BioScience Trends

International Research and Cooperation Association
for Bio & Socio-Sciences Advancement

Shanghai University of Traditional Chinese Medicine Library,
Shanghai, China

ISSN 1881-7815 Online ISSN 1881-7823
Volume 4, Number 6, December 2010
www.biosciencetrends.com

BioScience Trends

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: 03-5840-8764, Fax: 03-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

BioScience Trends is a peer-reviewed international journal published bimonthly by *International Research and Cooperation Association for Bio & Socio-Sciences Advancement* (IRCA-BSSA).

BioScience Trends publishes original research articles that are judged to make a novel and important contribution to the understanding of any fields of life science, clinical research, public health, medical care system, and social science. In addition to Original Articles, BioScience Trends also publishes Brief Reports, Case Reports, Reviews, Policy Forum, News, and Commentary to encourage cooperation and networking among researchers, doctors, and students.

Subject Coverage: Life science (including Biochemistry and Molecular biology), Clinical research, Public health, Medical care system, and Social science.

Language: English
Issues/Year: 6
Published by: IRCA-BSSA
ISSN: 1881-7815 (Online ISSN 1881-7823)
CODEN: BTIRCZ

Editorial Board

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Cover Photo of this issue

Shanghai University of Traditional Chinese Medicine Library, Shanghai, China

Shanghai University of Traditional Chinese Medicine is founded in 1956. It is one of the firstly established four Colleges and Universities of Tradition Chinese Medicine after the People's Republic of China has founded. In September 2003, the University was moved to Zhang Jiang campus which is a new campus located in Shanghai Pudong new area. The building in the picture is the library of Shanghai University of Traditional Chinese Medicine which is one of the most famous landmarks in the new campus. It looks like a Chinese ancient bronze vessel "Ding" (鼎) which is a symbol of ancient culture and civilization of China. More than 900,000 books have been preserved in the library and preservation of ancient Chinese medicine books is a major feature of this library with 32,000 books from Jin and Yuan dynasties to Republic of China.

(*Photo by* Fanghua Qi)

(This journal was partially supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.)

Policy Forum

The management of hepatocellular carcinoma in Asia: A guideline combining quantitative and qualitative evaluation

Peipei Song[1], Wei Tang[1,*], Sumihito Tamura[1], Kiyoshi Hasegawa[1], Yasuhiko Sugawara[1], Jiahong Dong[2], Norihiro Kokudo[1]

[1] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *Hepato-Biliary-Pancreatic Surgery Division, The General Hospital of PLA, Beijing, China.*

Summary **Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths around the world; Asian countries account for nearly 78% of the roughly 600,000 cases of HCC reported globally each year. Europe, the US, Asian-Pacific nations, South Korea, and Japan have published evidence-based guidelines for the management of HCC. The management of HCC in Japan has achieved remarkable results, which are attributed to a combination of quantitative and qualitative evaluation incorporated in the Japanese guidelines. However, many of the control methods and interventions in current HCC guidelines cannot be implemented in some Asian countries. The majority of HCC patients in Asia still present with advanced HCC and long-term outcomes following treatment are unsatisfactory because of a lack of effective adjuvant and systemic therapies. Asian countries should formulate evidence-based clinical practice guidelines and pay particularly close attention to combining quantitative and qualitative evaluation when drafting and implementing HCC guidelines. The guidelines should also be updated by incorporating new evidence.**

Keywords: Hepatocellular carcinoma, guideline, evidence-based, qualitative indicator

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related deaths around the world (*1*). Many countries have studied clinical practice guidelines for HCC in recent years (Table 1). The US National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology (NCCN Guidelines) (*2*), the British Society of Gastroenterology Guidelines (BSG Guidelines) (*3*), and the American Society of Clinical Oncology Consensus (ASCO Consensus) (*4*) have been acknowledged and cited around the world in the treatment of liver cancer. The European Association for the Study of the Liver published clinical practice guidelines for HCC (EASL Guidelines) in 2001 (*5*), the Korean Liver Cancer Study Group (KLCSG) and National Cancer Center (NCC) jointly published clinical practice guidelines for HCC (Korea Guidelines) in 2003 (*6*), the Japanese Ministry of Health, Labor, and Welfare published clinical practice guidelines for HCC (J-HCC Guidelines) in February 2005 (*7*), the American Association for the Study of Liver Disease published clinical practice guidelines for HCC (AASLD Guidelines) in November 2005 (*8*), and the Asian-Pacific Association for the Study of the Liver released the consensus recommendations for the treatment of HCC (APASL Guidelines) in 2008 (*9*). All of these guidelines provide an evidence-based approach to prevention, surveillance, diagnosis, staging, and treatment of HCC.

Asian countries account for at least two-thirds of the roughly 650,000 cases of HCC reported globally each year (*10*). HCC is prevalent in males, the incidence rates for men of the following countries and districts in Asia are > 25 per 100,000 persons: mainland China (58/100,000), Taiwan (53/100,000), South Korea

**Address correspondence to:*
Dr. Wei Tang, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
e-mail: TANG-SUR@h.u-tokyo.ac.jp

Table 1. Current standards for the management of hepatocellular carcinoma around the world

Area	Series of standards	Publishing	Reference
Europe	BSG Guidelines	British Society of Gastroenterology	(*3*)
	EASL Guidelines	European Association for the Study of the Liver	(*5*)
America	ASCO Consensus	American Society of Clinical Oncology	(*4*)
	AASLD Guidelines	American Association for the Study of Liver Disease	(*8*)
Asia	Korean Guidelines	Korean Liver Cancer Study Group and the National Cancer Center	(*6,13*)
	J-HCC Guidelines	Japanese Ministry of Health, Labor, and Welfare	(*7,14,18-20*)
	APASL Guidelines	Asian-Pacific Association for the Study of the Liver	(*9*)
	Chinese Consensus	Chinese Anti-Cancer Association Society of Liver Cancer, Chinese Society of Clinical Oncology, Chinese Society of Hepatology Liver Cancer Study Group	(*28*)

(45/100,000), Thailand (33.4/100,000), and Hong Kong (29.9/100,000) (*11*). Of particular note is the fact that China alone accounts for 55% of cases of HCC worldwide (*12*). In Asia, only the Korean Guidelines and J-HCC Guidelines have been published and widely adopted; other countries are still in the research stage and have not formulated a nationwide evidence-based clinical practice guideline for HCC.

Korean Guidelines for HCC: Korean Guidelines for HCC were published by the KLCSG and NCC in 2003 and were based on scientific evidence and forty-five experts who formed a special committee to develop strategies to diagnose and treat HCC (*6*). The KLCSG and NCC revised the Korean Guidelines in 2009 (*13*). About forty specialists in the fields of hepatology, general surgery, radiology, and radiation oncology participated in the revision, domestic and foreign literatures were meticulously reviewed, and opinions were solicited from advisory committee conferences. The revision summarized diagnosis, surgical resection, liver transplantation, local treatments, transcatheter arterial chemoembolization (TACE), radiation therapy, chemotherapy, preemptive antiviral treatments, and evaluation of response to HCC treatment. In South Korea, a nationwide surveillance program was launched in 2003, and patients over 40 years or who had hepatitis B virus (HBV)/hepatitis C virus (HCV) or liver cirrhosis were screened by the program.

J-HCC Guidelines: The management of HCC in Japan is characteristic of such management in Asian countries. Supported by the Japanese Ministry of Health, Labor, and Welfare, the J-HCC Guidelines were published in February 2005 and then widely adopted in HCC treatment in Japan. The set of guidelines covers six clinically important fields for HCC treatment, including prevention, diagnosis and surveillance, surgery, chemotherapy, TACE, and ablation therapy. For users' convenience, practical algorithms for the surveillance and treatment of HCC were also created (*14*).

In March 2006, approximately a year after publication of the J-HCC Guidelines, a questionnaire survey was conducted to investigate the level of awareness and influence of the guidelines among 2,279 members of the Liver Cancer Study Group of Japan and 689 primary care physicians in Osaka and Hyogo prefectures (*15*). Of the 1,175 respondents, 71.9% of hepatologists, 75.6% of liver surgeons, and 61.0% of primary care physicians have acknowledged the J-HCC Guidelines. After the introduction of the guidelines, 19-21% of hepatologists or liver surgeons changed their practices, 50-52% did not change but were convinced that their choice of treatment was similar to that recommended in the guidelines; 43% of primary care physicians changed their practices to follow the recommendations in the guidelines or paid closer attention to patient preferences.

Based on the surveillance algorithm and the diagnostic algorithm for HCC, patients with hepatitis HBV/HCV or liver cirrhosis in Japan have been closely followed with ultrasound, enhanced computed tomography (CT) scans, or enhanced magnetic resonance imaging (MRI) scans every 3-6 months before HCC develops; HCC nodules have been detected in the early stage in more than 60% of patients (*16*), and clinical diagnosis of HCC by diagnostic imaging is replacing pathological confirmation of the diagnosis (*17*). The treatment algorithm for HCC is based on three factors: degree of liver damage, number of tumors, and tumor diameter. The algorithm is easy to understand and can assist both physicians and patients in their decision-making regarding the treatment of HCC (*16*).

The management of HCC in Japan has achieved remarkable results, which are attributed to a combination of quantitative and qualitative evaluation incorporated in the J-HCC Guidelines:

(*I*) Quantitative evaluation was incorporated in the J-HCC Guidelines as the first evidence-based clinical practical guidelines for the treatment of HCC in Japan: (*i*) Medical literature on HCC in English was systematically reviewed. In total, 7,192 publications

on HCC were identified mainly from MEDLINE (1966-2002), and 334 articles were selected upon secondary selection. Of the selected articles, 44.2% were from Japan, 13.2% from elsewhere in Asia, and 42.6% from outside Asia (mainly from Europe) (*18*). (*ii*) With the incorporation of new evidence, a revised version of the J-HCC Guidelines was published in 2009 (*19*). A total of 2,950 articles were identified from MEDLINE (2002-2007). After the evaluation of evidence levels and content, 532 articles were ultimately selected. The next revision will be made in 2-3 years, and evidence reported after 2007 will be included in the new review. (*iii*) The algorithms in guidelines were established according to the current status of medical practices in Japan, where liver resection for HCC is regarded as safe with a mortality rate of less than 1% and cadaveric donors for liver transplantation are seldom available (*20*).

(*II*) The J-HCC Guidelines paid close attention to qualitative evaluation when the guidelines were formulated and implemented: (*i*) J-HCC Guidelines were compiled by an expert panel consisting of five surgeons, four internists, three radiologists, and one statistician. Most of the members were executive board members of the Liver Cancer Study Group of Japan. In order to assist in evidence collection and evaluation, a total of 26 physicians specializing in HCC also served as members of a task force for the establishment of the guidelines (*20*). (*ii*) The J-HCC Guidelines emphasized the evaluation of qualitative indicators. The 2005 J-HCC Guidelines incorporated 334 articles to form 58 pairs of research questions and recommendations; in the 2009 version, the 58 pairs of research questions were re-evaluated and 51 clinical questions were formulated. With these questions, physicians can better understand the guidelines and make the suitable clinical decisions for individual patients. (*iii*) Prior to publication, a draft of J-HCC Guidelines was submitted for internal evaluation (the 2005 guidelines were evaluated by 101 councilors of the Liver Cancer Study Group of Japan and the 2009 revision was evaluated by the 45th Japan Society of HCC) and external evaluation (the 2005 guidelines were evaluated by an external review board and the 2009 revision was available on the Web to seek public comments). In addition, a questionnaire survey was conducted to investigate the level of awareness and influence of the guidelines in 2006. (*iv*) While the guidelines are being followed, experts are researching problems encountered in actual clinical practice. For example, speakers at the 45th Annual Meeting of the Japan Society of Hepatology (2009) raised clinical questions regarding the remaining problems that needed to be clarified by the present guidelines, and HCC specialists (a total of 200 physicians) answered these questions using a question-and-answer analyzer system. Recommendations were approved when at least 67% of the HCC experts reached a consensus (*21*). This will greatly contribute to future improvement of the guidelines.

