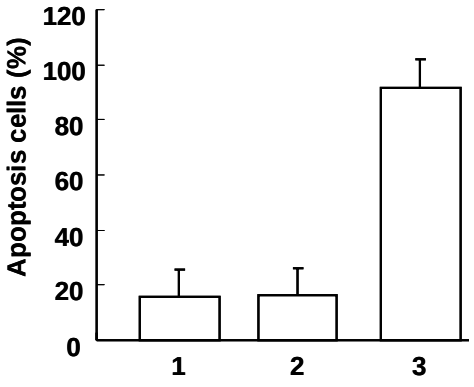

Figure 7. Detection of the effects of AdsiRNA-survivin on the apoptosis of HepG2 cells by TUNEL assay. 1, HepG2 cells; 2, HepG2 cells + AdU6-control; 3, HepG2 cells + AdsiRNA-survivin. The two stars indicate $P < 0.01$ in comparison to the control.

The fact that the number of apoptotic cells increased dramatically indicates that siRNA directed against survivin increases the spontaneous apoptosis rate of HepG2 cells. The number of apoptotic cells determined by TUNEL after infection with AdsiRNA is shown in Figure 7.

Figure 8 shows flow cytometrical detection of apoptotic cells. An obvious hypodiploid peak in the G0-G1 period, suggesting cell apoptosis, was observed in HepG2 cells infected with AdsiRNA-survivin, while the corresponding peaks in uninfected cells or cells infected with AdU6-control were significantly weaker. The apoptosis ratios for uninfected cells, cells infected with AdU6-control, and cells infected with AdsiRNA-survivin were 17.19%, 18.05%, and 87.68%, respectively ($P < 0.01$).

Discussion

RNA interference (RNAi) is an emerging method that provides a new platform for genome study and has been used in several fields, including gene therapy for viral disease and tumors (*11-13*). A number of studies have indicated that plasmid siRNA vectors have many shortcomings when used in experiments to inhibit target gene expression such as instable transfection efficiency and limited transfection cell types; moreover, their failure to act on target cells in animal experiments resulted in their limited use to experiments *in vivo* (*6*). The current study established an adenoviral vector-mediated siRNA expression system because the adenovirus has a wide-ranging host choice, including stationary and division-phase cells, and has a high and stable infection efficiency, so it can easily be used in animal experiments. Hepatocarcinoma cells were infected with AdsiRNA in order to examine the inhibition efficiency and influence of AdsiRNA on survivin gene expression in tumor cells. The results proved that this AdsiRNA system efficiently inhibited target gene expression at both gene and protein levels and can be used in other gene research. This study selected the IAP factor survivin as the target gene and found inhibition of survivin gene expression and obvious promotion of hepatocarcinoma cell apoptosis. Additionally, these results revealed survivin's anti-

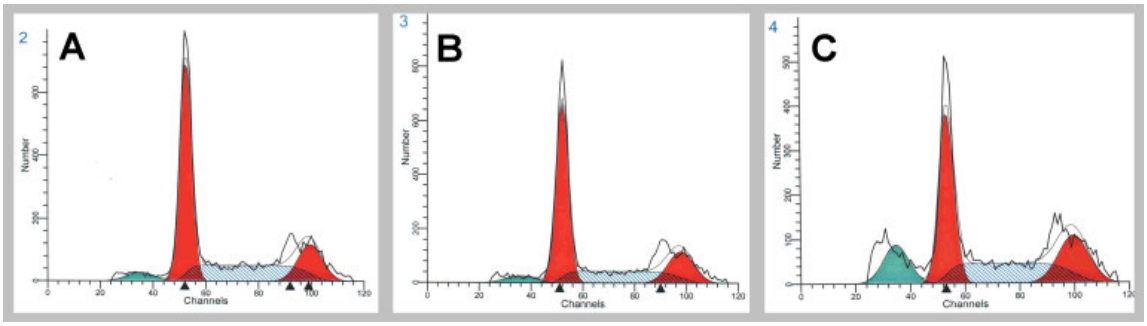

Figure 8. Detection of the effects of AdsiRNA-survivin on the apoptosis of HepG2 cells by cytometry. A, HepG2 cells; B, HepG2 cells + AdU6-control; C, HepG2 cells + AdsiRNA-survivin.

apoptotic role, which is consistent with previous studies (*3*). The current adenovirus siRNA system may provide technology to support future animal experiments with survivin, a popular and important tumor inhibitor, and at the same time offer an experimental base for research on the survivin gene signaling pathway.

This adenovirus siRNA vector system may be used in gene function research not only of the survivin gene but also of other target genes. An AdsiRNA-RhoA vector was also constructed and the same specific and efficient inhibition effect on the RhoA gene was achieved (paper in publication), indicating that this adenovirus siRNA vector may be widely used in siRNA construction and target gene research. The adenovirus siRNA vector system may provide a new investigation platform for gene function study, gene therapy for viral disease and tumor diseases, and drug screening tests, offering technological support for siRNA methods of studying target genes *via* animal experiments.

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