The management of HCC in China: Since the 1970s, China has taken measures to prevent hepatitis. In Taiwan, HBV immunization of newborns was introduced in 1984 (*22*), and follow-up results from the program have indicated a significant reduction in the incidence of HCC in children. The average annual incidence of HCC in children 6-14 years of age declined from 0.70/100,000 (1981-1986) to 0.57/100,000 (1986-1990) and to 0.36/100,000 (1990-1994) ($p < 0.01$) (*23*). Taiwan has a surveillance program in place but the program may not be widely available. There is no government-funded surveillance program for HCC in Hong Kong or other parts of China.

The national government and research institutes have researched the treatment of HCC in China. In 1989, the Medical Administration Department of the Ministry of Health of the People's Republic of China published "Treatment Standards for Common Malignant Tumors in China (Vol. 2, Hepatocellular Carcinoma)" (*24*). In 1990, the Drug Administration Department of the Ministry of Health of the People's Republic of China published "Guiding Principles for Clinical Research on Treatment of Hepatocellular Carcinoma Involving New Drugs/Traditional Chinese Medicines" (*25*). In 1999, the Chinese Anti-Cancer Association (CACA) published "New Treatment Standards for Common Malignant Tumors in China (Hepatocellular Carcinoma Section)" (*26*). In 2000, the 6th China Hepatic Surgery Academic Conference published "Choices for Hepatocellular Carcinoma Treatment Therapies" (*27*). In 2007 and 2008, the CACA Society of Liver Cancer (CSLC), Chinese Society of Clinical Oncology (CSCO), and Chinese Society of Hepatology (CMA) Liver Cancer Study Group jointly organized a consensus conference that met three times in Shanghai. More than sixty experts participated in the conference; they systematically reviewed the guidelines for and consensus opinion regarding the treatment of HCC around the world and they discussed the topics of diagnosis, surgery, interventional therapy, local ablation therapy, radiotherapy, biotherapy, molecularly targeted therapy, systemic chemotherapy, and combined treatments with traditional Chinese and western medicines. Based on the discussion, "The Expert Consensus on the Treatment Standards for Hepatocellular Carcinoma (the Chinese Consensus)" was published in 2009 (*28*) to promote the development of treatment standards for HCC. However, actual nationwide evidence-based clinical practice guidelines for HCC have yet to be formulated.

In conclusion, a greater number of Asian countries have paid close attention to the management of HCC and have achieved notable results over the past few years. South Korea and Japan have published and

widely adopted nationwide evidence-based clinical practice guidelines, but other Asian countries have not formulated a nationwide guideline until now. HCC prevention and surveillance in Asia have made some progress, but the majority of HCC patients in Asia still present with advanced HCC. Moreover, long-term outcomes following treatment of even early/intermediate or advanced disease are often unsatisfactory because of a lack of effective adjuvant and systemic therapies (*17*).

EASL Guidelines, AASLD Guidelines, APASL Guidelines, Korean Guidelines, and J-HCC Guidelines are all evidence-based guidelines that have outlined optimal approaches to managing HCC in resource-rich countries. However, many of the current HCC control methods and interventions can not be implemented in some Asian countries because these countries have limited resources. The current guidelines also fail to consider inconsistent resource distribution in areas with high overall standards of living and they fail to address inadequate infrastructure and resources in resource-poor Asian countries (*29*).

The practical effectiveness of J-HCC Guidelines have typically highlighted the importance of following clinical guidelines to treat HCC, and combination of quantitative and qualitative evaluation should be emphasized when drafting and implementing guidelines. In accordance with the current status of medical practices and medical methodologies, Asian countries should establish an expert panel with the support of government to formulate evidence-based clinical practice guidelines. Particularly close attention should be paid to combining quantitative and qualitative evaluation when drafting and implementing HCC guidelines. The guidelines should also be updated by incorporating new evidence.

Acknowledgements

This project was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

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(Received October 30, 2010; Revised November 22, 2010; Accepted November 29, 2010)

Review

The role of hypoxia in mental development and in the treatment of mental disorders: A review

Simon N. Basovich*

Life Sciences R&D, Melbourne, Australia.

Summary **The purpose of this review is to trace the trends in studying and applying hypoxia in the field of mental problems. A literature review was conducted using the PubMed database, with a time-frame extending to October 2010. According to the neurodevelopmental model of mental disorders, abnormalities in brain development during pre- and perinatal life lead to psychotic manifestation in adolescence or young adulthood. Studies show that hypoxia plays an important role in almost any risk factor related to brain development in early life: pre-eclampsia, infection/inflammation, hypoxia/ischemia, preterm birth, and asphyxia at birth. The cited data show trends in using hypoxia, especially in the form of intermittent hypoxic training, for the treatment and prevention of mental disorders, and trends in using it for increasing mental capacity in animals.**

Keywords: Hypoxia, mental disorders, treatment, prevention, mental capacity

1. Introduction

Mental disorders are devastating illnesses affecting millions worldwide, with significant financial and emotional burdens for patients, their families, and society. The total annual cost of depression in Europe was estimated at €118 billion in 2004 (*1*), the estimated total societal cost of schizophrenia for England was £6.7 billion in 2004/05 (*2*).

About 30 years ago, a neurodevelopmental model of mental disorders, as a hypothesis, was proposed (*3*). It has provided great impetus to the psychiatric research community and is now widely accepted and developed. This model suggests that abnormalities in brain development during pre- and perinatal life lead to psychotic manifestation in adolescence or young adulthood (*3-12*).

It is known that abnormalities in brain development during pre- and perinatal life arise under the influence of different stress factors. The role of hypoxia in stress factors such as hypoxia/ischemia and asphyxia is well known. The role of hypoxia in other stress factors, however, is not well understood, though hypoxia, according to modern conceptions, is included in the pathogenesis of almost any disorder. The literature is also scant on the use of hypoxia in the prevention and treatment of mental disorders. There are many poorly understood facts, connecting hypoxia and mental disorders; coordinating these facts with the neurodevelopmental model would be desirable.

The aim of this review is to trace the trends in studying and applying hypoxia in the field of mental problems, particularly: *i*) the role of hypoxia in abnormal brain development; *ii*) the possibilities of hypoxia for increasing mental capacity; and *iii*) the possibilities of hypoxia for treatment and prevention of mental disorders. A literature review was conducted using the PubMed database, with a time-frame extending to October 2010. More than 11,000 titles, about 400 abstracts, and some full-text articles were reviewed.

2. Hypoxia in the etiology of mental diseases

Environmental insults during early brain development may have long-lasting consequences for adult brain functioning. The following important stressful environmental risk factors that influence the future development of mental disorders were claimed in the studied literature: hypoxia (hypoxic hypoxia), ischemia, asphyxia, infection, inflammation, pre-eclampsia,

**Address correspondence to:*
Simon N. Basovich, Life Sciences R&D, 9/125 Thomas Street, Hampton, Victoria 3188, Australia.
e-mail: simonbas@tpg.com.au

preterm birth, and maternal psychological stress during pregnancy. Reviews for these risk factors have been done by (*13-16*).

Hypoxic hypoxia (breathing air with low oxygen content) and circulatory hypoxia (ischemia, asphyxia) are widely used in animal experiments to mimic preterm birth and other events resulting in neurodevelopmental disability, including schizophrenia and depression. Perinatal sublethal hypoxia, hypoxic/ischemic insults were usually used in experiments that show: *a*) significant alterations in corticogenesis: failure of brain growth, progressive cerebral ventriculomegaly, decreased subcortical white matter and corpus callosum size, and decreased cortical volume (*17*); *b*) alteration in the production and maintenance of glial and neuronal cells (*18*); *c*) white matter damage (*19*); *d*) loss of cortical neurons (*20*); *e*) modifications in corpus callosum, cingulum, and fimbria of the hippocampus (*21*); *f*) impaired development of neural processes and connections in the hippocampus, cerebellum, and visual cortex (*22*); *g*) reduced brain weight, ventriculomegaly, reduced basal ganglia volume and absence of astrogliosis (*23*); *h*) facilitation of proliferation of neural stem cells (*24*); *i*) lowered body and brain weights, as well as decreased cortical volumes of newborn rats; however, hypoxic rats had increased neuronal density and significantly more cortical neurons (*25*); and *j*) changes related to the subventricular zone, hippocampus, and dentate gyrus (*26-31*).

Several articles contained epidemiological data (*32-34*).

In a prospective cohort study of genetic and perinatal influences in the etiology of schizophrenia (*32*) was found that the odds of schizophrenia increased linearly with increasing number of hypoxia-associated obstetric complications and that this effect was specific to cases with an early age at onset/first treatment contact.

In a 19-year longitudinal study of hypoxic-ischemia-related fetal/neonatal complications was found (*33*) that these complications were associated with a doubling of the risk of developing a psychotic disorder.

Signs of asphyxia at birth are associated with an increased risk of schizophrenia in adults, according to a population-based case-control study (*34*). This study used 524 cases of schizophrenia and 1,043 controls.

Tissue (histotoxic, cytotoxic, cytopathic) hypoxia appears when tissues are unable to use oxygen despite normal oxygen delivery. This type of hypoxia is involved in infection/inflammation during pregnancy, which leads to pre-eclampsia and preterm birth. Each of these conditions leads to an increased risk for psychoses among adult offspring (*35-39*).

The main pathological process of infection/inflammation is widely recognized as the inflammatory response syndrome (*15,40,41*), which is based on tissue hypoxia. The cells under the tissue hypoxia behave as if there is too little oxygen because of an inflammation-induced alteration in cellular function, not because there is too little oxygen for cellular function (*42*). The studies show, in particular, that: *a*) hypoxia and the innate immune response are 2 adaptive mechanisms by which organisms respond to perturbation in organ function, playing a major role in spontaneous abortion, intrauterine growth restriction, pre-eclampsia, and preterm delivery (*43*); *b*) the placenta expresses a variety of pro- and anti-inflammatory cytokines, adipokines and cytokine-like angiogenic growth factors, production of which is altered in pre-eclampsia, driven (at least in part) by hypoxia (*44*); *c*) the underlying pathology of pre-eclampsia is thought to be a relatively hypoxic or ischemic placenta (*45*); *d*) infection-associated immunological events in early fetal life may have a stronger neurodevelopmental impact compared to late pregnancy infections (*46*); and *e*) in utero exposure to bacterial infection can severely alter fetal cardiovascular function, resulting in dysregulation of cerebral blood flow and subsequent hypoxic-ischemic brain injury (*16*).

Maternal psychological stress during pregnancy is probably also accompanied by hypoxia (*47-49*), but this association should be analyzed more scrupulously.

The earlier cited data show that hypoxia is involved in almost any important stressful environmental risk factor during early life, leading to excessive pathologic neurogenesis. Such pathologic neurogenesis becomes apparent from the changing size of the defined neuronal network zones or their structure, for example, increased neuronal density. Furthermore, pathologic neurogenesis in mental disorders is clearly routinely seen in psychosurgery, the task of which is just to ablate pathologically changed brain zones. Pathologic neurogenesis is probably connected with the abnormal formation of neural network elements. For example, "the specificity of synapse formation requires the precise execution of multiple developmental events, including cell fate specification, cell migration, axon guidance, dendritic growth, synaptic target selection, and synaptogenesis" (*50*). The changes in the brain stimulated by pathologic neurogenesis may lead to abnormal communications in the neural network; which causes abnormal associations, ideas, and acts, *i.e.*, mental disorders.

3. Hypoxia in increasing mental capacity

It was shown earlier that acute hypoxia in early life is a trigger for anomalous brain development, which leads to psychotic manifestation in adolescence or young adulthood. The neurodevelopmental model clearly suggests that moderate hypoxia in early life may be a trigger for a moderate increase in brain development leading to increased mental capacity, probably to the level of genius and, sometimes, to madness.

Proverbs, adages and quotes that make the connection between genius and madness have existed in different languages for hundreds or even thousands of years as the following examples demonstrate: "There is a fine line between genius and insanity"; "There is just one step from genius to madness" (Pushkin); "There is no great genius without a mixture of madness" (Aristotle).

The phenomenology and psychopathology of genius was considered (*51*). The author noted that the relationship between genius and madness has been a subject of interest since the beginning of critical and philosophical thinking. Thus, Aristotle, in the Problemata, asks himself "Why are all extraordinary men in the fields of philosophy, politics, poetry and art melancholic?" adding afterwards "... and some of them in such a way that they may suffer from pathologic manifestations whose origin is in the black bile". In the past decades the German author Tellenbach studied the personalities of several geniuses, both from fiction, such as Hamlet, and from reality, such as the writer von Kleist, concluding that they suffered from a specific form of depression that he called "Schwermut" (melancholy), which was supposedly different from the narrowly defined illness of depression. Other work done on this subject is the extensive study by the North American author Kay Jamison, who, after researching the biography and the tree of a long list of writers, composers and musicians, concluded that all of them had suffered to some degree from a bipolar disorder. This author finds that, together with other essential features, the geniuses always show forms of experiencing and/or of behaving which do not fall within the range that is considered normal, although they can not always be classified as "pathological".

There are many other examples of geniuses who had suffered from mental malfunctions: Rembrandt (*52*), Vincent van Gogh (*53*), and Leo Tolstoy (*54*).

Increased mental capacity in humans may be modeled in animal experiments, where it should be reflected as increased development of conditioned reflexes. Some articles (*55-59*) suggest this possibility.

As was shown by Meerson *et al.* (*55*), regular training of adult rats in the hypobaric chamber to intermittent effect of altitude hypoxia causes a pronounced activation of protein synthesis and an increase of the DNA concentration in the brain. This activation is accompanied by a better preservation of developed conditioned reflexes of passive avoidance and an increase in the resistance of time relations to the electric shock effect.

It was found that neonatal exposure to intermittent hypoxia enhances the performance of mice in water maze and in 8-arm radial maze tasks (*56*). Intermittent hypoxia was simulated in a hypobaric chamber at 2 km (16.0% O_2) or 5 km (10.8% O_2) for 4 h/day from birth to 1, 2, 3, or 4 week(s), respectively.

Lu *et al.* (*57*) state that mild intermittent hypoxia (16.0% O_2, 4 h/day for 4 weeks) is known to markedly enhance spatial learning and memory in postnatal developing mice. From this observation, they found that Spine-associated Rap-specific GTPase-activating protein is functionally required for synaptic plasticity and contributes to this intermittent hypoxia-induced enhancement.

Shao *et al.* (*58*) investigated the effect of hypoxic preconditioning on spatial cognitive ability in mice after acute and repeated hypoxic exposures. The tolerance time was progressively prolonged as exposure went on.

It was found that conditioning-like brief neonatal hypoxia (100% N_2, 5 min) improved cognitive function and brain tissue properties (*59*). Marked gender dimorphism in adult rats was observed. It was proposed that brief neonatal hypoxia may exert long-term beneficial effects through stimulation of neurogenesis.

The cited experimental animal data may indicate that the stimulating influence of hypoxia is the basis for increased mental capacity, probably, to the level of genius. This would occur very rarely, when the numerous parameters of hypoxic influence combine in an optimal manner. Unfortunately, none of these studies (*55-59*) made even casual mention of the connection between increased mental capacity and mental disorders. No articles were found in which direct research was done on the dependence of mental development (normal, increased, mad) as a function of the power of hypoxia. Such a research could be useful, for example, in breeding smart dogs for special services.

4. Hypoxia in the treatment and prevention of mental diseases

The effects of rarefied air on an organism have been known for a centuries, but the first scientific studies appeared at the end of the 19th century.

It was found much later that breathing air with low oxygen content can be used as a method of hypoxic stimulation. This method is also known as intermittent (interrupted) hypoxic training (therapy) (IHT), normobaric hypoxic training (therapy), hypoxytherapy, and has been used by about 2 million patients for the last 30 years. This drug-free method is almost without contraindications and is applied to help recover from disorders such as bronchial asthma, insomnia, cardiovascular, obstetric, and gynecological disorders, and depression (*60-62*). This method was officially recommended in medicine (*63*) and also applied to increase physical working capacity and endurance, especially in sports (*64,65*). Much literature may be found on the web sites *www.go2altitude.com* (mostly sport) and *www.bionova.ru* (mostly medicine).

The main idea of this method is to fight with hypoxia through hypoxia, *i.e.*, through previous

adaptation to hypoxia by minor, harmless hypoxia. Generally, this approach is similar to the idea of training for any harmful factor, for example, to infection through vaccination. Another training example is jogging, where a demand (load) hypoxia is created. Hypoxia accompanies almost any pathological process and thus hypoxic training to hypoxia is very important.

There are three basic mechanisms underlying the beneficial effects of IHT (*62*): regulation of respiration, free-radical production, and mitochondrial respiration. It was found that IHT induces increased ventilatory sensitivity to hypoxia, as well as other hypoxia-related physiological changes, such as increased hematopoiesis, alveolar ventilation and lung diffusion capacity, and alterations in the autonomic nervous system. Due to IHT, antioxidant defense mechanisms are stimulated, cellular membranes become more stable, Ca^{2+} elimination from the cytoplasm is increased, and O_2 transport in tissues is improved. IHT induces changes within mitochondria, involving NAD-dependent metabolism that increase the efficiency of oxygen utilization in ATP production.

Hypoxic training is not, however, a method of treatment a specific disorder. Rather, hypoxic training is a method to improve general resistance the organism, increasing the possibility of resisting unfavorable factors. For example, the effects of hypoxic training (10% O_2) on increasing the compensatory capabilities of organisms were researched by Strelkov *et al.* (*64*). Experimental data on animals were provided for asphyxia, acute hypoxia with hypercapnia, hemorrhagic shock, physical load, tick-borne encephalitis virus infection, and intoxication by 8 different poisons. Clinical data were provided for gynecological and oncological patients. All data show significant and reliable increase in compensatory capabilities after previous hypoxic training/preconditioning.

The protecting effect of the hypoxic training as a preconditioning procedure was studied in different fields by other researches as well (*66-68*).

The following sections examine issues related to the use of different versions of hypoxic hypoxia for therapy/prevention of mental disorders.

4.1. *Sojourns in the high mountains*

In 1952-1954, expeditions with patients with schizophrenia were conducted in the area of Elbrus (Caucasus) (*69*). Positive but insufficiently strong results were obtained. The study author mentioned that the better result was obtained not when patients were stayed at the mountains, but when they climbed down. In the author's opinion, it was because patients, after acclimatization to the high mountains climate, were better able to use oxygen.

In 1961-1963, expeditions with patients with schizophrenia were conducted in Kirgizia, in the area of Tien Shan (*70-72*). A temporary summer hospital was organized at 3,540 m. Positive results were found for some forms of schizophrenia, but even better effects were observed when patients climbed to 4,000-4,200 m. Of interest among the reasons for those expeditions was the following: "We have noticed that there are extremely few psychiatric patients among the residents of the high mountains".

The effects of high altitude stay on the incidence of common disorders in man were described by Singh *et al.* (*73*). The study involved 130,700 men stationed on the plains between 760 m and sea level, and 20,000 men stationed at altitudes between 3,692 and 5,538 m from 1965 to 1972 (during the Indian-Chinese conflict). A significantly lower number of cases of most disorders, including psychiatric disorders, were found among the men at high altitude than among those at sea level.

4.2. *Hypobaric chamber*

A few articles have been published on studies in the hypobaric chamber. The use of such a chamber instead of sojourns in the high mountains was an attractive factor for the researchers.

Patients entered the hypobaric chamber together with the doctor (*74*). The pressure in the hypobaric chamber decreased gradually up to a "height" of 10,500 m. The doctor was forced to use an oxygen apparatus beginning at a "height" of 5,000 m, whereas patients tolerated the following rarefying easily. There was no "lifting" above 10,500 m, though there were no signs of unconsciousness in the patients. Each session lasted for 1-2 h, and occurred three times a week, for a total of 6-8 sessions on average. There were 16 patients, all with schizophrenia. Only transitory improvement occurred, mainly short-time disinhibition. (*Note*: The patients' amazing tolerance to hypoxia shows that their brains are probably in a condition of strong hypoxia. This important circumstance may be due to excessive pathologic neurogenesis, and further research is needed. – S.B.).

Similar research was performed by Kantorovich (*72*) with the same results.

It was found that intermittent hypobaric hypoxia promotes hippocampal neurogenesis and produces antidepressant-like effects in adult rats (*75*).

4.3. *Normobaric hypoxytherapy*

Normobaric hypoxytherapy as an IHT is the most widespread version of hypoxic training found in general therapy and sport due to its availability and usability.

The first trials of this method in psychiatry were conducted in the United States (*76-80*), mostly with patients who were diagnosed with schizophrenia. The results were initially insignificant but encouraging, but eventually became negligible.

The detailed analysis of the procedure and equipment used in these trials (*81*) showed that the reason for the unsuccessful results was a weak hypoxic influence; improvements to the procedure and equipment were proposed. This conclusion should be considered in any future research on the treatment of mental diseases.

A positive experience in the treatment of depression by mild (9% O_2 and above) hypoxic hypoxia was reported (*82*).

The use of IHT in therapy of endogenous depressions was described by Karimulaev (*83*). The therapeutic procedure was as follows: breathing a hypoxic gas mixture of 10% O_2 through the mask for 3-5 min and then breathing atmospheric air for 3-5 min; this pattern continued for up to 120 min. This procedure has been used as a monotherapy in a group of 51 patients; a positive effect was achieved in 36 patients (71%). Therapeutic effectiveness was positively correlated with the speed of an approach of the therapeutic effect. Sufficient improvement was achieved after 3-4 weeks of treatment in the majority of the participants.

The effect of IHT on postschizophrenic depression has also been studied (*83,84*) with therapeutic effectiveness being achieved in 57% of patients.

4.4. *Hypoxic hypoxia as a preventive means*

Numerous studies have provided evidence for the brain-protective features of hypoxic hypoxia.

The effects of preconditioning using mild repetitive hypobaric hypoxia (360 Torr for 2 h on each of 3 days) have been studied in the learned helplessness model of depression in rats (*85*). The hypoxic preconditioning had a clear antidepressive action returning the behavioural and hormonal parameters to the control values and was equally effective as the antidepressant. The study authors considered the findings to suggest that hypoxic preconditioning is an effective tool for the prophylaxis of post-stress affective pathologies in humans.

The protective effects of hypoxic preconditioning on the development of depressive states in rat models were studied by Rybnikova *et al.* (*86*). Three episodes of intermittent preconditioning using hypobaric hypoxia (360 mmHg, 2 h) prevented the onset of depressive behavioural reactions, hyperfunction of the hypophyseal-adrenal system and impairments in its suppression in the dexamethasone test in rats following unavoidable aversive stress in a model of endogenous depression. The anxiolytic and antidepressant actions of hypoxic preconditioning in experiments on rats were no less marked than those of the tetracyclic antidepressant Ludiomil. The results obtained provide evidence that preconditioning with intermittent hypobaric hypoxia increases resistance to psychoemotional stresses, has marked anxiolytic and antidepressant effects and can be used for the prophylaxis of depressive episodes.

Hormonal mechanisms of neuroprotective effects of the mild hypoxic preconditioning in rats were also studied by Rybnikova *et al.* (*87*).

Hypoxic hypoxia has been found to prevent brain injury and to have a protective role in the central nervous system (*24*). Adult rats were exposed to "high altitudes" of 3,000 and 5,000 m for 4 h per day for 2 consecutive weeks. The study authors were convinced that the proliferation of neural stem cells in the subventricular zone and dentate gyrus may contribute to adaptive changes following intermittent hypoxia.

Regarding the routine practice in public health for the prevention of mental disorders, the most developed type of prevention is secondary prevention, *i.e.*, early intervention (*88-94*). Vaccination (*95,96*), improved prenatal nutrition (*95*) and prenatal multivitamin use (*96*) may be beneficial in primary prevention, but these means have not been shown to substantially prevent mental diseases. The pessimistic conclusion that has been drawn is that "primary prevention is beyond capacity of our present knowledge" (*88*). However, a trend is developing for solving the problem of effective primary prevention of mental diseases by the use of IHT.

As was examined earlier, pre-eclampsia and infection/inflammation during the prenatal period, as well as asphyxia at birth, are closely connected with hypoxia and are the most important triggers of future mental disorders. Therefore, successful treatment or prevention of these conditions will simultaneously prevent mental disorders; *i.e.*, IHT may be considered as a means for the primary prevention of mental disorders.

The research of the development of children born to mothers with pre-eclampsia who have been treated by normobaric hypoxia was conducted by Verbonol and Chizhov (*97*). A hundred women cured by IHT and 50 control women (given conventional treatment) were under care. IHT was carried out at 16-35 weeks of pregnancy and consisted of 8-30 sessions. Each session included 5 min of breathing a hypoxic gas mixture (10% O_2) through mask, interrupted by 5 min of breathing atmospheric air, with a total of 6 cycles in 1 h. All children were under care at birth and monthly during the first year of life. The following parameters were measured: percentage of premature births, Apgar scores, characteristics of physical and neuropsychic development, breastfeeding duration, percentage of children with allergic diathesis, hemoglobin content in child's peripheral blood, and prevalence of acute respiratory disorders. All measured parameters were significantly better in children whose mothers had been treated by IHT.

Oxygen metabolism kinetics was investigated in 90 pregnant females at high risk for pre-eclampsia and associated vascular disorders (*98*). Patients were

treated with IHT. The study revealed that initial disorders of tissue respiration featured compensatory stimulation of tissue oxygen consumption. In early signs of pre-eclampsia the consumption intensity was found to be diminished. During treatment there was evidence of normalization in oxygen metabolism. This treatment proved to be an efficient drug-free method of pre-eclampsia prevention.

The efficiency of preventive use of IHT in pregnant women at high risk of developing of pre-eclampsia was studied by Evgen'eva *et al.* (*99*). The authors focused on a decrease in the incidence of pre-eclampsia, especially its severe patterns, and perinatal mortality.

The use of IHT with 10% O_2 is not only absolutely harmless for the fetus and has no unfavourable effects on the course of the pregnancy or its outcome, but it is also accompanied by a significant increase in the mass of the placenta by 26.9-33.2% and the mass of the fetus by 8.5-12.2% (*60*). Many other clinical data to support the harmlessness of IHT are provided.

The use of IHT in obstetrics was reviewed by Tsyganova (*100*). The literature and the researcher's own investigations showed more successful delivery, less frequent occurrence of nephropathy, fetal hypoxia, premature labor, and better physical condition of newborns.

The use of IHT in obstetrics and gynecological practice was recommended by Russian Health Ministry (*101*).

Hypoxic influence was studied in experiments on pregnant animals (rabbits, rats) conducted during the last third of pregnancy using a hypobaric chamber (*102*). It was established that a moderate hypoxic influence during this period promotes the physiological maturing of the fetus, and the mass of the newborn animals was appreciably increased.

Infection/inflammation during pregnancy, as described earlier, is an important hypoxia-connected risk factor for future mental disorders. In the paper (*64*) infection was mentioned among the numerous harmful influences toward which IHT can increase resistance. Experimental data on mice infected with tick-borne encephalitis virus showed a survival rate of 51.7 ± 5.4% in the main group *versus* 33.3 ± 5.1% in the control group. Therefore, use of IHT for increasing resistance to infection is simultaneously a means for the prevention of mental disorders caused by infection.

Data from the literature (*60,97-101*) related to the general IHT procedure, particularly in obstetrical applications, suggest the following IHT procedure for prevention of mental diseases: one IHT session before pregnancy and one or two sessions during pregnancy after the 16th week. These data clearly show a trend for the successful use of IHT in the primary prevention of mental diseases, but additional studies are needed.

5. Conclusion

Hypoxia plays an important role in almost all environmental risk factors for future mental disorders, acting during early development and capable to stimulate mental disorders in adolescence or young adulthood as a result of pre-eclampsia, infection/inflammation, hypoxia/ischemia, preterm birth, or asphyxia at birth.

Hypoxia stimulates neurogenesis. Excessive pathologic neurogenesis becomes apparent from the changing size of the defined neuronal network zones or from changing their structure, for example, increased neuronal density. The changes in the brain stimulated by pathologic neurogenesis may lead to abnormal communications in the neural network, which causes abnormal associations, ideas, and acts, *i.e.*, mental disorders.

Although more studies need to be done, hypoxic hypoxia, especially in the form of IHT, may have applications in increasing mental capacity of animals and in the treatment and primary prevention of mental disorders.

Acknowledgements

The author thanks Violetta Bassovitch and Barbara Every for their editorial assistance.

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(Received November 4, 2010; Revised November 23, 2010; Accepted November 24, 2010)

Review

Chinese herbal medicines as adjuvant treatment during chemo- or radio-therapy for cancer

Fanghua Qi[1,2], Anyuan Li[1,*], Yoshinori Inagaki[2], Jianjun Gao[2], Jijun Li[1], Norihiro Kokudo[2], Xiao-Kang Li[3], Wei Tang[2,*]

[1] *Department of Traditional Chinese Medicine, Shandong Provincial Hospital affiliated with Shandong University, Ji'nan, China;*
[2] *Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[3] *Division of Radiation Safety and Immune Tolerance, National Research Institute for Child Health and Development, Tokyo, Japan.*

Summary **Numerous studies have indicated that in cancer treatment Chinese herbal medicines in combination with chemo- or radio-therapy can be used to enhance the efficacy of and diminish the side effects and complications caused by chemo- and radio-therapy. Therefore, an understanding of Chinese herbal medicines is needed by physicians and other health care providers. This review provides evidence for use of Chinese herbal medicines as adjuvant cancer treatment during chemo- or radio-therapy. First, Chinese herbal medicines (*e.g.* Astragalus, Turmeric, Ginseng, TJ-41, PHY906, Huachansu injection, and Kanglaite injection) that are commonly used by cancer patients for treating the cancer and/or reducing the toxicity induced by chemo- or radio-therapy are discussed. Preclinical and clinical studies have shown that these Chinese herbal medicines possess great advantages in terms of suppressing tumor progression, increasing the sensitivity of chemo- and radio-therapeutics, improving an organism's immune system function, and lessening the damage caused by chemo- and radio-therapeutics. Second, clinical trials of Chinese herbal medicines as adjuvant cancer treatment are reviewed. By reducing side effects and complications during chemo- and radio-therapy, these Chinese herbal medicines have a significant effect on reducing cancer-related fatigue and pain, improving respiratory tract infections and gastrointestinal side effects including diarrhea, nausea, and vomiting, protecting liver function, and even ameliorating the symptoms of cachexia. This review should contribute to an understanding of Chinese herbal medicines as adjuvant treatment for cancer and provide useful information for the development of more effective anti-cancer drugs.**

Keywords: Chinese herbal medicine, adjuvant treatment, chemotherapy, radiotherapy

1. Introduction

Cancer has emerged as a major global public health problem (*1*). Its incidence and mortality rates continue to rise. A report released by the World Health Organization (WHO) shows that in 2008 an estimated 12.7 million people were diagnosed with cancer and 7.6 million people died from cancer worldwide. The WHO predicts that by 2030 an estimated 21.4 million new cases of cancer and 13.2 million cancer deaths will occur annually around the world (*2*). Surgery, chemotherapy, and radiotherapy are still the major conventional cancer therapies. However, these therapies have numerous limitations and drawbacks: *i*) given poor diagnosis and other factors, most cancer patients are diagnosed too late to undergo surgery; *ii*) most cancers have a postoperative survival rate of less than 5 years and recurrence is quite common in patients who have had a resection; *iii*) although chemotherapy and radiotherapy are effective against cancer, they also have serious side effects and

**Address correspondence to:*
Dr. Anyuan Li, Department of Traditional Chinese Medicine, Shandong Provincial Hospital, Shandong University, No. 324, Jingwuweiqi Road, Ji'nan 250021, Shandong, China.
e-mail: sdslyy999@163.com
Dr. Wei Tang, Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
e-mail: TANG-SUR@h.u-tokyo.ac.jp

complications (*e.g.* fatigue, pain, diarrhea, nausea, vomiting, and hair loss); and *iv*) since some cancers are relatively chemo- or radio-resistant and highly refractory to cytotoxic chemotherapy or radiotherapy, systemic cytotoxic chemotherapy and radiotherapy are minimally effective at improving patient survival (*3,4*). Therefore, more effective therapies or combination therapies must soon be developed to treat cancer.

Over the past few years, use of complementary and alternative medicine (CAM) has become increasingly popular among cancer patients in Western countries with a prevalence as high as 80% (*5,6*). Traditional Chinese medicine (TCM) and herbal medicines in particular have been used in the treatment of cancer for thousands of years in China, Japan, and other Asian countries. These medicines are widely accepted as current forms of CAM in cancer treatment in the United States and Europe (*7,8*). As recent pre-clinical and clinical studies have shown, TCM combined with conventional Western medicine (chemotherapy and radiotherapy) can provide effective supportive care for cancer patients. TCM has great advantages in terms of increasing the sensitivity of chemo- and radio-therapeutics, reducing the side effects and complications associated with chemotherapy and radiotherapy, and improving patient quality of life and survival time (*9*).

Therefore, an understanding of Chinese herbal medicines is needed by physicians and other health care providers. This review provides evidence for use of Chinese herbal medicines as adjuvant cancer treatment during chemo- or radio-therapy. First, some Chinese herbal medicines (*e.g.* Astragalus, Turmeric, Ginseng, TJ-41, PHY906, Huachansu injection, and Kanglaite injection) that are commonly used by cancer patients to treat the cancer and/or reduce the toxicity induced by chemo- or radio-therapy are discussed. Second, clinical trials of Chinese herbal medicines as adjuvant cancer treatment to reduce the side effects and complications during chemo- and radio-therapy are reviewed. This review should contribute to an understanding of Chinese herbal medicines as adjuvant treatment for cancer and provide useful information for the development of more effective anti-cancer drugs.

2. Chinese herbal medicines commonly used as adjuvant treatment in cancer therapy

Chinese herbal medicines have been used in the treatment of a variety of diseases in China, Japan, South Korea, and other Asian countries for thousands of years (*10*). The biological ingredients of herbal medicines are mainly extracted from plants, animal parts, shells, insects, and even stones and minerals (*11*). The herbal medicines in current use are usually classified as single herbs, compound formulations (a combination of several herbs), and Chinese medicine preparations (*12*). In recent decades, a large number of herbal medicines including single herbs, traditional herbal formulations, and Chinese medicine preparations have been used by cancer patients around the world, and especially in China. Numerous basic and clinical studies have been conducted in order to identify effective anticancer agents in Chinese herbal medicines and ascertain their properties as relate to the treatment of cancer. Several herbal medicines have been found to have potentially beneficial effects on cancer progression and may ameliorate chemotherapy- or radiotherapy-induced complications and side effects (*9,13*). Therefore, the anticancer pharmacology of the Chinese herbal medicines most commonly used as adjuvant treatment in cancer therapy must be understood.

2.1. *Single herbs*

Several single herbs have been found to have a potentially beneficial effect at treating cancer. A brief outline on the oncologic pharmacology of the most commonly used ingredients is presented below (Table 1).

Radix Astragali (*Astragalus propinquus*, huangqi) has been used in China for thousands of years. It is traditionally considered to be a tonic that can improve the functioning of the lungs, adrenal glands, and the gastrointestinal tract, increase metabolism, promote healing, and reduce fatigue (*14*). Currently, much of the pharmacological research has shown that Astragalus has potent immunomodulatory properties that include increasing the production of interferon, tumor necrosis factor (TNF), activating lymphocytes, natural killer cells, and macrophages (*15*). Astragalus has also been shown to be an adjunct anticancer agent that increases resistance to the immunosuppressive effects of chemotherapy drugs while stimulating macrophages to produce interleukin (IL)-6 and TNF (*16*). Astragalus in combination with recombinant IL-2 is capable of enhancing the anticancer activity of recombinant IL-2-generated lymphokine-activated killer (LAK) cells on murine renal carcinoma cells and reducing the severe side effects of recombinant IL-2 therapy (*e.g.* acute renal failure, capillary leakage syndrome, myocardial infarction, and fluid retention) in the treatment of cancer patients (*17*).

Turmeric (*Curcuma longa*, jianghuang), a rhizomatous herbaceous perennial plant of the ginger family (Zingiberaceae), has a long history of use in Asia as a treatment for inflammatory conditions. Curcumin, a yellow natural polyphenol, is the primary active constituent of turmeric and has been noted to have numerous pharmacological activities including anti-inflammatory, antioxidant, and anticancer properties (*18*). Pre-clinical cancer research using curcumin has shown that it inhibits carcinogenesis

Table 1. Single herbs commonly used in cancer treatment

Common name	Other names	Latin name	Major active ingredients	Biological activity	Evidence of anticancer activity	*Ref.*
Radix astragali	Huang qi; Milk vetch	*Astragalus membranaceus*	Polysaccharides, saponins, flavonoids	Immunomodulatory, anticancer, antiviral	*Precinical:* Stimulates the production of IL-6 and TNF and enhances the activity of LAK cells *Clinical:* Potentiates the activity of chemo-therapeutic agents, prolongs survival, reduces the adverse toxicity of chemo- or radio-therapy	*15-17*
Turmeric	Jianghuang	*Curcuma longa*	Curcumin, demethoxycurcumin, bisdemethoxycurcumin	Anti-inflammatory, antioxidant, anticancer	*Precinical:* Inhibits carcinogenesis and modulates chemo-resistance and radio-resistance *Clinical:* Potentiates the anti-tumor effect of chemotherapeutic agents	*18-21*
Ginseng	Rensen; Panax	*Panax Ginseng*	Triterpene glycosides, ginsenosides	Immune-modulation, vasorelaxation, antioxidation, anti-inflammation, anticancer	*Precinical:* Inhibits cancer growth and potentiates the anti-tumor effect of chemotherapeutic agents *Clinical:* Potentiates the anti-tumor effect of chemotherapeutic agents and attenuates the adverse toxicity of chemo- or radio-therapy	*22-25*
Garlic	Dasuan	*Allium sativum*	Allicin, alliin	Anti-bacterial, anti-hypertensive, anti-thrombotic, anticancer, immuno-stimulant, hyperglycemic	*Precinical:* Inhibit cancer growth by inducing differentiation and apoptosis and scavenging carcinogen-induced free radicals *Clinical:* Garlic reduces the incidence of stomach, esophageal, and colorectal cancer	*26-28*
Mylabris	Banmao	*Mylabris phalerata*	Cantharidin	Anticancer	*Precinical:* Inhibit cancer growth by inducing cell apoptosis and regulating the immune system *Clinical:* No reports in English	*29,30*
Toad venom	Chansu	*Bufo bufo gargarizans* Cantor	Bufadienolides	Cardiotonic, antimicrobial, local anesthetic, analgesic, anticancer	*Precinical:* Inhibits cancer growth by inhibition of cell proliferation and induction of cell apoptosis *Clinical:* No reports in English	*12,31-33*

in a number of cancers (*e.g.* colorectal, pancreatic, gastric, prostate, and hepatic) and at various stage of carcinogenesis (*e.g.* proliferation, angiogenesis, and metastasis) (*19*). Curcumin has also been found to be a chemo-sensitizer that enhances the activity of other anticancer agents in the treatment of chemo-resistant and multidrug-resistant (MDR) cancer (*20*). Furthermore, curcumin has a radio-sensitizing and radio-protective effect on cancer cells. Curcumin in combination with radiation has been found to significantly enhance radiation-induced inhibition and apoptosis of the prostate cancer cell line PC3 (*21*).

Ginseng (*Panax ginseng*) is one of the most widely used herbal medicines and has been used as a restorative tonic in China, Japan, and South Korea for thousands of years. It can improve circulation, increase blood supply, and aid recovery from weakness after illness (*22*). Currently, much of the pharmacological research has shown that Ginseng has potent immune modulation, vasorelaxation, anti-oxidation, anti-inflammation, and anticancer properties. Ginseng has potential as a chemo-preventive agent or adjuvant treatment in stomach, liver, pancreas, and colon cancer by inhibiting the inflammation-to-cancer sequence (*23*). Ginseng appears to be a promising radio-protector and is capable of attenuating the deleterious effects of radiation on normal human tissue, and especially for cancer patients undergoing radiotherapy. This activity may be associated with its anti-oxidation and immune modulation properties (*24*). In addition, an epidemiological study indicated that patients taking ginseng had a 50% lower risk of cancer recurrence compared to patients not taking ginseng (*25*).

Garlic (*Allium sativum*) has been used for medicinal purposes for thousands of years. Historically, garlic has been used to treat infections, diarrhea, rheumatism, and snakebites. Currently, much of the pharmacological research has shown that garlic has anti-bacterial, anti-hypertensive, anti-thrombotic, anticancer, immuno-stimulant, and hyperglycemic activity (*26*). Garlic contains a high concentration of sulfur-containing compounds (*e.g.* allicin and alliin) that appear to be the active substances in garlic. Numerous preclinical studies have shown that garlic and its active constituents have an anticancer effect on various tumors and especially on colon tumors by

controlling DNA repair, inhibiting cell proliferation and angiogenesis, inducing differentiation and apoptosis, inhibiting metabolism, and scavenging carcinogen-induced free radicals (*27,28*). In addition, epidemiological studies have shown a decreased risk of stomach, esophageal, and colorectal cancer with increased consumption of garlic (*27*).

Mylabris (*Mylabris phalerata*) is the dried body of the Chinese blister beetle and has been used as a Chinese herbal remedy for more than 2,000 years. The active constituent of mylabris is cantharidin (*29*). Recent preclinical studies have shown that mylabris and cantharidin have anticancer activity on various cancers and especially on liver and esophageal cancer by inducing cell apoptosis and regulating the immune system (*29,30*). However, their toxicity on the renal system and suppression of bone marrow limits their clinical usage. Therefore, several modified cantharidin analogues (*e.g.* norcantharidin) have been synthesized chemically in order to achieve anticancer properties comparable to the original compound but with less of a toxic effect on non-cancer cells. Such analogues may be more suitable for medical investigation than cantharidin itself (*30*).

Toad venom, known as Chansu in China, is obtained from the postauricular and skin glands of the toad (*Bufo bufo gargarizans* Cantor). Chansu has been widely used as an anodyne, cardiotonic, antimicrobial, local anesthetic, and antineoplastic agent in China and other Asian countries for thousands of years (*12*). It is the major component of several popular traditional Chinese medications such as Liushenwan, Shexiangbaoxinwan, and Niuhuangxiaoyanwan. These Chinese medications have long been used in China, Japan, Korea, and other Asian countries and are currently used as alternative medicines. Bufadienolides, such as bufalin, cinobufagin, resibufogenin, and telocinobufagin, are the major active constituents derived from Chansu (*31*). Preclinical studies of Chansu and its constituents have shown they have a potent anticancer effect on leukemia and liver, lung, and prostate cancers by inhibiting cell proliferation and inducing cell apoptosis (*32*). In addition, some Chinese medicine preparations (*e.g.* Huachansu) containing Chansu have been prepared and are widely used in clinical cancer treatment in China (*33*).

2.2. *Traditional herbal formulations*

Traditional herbal formulations (or Kampo in Japanese) are compound formulations that mostly come from Shang Han Lun and Jin Gui Yao Lue, two classics of traditional medicine edited by Zhang Zhongjing, a well-known Chinese physician during the Han Dynasty (*34*). A brief outline on the oncologic pharmacology of the most commonly used traditional herbal formulations is presented below (Table 2).

TJ-41 (Bu-Zhong-Yi-Qi-Tang in Chinese, Hochu-ekki-to in Japanese or Bojungikki-Tang in Korean) is a traditional herbal formulation widely used in China, Japan, and South Korea. It contains 7 herbs including *Pinellia tuber*, *Scutellaria baicalensis*, *Zingiberis rhizoma*, *Zizyphi fructus*, *Coptidis rhizoma*, *Glycyrrhiza radix*, and *Panax ginseng* (*35*). Currently, much of the pharmacological research has shown that TJ-41 has potent immunomodulatory and anticancer properties. TJ-41 has a significant chemo-preventative effect on ovarian and liver cancer lines by inducing apoptosis and arresting the cell cycle (*36,37*). The oral administration of TJ-41 is able to enhance concomitant immunity against tumor development and restore the antitumor T cell response in tumor-bearing mice (*38*). In addition, TJ-41 has been shown to reduce the extent of side effects such as leucopenia and intestinal damage and fatigue occurring as a result of radiation or chemotherapy to treat malignant tumors (*39,40*).

TJ-48 (Shi-Quan-Da-Bu-Tang in Chinese and Juzen-taiho-to in Japanese) is a famous traditional herbal formulation that has long been used to treat anemia, anorexia, extreme exhaustion, and fatigue. It contains 10 herbs including *Angelica sinensis*, *Paeonia lactiflora*, *Atractylodes macrocephala*, *Poria cocos*, *Cinnamomum cassia*, *Astragalus membranaceus*, *Liqusticum wallichii*, *Glycyrrhiza inflate*, and *Rehmannia glutinosa* (*41*). Currently, TJ-48 has been shown to have an antitumor effect on various cancers (*e.g.* endometrial carcinoma and malignant glioma) by regulating estrogen receptors or enhancing systemic immunological function (*42,43*). Furthermore, TJ-48 has the advantage of minimal toxicity in combination with chemotherapy or radiation therapy. The combination of TJ-48 and mitomycin C (MMC) resulted in significantly longer survival in p-388 tumor-bearing mice than MMC alone, and TJ-48 decreased the diverse effects of MMC such as leukopenia, thrombopenia, and weight loss (*41*).

PHY906 is a modified pharmaceutical preparation derived from the traditional herbal formulation Huang-Qin-Tang, which has been used for over 1,800 years in the Orient to treat a wide range of gastrointestinal symptoms, including nausea, vomiting, cramping, and diarrhea. PHY906 consists of four commonly used herbs, *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall, *Glycyrrhiza uralensis* Fisch, and *Ziziphus jujube* Mill, at a ratio of 3:2:2:2 (*44*). Numerous studies have shown that PHY906 not only reduces gastrointestinal toxicity and enhances the antitumor efficacy of some anticancer drugs but also alleviates chemotherapy-induced side effects, such as diarrhea. Clinical trials indicate that PHY906 can serve as an adjuvant to CTP-11, 5-fluorouracil (5-FU), leucovorin (LV), and capecitabine in the treatment of advanced colorectal, pancreatic, and liver cancer (*45-47*). PHY906 treatment results in a significant decrease in patient nausea and diarrhea, and no PHY906-associated toxicity has been observed.

Table 2. Traditional herbal formulations commonly used in cancer treatment

Common name	Other names	Composition	Biological activity	Evidence of anticancer activity	*Ref.*
TJ-41	Bu-Zhong-Yi-Qi-Tang; Hochu-ekki-to; Bojungikki-Tang	Includes 7 herbs: *Pinellia tuber*, *Scutellaria baicalensis*, *Zingiberis rhizoma*, *Zizyphi fructus*, *Coptidis rhizoma*, *Glycyrrhiza radix*, *Panax ginseng*	Immunomodulatory, anticancer	*Precinical:* Inhibits cancer growth by inducing apoptosis, arresting the cell cycle, and enhancing immunity *Clinical:* Attenuates the adverse toxicity of chemo- or radio-therapy	*35-40*
TJ-48	Shi-Quan-Da-Bu-Tang; Juzen-taiho-to	Includes 10 herbs: *Angelica sinensis*, *Paeonia lactiflora*, *Atractylodes macrocephala*, *Poria cocos*, *Cinnamomum cassia*, *Astragalus membranaceus*, *Liqusticum wallichii*, *Glycyrrhiza inflata*, *Rehmannia glutinosa*	Immunomodulatory, anticancer	*Precinical:* Inhibits cancer growth by regulation of estrogen receptors or enhancement of systemic immunological function *Clinical:* Improves quality of life and survival	*41-43*
PHY906	Huang-Qin-Tang	Includes 4 herbs: *Scutellaria baicalensis* Georgi, *Paeonia lactiflora* Pall, *Glycyrrhiza uralensis* Fisch, *Ziziphus jujube* Mill	Anti-inflammatory, anticancer	*Precinical:* Enhances the antitumor efficacy of some anticancer drugs and alleviates chemotherapy-induced side effects, such as diarrhea *Clinical:* Potentiates the anti-tumor effect of chemotherapeutic agents and attenuates chemotherapy-induced side effects	*44-47*

2.3. *Chinese medicine preparations*

Chinese medicine preparations are a form of Chinese herbal medicine that are isolated from single herbs or traditional herbal formulations and that are prepared using modern advanced pharmaceutical technology. There are various dosage forms including injections, tablets, pills, capsules, and liquids. Compared to traditional decoctions, Chinese medicine preparations are safer, more effective, and easier to use (*12*). Thus, Chinese medicine preparations are becoming increasingly popular in China and are attracting worldwide attention. A brief outline on the oncologic pharmacology of the most commonly used Chinese medicine preparations that have been approved by the State Food and Drug Administration (FDA) of China is briefly presented below (Table 3).

Huachansu (Cinobufacini) injection, an aqueous extract from the skin and parotid venom glands of the toad (Bufo bufo gargarizans Cantor) that contains Chansu, is a Chinese medicine preparation widely used in clinical cancer therapy in China (*33*). Cardiac glycosides including bufalin, resibufogenin, and cinobufagin are the three major active constituents to which the antitumor activity of huachansu may be attributed (*48,49*). Pre-clinical studies have shown that huachansu effectively inhibits growth and has anti-hepatitis B virus (HBV) activity on human hepatocellular carcinoma (HCC) cells (*50-52*). Huachansu induces apoptosis of HCC cell lines HepG2 and Bel-7402 *via* a mitochondria-mediated apoptotic pathway (*51*). Clinical data showed that Cinobufacini used alone or in combination with other chemotherapeutic agents (*e.g.* gemcitabine and oxaliplatin) had significant anticancer activity against human cancers, such as HCC, non-small-cell lung cancer, pancreatic cancer, and gallbladder carcinoma (*33,53*). A pilot study of huachansu in patients with HCC, non-small-cell lung cancer, and pancreatic cancer showed that huachansu improved the quality of life of patients and even reduced tumor shrinkage with little toxicity (*33*). Another clinical study using huachansu in combination with gemcitabine and oxaliplatin in treating gallbladder carcinomas showed that huachansu substantially enhanced the antitumor efficacy of gemcitabine and oxaliplatin and improved the quality of life of patients (*53*).

Kanglaite injection is an acetone extract of herbal medicine coix seed (Semen Coicis Yokuinin) prepared as an herbal medicine using modern advanced pharmaceutical technology. The injection has been approved for the treatment of lung and hepatic cancer in China (*35*). Preclinical experiments have shown that kanglaite may inhibit tumor cell mitosis at the boundary of the G2/M phase of the cell cycle and induce apoptosis through activation of the Fas/FasL pathway (*54*). A phase I study of Kanglaite in 16 patients with refractory solid tumors (*e.g.* lung, colon, prostate, and esophageal cancer) was conducted at the Huntsman Cancer Institute (Salt Lake City, UT, USA) and no dose-limiting hematologic or symptomatic toxicity was observed in the first cycle at a maximum dose of up to 50,000 mg/day (*55*). A recent randomized phase II study at the Shanghai Cancer Hospital of Fudan University

Table 3. Chinese medicine preparations commonly used in cancer treatment

Common name	Composition	Biological activity	Evidence of anticancer activity	*Ref.*
Huachansu injection	*Bufo bufo gargarizans* Cantor	Anticancer, anti-HBV	*Precinical:* Induces cancer cell apoptosis through a mitochondria-mediated apoptotic pathway	*33,48-52*
			Clinical: Potentiates the anti-tumor effect of chemotherapeutic agents, attenuates chemotherapy-induced side effects, improves quality of life and survival	
Kanglaite injection	*Semen Coicis*	Anticancer	*Precinical:* Inhibits the cell cycle in the G2/M phase and induces apoptosis through activation of the Fas/FasL pathway	*35,53-55*
			Clinical: Potentiates the anti-tumor effect of chemotherapeutic agents	
Shenqi fuzheng injection	*Astragalus propinquus*, *Codonopsis pilosula*	Immunomodulatory, anticancer	*Precinical:* Potentiates the anti-tumor effect of chemotherapeutic agent and attenuates chemotherapy-induced side effects	*56,57*
			Clinical: Potentiates the anti-tumor effect of chemotherapeutic agents, attenuates chemotherapy-induced side effects, improves quality of life and survival	

(Shanghai, China) used an MMC/cisplatin (DDP) regimen in combination with kanglaite as a salvage treatment for patients with advanced breast cancer (n = 60) (*56*). Results indicated that there was no additional benefit when kanglaite was added to the MMC/DDP combination in the management of breast cancer, so the effect of kanglaite on cancer will be evaluated further in a phase II study.

The Shenqi fuzheng injection is isolated from two kinds of herbal medicines, Astragalus propinquus (huangqi) and Codonopsis pilosula (dangshen), using modern advanced pharmaceutical technology. The injection has been approved by China's FDA primarily as an antitumor injection since the 1990s (*57*). Currently, many trials have studied the shenqi fuzheng injection in combination with chemotherapy (*e.g.* 5-FU and cisplatin) in patients with lung, breast, and colorectal cancer; some have shown that the shenqi fuzheng injection may play an important role in the treatment of advanced cancers by improving tumor response and reducing the toxicity of chemotherapy (*57,58*). However, most of these trials were conducted in China and little is known about use of the shenqi fuzheng injection outside of China. Thus, the mechanisms of the injection's action must be investigated and the injection must be clinically evaluated further.

3. Clinical trials of Chinese herbal medicines as adjuvant treatment in cancer therapy

In conventional Western medicine, chemotherapy and radiotherapy are major conventional cancer therapies. These therapies are directed at killing or eradicating cancer cells. Unfortunately, distinguishing between cancer cells and normal healthy cells is difficult for most cancer treatments, leading to the damage of normal cells (*59,60*). The results of this damage are called complications and side effects of cancer treatment and mainly include fatigue, pain, infection/fever, anemia, diarrhea, nausea and vomiting, hair loss, and bone marrow suppression (*61*). These complications and side effects inconvenience and cause discomfort to patients and they may also limit or prevent the delivery of therapy at its optimal dose and time, potentially causing fatalities (*62*). Thus, more effective therapies to help prevent and control the complications and side effects of conventional cancer therapy must soon be developed. Several Chinese herbal medicines have been found to be adjunctive in chemo- and radio-therapy. However, numerous clinical trials have been published only in China or other Asian countries and cannot be found on PubMed. Thus, reports of clinical trials published on PubMed were searched to provide a brief outline on the use of Chinese herbal medicines to reduce complications and side effects associated with conventional cancer therapy (Table 4).

3.1. *Fatigue*

Fatigue is regarded as a highly prevalent and unavoidable side effect experienced during the course of cancer and chemo- or radio-treatment. Many studies have found that the prevalence rates of fatigue in cancer patients exceed 60% (*63*). Cancer-related fatigue significantly interferes with patients' daily activities and decreases their quality of life. However, it remains under-recognized and under-treated, partly because of limited understanding of its pathophysiology and lack of effective treatments (*64*). Several Chinese herbal medicines may have beneficial effects on cancer-related fatigue and quality of life for cancer patients. In a pilot randomized clinical trial, 40 patients with cancer-related fatigue were randomized into an experimental group treated with Bojungikki-tang for 2 weeks and a control group without any intervention (*40*). The participants in the trial included breast cancer patients (n = 11, 27.5%), colon cancer patients (n = 5, 12.5%),

Table 4. Clinical trials of Chinese herbal medicines as adjuvant treatment to reduce complications and side effects

Complications and side effects	Patients	Experimental group	Control group	Outcomes	*Ref.*
Fatigue	$n = 40$ (breast, stomach, and colon cancer)	TJ-41 + chemotherapy or radiotherapy	Chemotherapy or radiotherapy	Fatigue level increased (experimental group *versus* control group, $p < 0.05$).	*40*
Pain	$n = 250$	Kang-Fu-Zhi-Tong adhesive plaster	Morphine	The analgesic effect was equivalent in the 2 groups ($p > 0.05$). The analgesia time was prolonged significantly (experimental group *versus* control group, $p < 0.001$).	*66*
Radiation pneumonitis	$n = 100$ (lung cancer)	Liangxue Jiedu Huoxue Decoction + radiotherapy	Radiotherapy	The incidence rate of radiation pneumonitis was lower in the treatment group than in the control group (13.04% *versus* 33.33%, $p < 0.05$).	*66*
Gastrointestinal side effects (diarrhea, nausea, and vomiting)	$n = 24$	PHY906	Chemotherapy (capecitabine)	Some gastrointestinal side effects such as diarrhea were reduced.	*70*
Hepatotoxicity	$n = 84$	Chinese herbal formulations (Xiao-Chai-Hu-Tang, Huang-Lian-Jie-Du-Tang or Yin-Chen-Wu-Ling-San) + chemotherapy	Chemotherapy	The serum levels of ALT and AST in combination treatment group were lower than in the control group.	*72*
Cachexia	$n = 22$	Atractylenolide I	Fish-oil	Atractylenolide I ameliorated the symptoms of gastric cancer cachexia	*74*
Idiopathic sweating	$n = 32$	Yu-Ping-Feng-San	Nothing	Twenty-six patients (81.3%) had cessation of sweating during or after treatment	*75*

stomach cancer patients ($n = 5$, 12.5%), lung cancer patients ($n = 5$, 12.5%), and patients with other cancers ($n = 14$, 35.0%). Before the trial started, participants had undergone chemotherapy or radiotherapy for nearly one and a half years. Results showed that the fatigue level in the experimental group improved significantly compared to that in the control group ($p < 0.05$). Furthermore, liver and kidney function (the serum levels of alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine) were measured to test the toxicity of Bojungikki-tang and no serious adverse effects occurred during the trial. However, more rigorous trials are needed to confirm the efficacy of Bojungikki-tang and other herbal medicines on cancer-related fatigue.

3.2. *Pain*

Pain is a common symptom of cancer and the causes of pain can be disease or treatment-related (*e.g.* surgery, chemotherapy, or radiotherapy). The prevalence of pain in patients with cancer has been reported to be between 50% and 70% during cancer treatment (*65*). As indicated in current WHO guidelines, combined treatments are the standard of care for cancer pain (*66*). Trials have suggested that Chinese herbal medicines may be effective at treating cancer pain and that their effects are similar to those of Western analgesics. Chinese herbal medicines may reduce the side effects of conventional analgesics, thus enhancing cancer patients' quality of life (*67*). In a randomized controlled clinical trial, 250 patients with cancer pain were randomized into an experimental group treated with a Chinese medicine preparation in the form of Kang-Fu-Zhi-Tong adhesive plaster ($n = 182$) and a control group treated with morphine ($n = 68$) (*67*). The results showed the analgesic effect in the 2 groups was equivalent after 3 days of treatment ($p > 0.05$). However, the analgesia time was significantly prolonged in the herbal treatment group compared to that in the morphine group ($p < 0.001$).

3.3. *Respiratory tract infections*

Radiation pneumonitis is one of the most common complications during radiotherapy for thoracic tumors. It impacts the quality of life of patients and is life-threatening. Although corticosteroid therapy is useful in the treatment of acute pneumonitis, it causes numerous side effects (*68*). Clinical trials have suggested that Chinese herbal medicines may be effective at treating radiation pneumonitis with few side effects. In a prospective randomized clinical study, 100 lung cancer patients scheduled to receive radiotherapy were randomly divided into a treatment group (Liangxue Jiedu Huoxue Decoction

+ radiotherapy) and control group (radiotherapy) with 50 patients in each group (*69*). Results showed that the incidence rate of radiation pneumonitis was lower in the treatment group than in the control group (13.04% *versus* 33.33%, $p < 0.05$). Furthermore, the extent of lung injures and the symptoms of radiation pneumonitis improved in the treatment group.

3.4. *Gastrointestinal side effects*

Gastrointestinal side effects including diarrhea, nausea, and vomiting are the most common symptoms occurring in patients receiving chemo- or radio-therapy (*70*). However, there is still no effective treatment to ameliorate diarrhea, nausea, or vomiting in cancer patients. Recently, clinical trials have suggested that Chinese herbal medicines may be effective at treating these side effects. A phase study was conducted using PHY906 in combination with capecitabine in patients with advanced pancreatic and gastrointestinal malignancies (*71*). Twenty-four cancer patients were randomly divided into 4 groups and treated with different concentrations of capecitabine (1,000, 1,250, 1,500, and 1,750 mg/m^2, bid) in combination with PHY906 (800 mg, bid) for 14 days. Results showed that there was no dose-limiting toxicity at the maximum dose level of 1,750 mg/m^2 and some gastrointestinal side effects such as diarrhea were reduced. These findings suggest that PHY906 increased the therapeutic index of capecitabine in patients by reducing its side effects.

3.5. *Hepatotoxicity*

Hepatotoxicity is a common side effect of chemotherapy. Its prevalence ranges from 33 to 65.6% among patients with cancer, and up to 30% of patients have grade III or IV hepatotoxicity (*72*). If hepatotoxicity is severe (as indicated by AST and ALT levels), chemotherapy may be canceled or delayed for some cancer patients. However, there are no drugs that effectively protect liver function and elevation of liver enzymes is sometimes accompanied by severe thrombocytopenia and other aggravated side effects (*73*). Chinese herbal medicines may be effective at improving hepatotoxicity. A case-control study was conducted using the medical records of 89 patients with cancer who received a total of 184 courses of chemotherapy. Of the 184 courses, 42 in which Chinese herbal formulations (Xiao-Chai-Hu-Tang, Huang-Lian-Jie-Du-Tang or Yin-Chen-Wu-Ling-San) were used in combination with chemotherapy served as the experimental group while the remaining 142 courses served as the control group (*73*). Results showed that the combined treatment group had lower serum levels of ALT and AST than did the control group. This suggests that use of Chinese herbal medicines might result in the protection of liver function during chemotherapy.

3.6. *Other complications or side effects*

Cachexia is a syndrome characterized by body weight loss and metabolic abnormalities. It is responsible for 22% of all cancer patients' deaths and is associated with a shorter survival period and reduced quality of life (*74*). However, no effective systemic anticancer therapy is available, and the toxicity of conventional chemotherapy may diminish a patient's nutritional status. Therefore, a novel agent to improve the symptoms of cachexia and quality of life for patients with advanced cancer must be identified. A randomized pilot study of atractylenolide I (the main bioactive chemical compound of the Chinese herb *Rhizoma atractylodis*) was conducted in patients with gastric cancer-related cachexia (*75*). A total of 22 cancer patients were randomly divided into 2 groups: a group given atractylenolide I and a control group given fish oil for 7 weeks. Results showed that atractylenolide I ameliorated the symptoms of gastric cancer-related cachexia (as gauged by parameters such as appetite, body weight, and mid-arm muscle circumference) presumably by mediating cytokine (IL-1, IL-6, and TNF-α) production and inhibiting proteolysis-inducing factor.

End-stage cancer patients frequently suffer from idiopathic sweating that may be associated with greater susceptibility to upper airway infections and subsequent sepsis. The traditional Chinese herbal formulation Yu-Ping-Feng-San includes three herbs (*Saposhnikovia divaricata*, *Rhizoma atractylodis*, and *Astragalus propinquus*) and has been used in traditional Chinese medicine for more than 400 years to manage sweating. A prospective clinical study was conducted to evaluate the effect of Yu-Ping-Feng-San on end-stage cancer patients ($n = 32$) with idiopathic sweating and adverse reactions (*76*). Quantitative measurement of sweating showed that 26 patients (81.3%) had ceased idiopathic sweating during or after treatment. This study indicated that Yu-Ping-Feng-San may be effective at relieving idiopathic sweating in end-stage cancer patients. However, a randomized, double-blinded clinical trial should be conducted to verify this therapeutic effect.

4. Conclusion

In conclusion, Chinese herbal medicines substantially influence cancer therapy as adjuvant treatment. In cancer treatment, Chinese herbal medicines in combination with chemo- or radio-therapy are capable of enhancing the efficacy of and diminishing the side effects and complications caused by chemo- and radio-therapy. Chinese herbal medicines (*e.g.* Astragalus, Turmeric, Ginseng, TJ-41, PHY906, Huachansu injection, and Kanglaite injection) have great

advantages in terms of suppressing tumor progression, increasing the sensitivity of chemo- and radio-therapeutics, improving an organism's immune system function, and lessening the damage caused by chemo- and radio-therapeutics. They have a significant effect on reducing cancer-related fatigue and pain, improving respiratory tract infections and gastrointestinal side effects including diarrhea, nausea, and vomiting, protecting liver function, and even ameliorating the symptoms of cachexia. This review of those medicines should contribute to an understanding of Chinese herbal medicines as adjuvant treatment for cancer and provide useful information for the development of more effective anti-cancer drugs. However, randomized, effective Chinese herbal medicines must be further examined in controlled clinical trials involving cancer patients.

Acknowledgements

This project was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.

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(Received October 1, 2010; Revised November 16, 2010; Accepted November 29, 2010)

Brief Report

Preparation of asialo-agalacto-glycophorin A for screening of anti-Tn antibodies

Tomoyuki Koizumi[1], Ayano Matsumoto-Takasaki[1], Hiroshi Nakada[2], Munehiro Nakata[1], Yoko Fujita-Yamaguchi[1,*]

[1] *Department of Applied Biochemistry, Tokai University, Kanagawa, Japan;*
[2] *Department of Molecular Bioscience, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Japan.*

Summary **Oncogenic antigens such as Tn-antigen (GalNAcα-Ser/Thr) are involved in metastatic processes and are associated with a poor prognosis, thus representing excellent targets for cancer intervention. Available anti-Tn antibodies which can be applied for therapeutics or diagonostics are severely limited mostly because the Tn-antigen epitope by itself is too small to be antigenic in addition to the fact that many carbohydrates are self-antigens. To characterize anti-Tn monoclonal antibodies as well as to perform panning and screening for isolation of anti-Tn single chain variable fragments from phage-display libraries, a large quantity of inexpensive Tn-antigens are needed. In this study, thus, glycophorin A which is a highly glycosylated sialoglycoprotein with approximately 12 *O*-glycans was sequentially treated with sialidase and β-galactosidase to remove sialic acid and galactose residues. The resulted product was shown to be an asialo-agalacto-glycophorin A which is reactive to an anti-Tn-antigen antibody. The simple preparation procedures described here would greatly help production and characterization of potentially valuable anti-Tn-antigen antibodies, which can be readily developed for cancer therapeutics and diagnostics.**

Keywords: Glycophorin A, anti-Tn antibodies/Tn-antigen, oligosaccharides, glycotechnology

1. Introduction

Tumor-associated carbohydrate antigens, so-called oncogenic antigens, are involved in metastatic processes and are associated with a poor prognosis, thus representing excellent targets for cancer intervention. Tn-antigen (GalNAcα-Ser/Thr) and T-antigen (Galβ1-3GalNAcα-Ser/Thr) are such antigens associated with carcinomas and are generally masked by covalently linked terminal carbohydrate moieties in normal human tissues but are exposed in most primary and metastatic epithelial malignant tumors (*1*). Two anti-Tn-antigen specific monoclonal antibodies (mAbs) have been independently produced as a result of immunizing mice with either colon cancer cells or primary breast tumors (*2*,*3*). MLS128 is an IgG_3 that recognizes the structure of three consecutive Tn antigens (Tn3) whereas 84D4 is an IgM that recognizes two and three consecutive GalNAcα-Ser/Thr residues (Tn2 and Tn3, respectively) with similar affinity (*4*,*5*).

Our recent studies revealed that MLS128 treatment significantly inhibited colon and breast cancer cell growth by binding to 110-210 kDa glycoproteins on the cell surface, and that MLS128 treatment caused down-regulation of insulin-like growth factor-I receptor and epidermal growth factor receptor in LS180 colon cancer cells, suggesting that MLS128-inhibited cancer cell growth is in part mediated by down-regulation of growth factor receptors (*6*). Based on these findings, human-type antibodies against Tn3, which is the epitope for MLS128, were screened from a phage library displaying human single-chain variable fragments (scFvs) (*7*). For characterization of anti-Tn mAbs as well as panning and screening in isolation of anti-Tn scFvs, synthetic Tn3- or Tn2-peptide and the backbone peptide have been used (*5*,*7*). These peptides are suitable for precise measurements of affinities for either Tn2 or Tn3 epitope, but are rather costly when used for screening of anti-Tn-antigen antibodies. Tn2- and/or Tn3-containing glycoproteins are obviously alternative

**Address correspondence to:*
Dr. Yoko Fujita-Yamaguchi, Department of Applied Biochemistry, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan.
e-mail: yamaguch@keyaki.cc.u-tokai.ac.jp

choices for screening purposes. Glycophorin A (GPA) contains major *O*-linked oligosaccharide structures, which have been extensively characterized (*8*). GPA is a highly glycosylated sialoglycoprotein containing approximately 12 *O*-glycans and one *N*-glycan. GPA peptides were previously isolated by HPLC and treated with neuraminidase and β-galactosidase after being immobilized on plastic plates for determination of specificity of MLS128 (*4*). Here, we report a bulk preparation of asialo-agalacto-GPA which can readily be used for screening anti-Tn antibodies.

2. Materials and Methods

2.1. *Materials*

GPA and β-galactosidase from bovine testes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sialidase from *Arthrobactor ureafacience* was obtained from Nacalai Tesque (Kyoto, Japan). Biotinylated *Arachis hypogaea* (PNA) and *Maackia amurensis* (MAM) lectins were purchased from J-OIL MILLS (Tokyo, Japan). Streptavidin-horseradish peroxidase (streptavidin-HRP) was purchased from GE Healthcare Biosciences (Piscataway, NJ, USA). Anti-mouse IgG-HRP was obtained from Medical & Biological Laboratories (Nagoya, Japan). Anti-mouse secondary antibody labeled with biotin was from Kierkegaard & Perry Lab (Gaithersburg, MD, USA). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)/H_2O_2 was from Roche Diagnostics (Mannheim, Germany). Production and characterization of MLS128 were previously described (*2*,*4*).

2.2. *Sialidase treatment of GPA and detection of T-antigen by a lectin*

One mg of GPA was dissolved in 500 μL of distilled water. Four hundred fifty μL containing 0.9 mg of GPA were used for sialidase digestion at 37ºC for 24 h in a final volume of 790 μL of 0.2 M sodium acetate buffer, pH 5.0, containing 0.2 units of sialidase. After 2 h digestion, an additional 0.2 units of the enzyme were added to assure complete digestion. PNA-biotin was used to assess the removal of sialic acid residues from GPA by detection of T-antigen epitopes on GPA (*9*). Briefly, 5 μL were each taken from the reaction mixture at different time points, mixed with 45 μL of 10 mM phosphate buffered-saline, pH 7.4 (PBS), and plated in wells of a 96-well plate. After incubation for 1 h at room temperature, the wells were blocked by incubating with 50 mM Tris-buffered saline, pH 7.4 (TBS) containing 3% BSA at 37ºC for 1 h. To each well, PNA-biotin (50 μL of 5 μg/mL of TBS containing 1% BSA) was allowed to react for 1 h at room temperature. After washing with 200 μL TBS 5 times, the wells were mixed with 50 μL of 1,000-fold diluted streptavidin-HRP. After 1 h incubation at room temperature, the wells were washed with 200 μL TBS 5 times. The bound HRP was detected using ABTS as a substrate. After 30 min incubation in the dark, absorbance at 405 nm was measured using a plate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA).

2.3. *β-galactosidase treatment of asialo-GPA and enzyme-linked immunosorbent assay (ELISA)*

The resulting asialo-GPA (~ 0.83 mg) was further treated with 0.1 units of β-galactosidase for 24 h at 37ºC in 3 mL of 50 mM acetate buffer, pH 4.6, containing 0.15 M NaCl. After 2 h incubation, an additional 0.1 units of β-galactosidase were added to assure complete digestion. The resulting asialo-agalacto-GPA solution was aliquoted and stored at −80ºC.

Reactivity of uncovered Tn-antigen epitopes on GPA to MLS128 was determined by ELISA. Briefly, 10 μL (containing ~ 2.8 μg of asialo-agalacto-GPA) before and after 24 h β-galactosidase digestion was placed in wells of a 96-well plate and incubated for 1 h at room temperature. The wells were blocked with 3% BSA/TBS as described above. To the wells, MLS128 (50 μL of 5 μg/mL 1% BSA/PBS) was added and then incubated for 1 h at room temperature. After washing as described above, 50 μL of 1,000-fold diluted anti-mouse IgG-HRP were added, followed by incubation for 1 h at room temperature. The procedures for HRP activity measurement were the same as described above.

2.4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting*

Four μg each of GPA, asialo-GPA, and asialo-agalacto-GPA were separated by SDS-PAGE on a 10% gel and stained with Coomassie Brilliant Blue (CBB). The same sets of samples separated by SDS-PAGE were transferred to an Immobilon-P transfer membrane (Millipore Co., Bedford, MA, USA). The membrane was blocked with 5% skim milk in TBS for 1 h at room temperature. After incubation with MLS128 (6.25 μg/mL) for 16 h at 4ºC, bound MLS128 was detected using the biotin-labeled anti-mouse IgG antibody, the Vectastain ABCAmPTM kit, and the Vector substrate kit (Vector Lab., Inc., Burlingame, CA, USA) as previously described (*6*).

3. Results and Discussion

The PNA reactivity of either 2 h- or 24 h-sialidase digested GPA was the same (Figure 1A). The results suggested that under the conditions used, the removal of sialic acid residues from GPA apparently was completed after 2 h digestion. Treatment of this asialo-GPA preparation with β-galactosidase for 24 h resulted

in production of MLS128 reactive asialo-agalacto GPA (Figure 1B). The stepwise removal of sialic acid and galactose residues from GPA was confirmed by SDS-PAGE/CBB-staining analysis (Figure 1C), which clearly showed reduction of apparent molecular sizes of the original, asialo-, and asialo-agalacto-GPAs. The appearance of Tn-antigen epitopes on the asialo-agalacto-GPA was clearly seen as a MLS128-reactive band (Figure 1D, lane 3). In contrast, the original and asialo-GPAs were not detected by MLS128 (Figure 1D, lanes 1 and 2, respectively). The LS180 colon cancer cell lysate (*6*) was included as a positive control (Figures 1C and 1D, lane4). These results demonstrated that Tn3 epitopes on GPA are exposed after treatments with sialidase and β-galactosidase. Assays carried out to examine the reactivity of GPA, asialo-GPA, and asialo-agalacto-GPA to MAM-lectin, PNA-lectin, and MLS128 revealed that sialic acid residues were not detected in asialo- and asialo-agalacto-GPA preparations by MAM-lectin and that Tn3/Tn2 antigens were not detected in BPA and asialo-GPA by MLS128, but that PNA-lectin, which did not bind to GPA as expected, bound to both asialo- and asialo-agalacto-GPA preparations (data not shown). The results obtained with PNA-lectin suggested the presence of T-antigens on the asialo-agalacto-GPA preparation.

Next, to determine optimum conditions for assaying anti-Tn-antigen antibodies, ELISA was carried out using MLS128 under reduced concentrations of either the antibody or the asialo-agalacto-GPA preparation in combination with increased ABTS incubation times. The results indicated that 5 μL of asialo-agalacto-GPA with 0.2 μg/mL of MLS128 (Figure 2A) or 5 μg/mL of MLS128 with < 0.625 μL of asialo-agalacto-GPA (Figure 2B) would be enough to detect the binding activity. Thus, asialo-agalacto-GPA prepared here would allow more than 3,000 assays for screening anti-Tn-antigen antibodies with similar affinity to that of MLS128. For anti-Tn-antigen single-chain antibodies with typically 1/100 less affinity than mAbs, however, 5 μL/well of asialo-agalacto-GPA and 6 h of incubation with ABTS should be tested first. The assay conditions

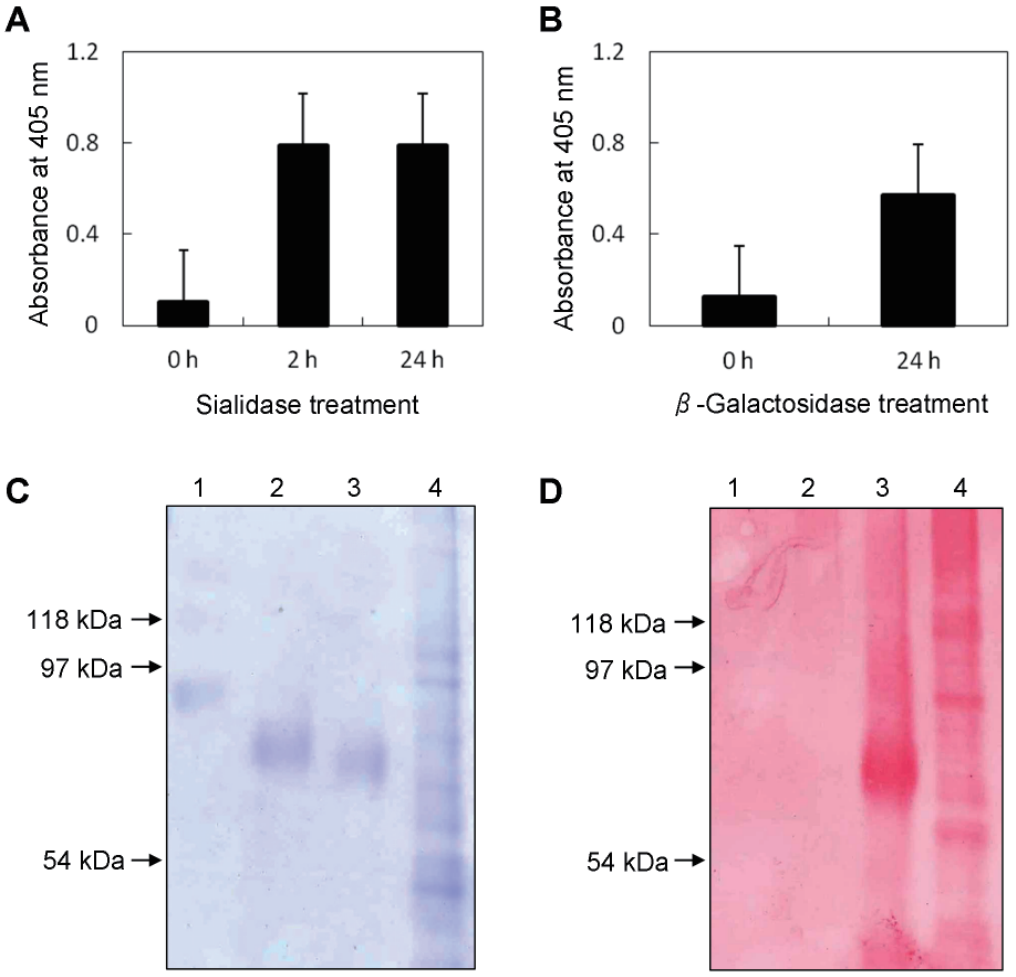

Figure 1. T- and Tn-antigenicity of GPA treated with sialidase (A) and β-galactosidase (B), and molecular size and Tn antigenecity analyzed by SDS-PAGE (C) and Western blotting (D). (A) T-antigen reactivity of GPA after sialidase digestion was monitored by binding to PNA as described in Materials and Methods. **(B)** Tn-antigen reactivity of GPA after β-galactosidase digestion was monitored by ELISA using MLS128 as described in Materials and Methods. **(C)** and **(D)** SDS-PAGE gel stained with CBB and Western blotting with MLS128, respectively. Lanes 1, 2, 3, and 4 contained GPA, asialo-GPA, asialo-agalacto-GPA, and LS180 cell lysate (as a positive control, *Ref. 6*), respectively.

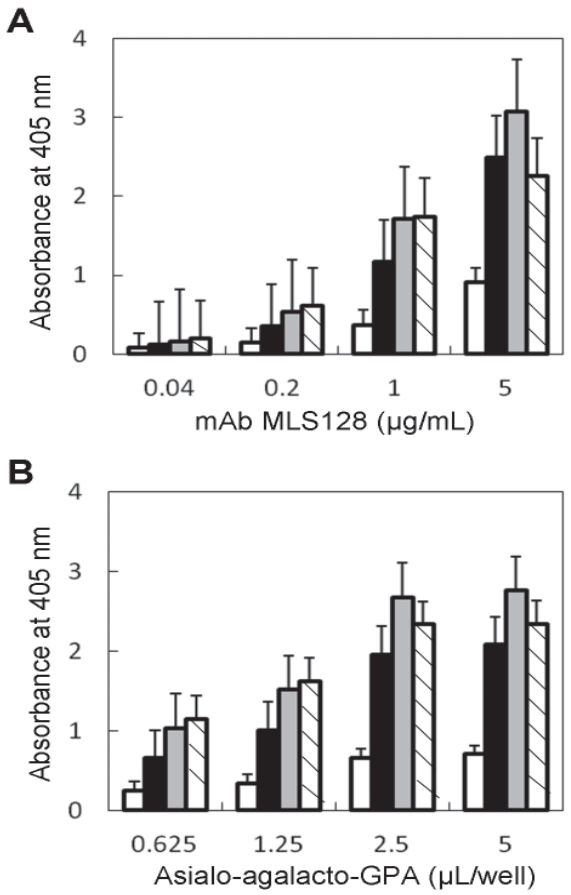

Figure 2. ELISA with various concentrations of MLS128 (A) or asialo-agalacto-GPA (B) in combination with different ABTS incubation times. (A) Five μL of asialo-agalacto-GPA per well were analyzed with 0.04, 0.2, 1, and 5 μg/mL of MLS128. **(B)** Various asialo-agalacto-GPA amounts (0.626, 1.25, 2.5, and 5 μL) were analyzed with 5 μg/mL of MLS128. The reactivity after ABTS incubation times of 15 min (open bar), 1 h (black bar), 2 h (gray bar), or 6 h (hatched bar) is shown.

then need to be optimized for the type of antibodies to be examined. Although enzymes used for digestions remained in the final asialo-agalacto-GPA solution, reproducible ELISA results have been obtained as shown in Figures 1 and 2, which indicated that the antibody-Tn-antigen reaction was not affected by the freed carbohydrates or traces of enzymes under the conditions used.

The simple preparation procedures described here would greatly help production and characterization of potentially valuable anti-Tn-antigen antibodies, which can be readily developed for cancer therapeutics and diagnostics.

Acknowledgements

This work was supported by Grant 22570125 from the Japan Society for the Promotion of Science. Dr. Takasaki-Matsumoto is a recipient of the RPD from the Japan Society for the Promotion of Science.

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(Received September 29, 2010; Revised November 8, 2010; Accepted November 8, 2010)

Brief Report

Identification of mouse mutant cells exhibiting plastic mutant phenotype II; Ionizing radiation-induced mutant phenotype plasticity is not dependent on DNA methylation of the *hypoxanthine phosphoribosyl transferase* gene in mouse FM3A cells

Masatake Yamauchi[1,*], Kumiko Fukutsu[2], Mari Sakagami[1], Taeko Miyazaki[3], Yuji Yamada[2]

[1] *Laboratory of Precancerous Mutation, Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, Inage, Chiba, Japan;*
[2] *Department of Radiation Dosimetry, Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences, Inage, Chiba, Japan;*
[3] *Graduate School of Bioresources, Mie University, Tsu, Mie, Japan.*

Summary **As we previously reported, we isolated and examined mouse mutant cells exhibiting phenotypic plasticity. Approximately 10% of 6-thioguanine resistant ($6TG^R$) cells derived from the irradiated cell population exhibited phenotypic plasticity and reverted to wild type HAT resistance (HAT^R). Similar mutant cells were also identified in an un-irradiated wild type cell population, but at a lower frequency. Ionizing irradiation enhanced the frequency of the plastic mutation approximately 24 times in our experiments. Treatment with 5-aza-cytidine did not affect phenotypic plasticity. In this study, we further performed detailed molecular analysis of the promoter region of the *hypoxanthine phosphoribosyl transferase* (*Hprt*) gene. The analysis revealed that most cytidine residues were not methylated, even in $6TG^R$ mutant cells, in which Hprt activity must be down-regulated. These results suggested that DNA methylation was not involved in mutant phenotype plasticity, a new type of genomic instability induced by ionizing radiation. Plasticity in gene regulation may play an important role in radiation carcinogenesis, which is a multiple-stage process.**

Keywords: Phenotype plasticity, genomic instability, ionizing radiation, DNA methylation, hypoxanthine phosphoribosyl transferase (Hprt), mouse FM3A cells

1. Introduction

Accumulating evidence has made it clear that genomic instability plays an important role in mutagenesis (*1-3*) and carcinogenesis (*4-6*) in mammalian cells. Genomic instability is not specific to irradiated cells; normal un-irradiated cells exhibit the same characteristics at lower frequencies. Ionizing radiation (IR) increases the genomic instability of irradiated cells as well as neighboring cells. The latter effect is known as the bystander effect (*7-9*). The bystander effect of IR is controversial, as some researchers claim that observations of the bystander effect may simply be due to experimental error (*10-12*).

Genomic instability induced by IR is characterized by an increased rate of genome alterations such as chromosomal aberrations, micro-nucleation, mutations, microsatellite instability, and cell death (*13*). Increased genomic instability caused by IR is of great concern in the age of advanced medical technologies which use IR, not only chest X-rays and mammography, but also computed tomography (CT) and positron emission tomography (PET), in which higher X-ray doses are often employed. Although IR is currently recognized as a relatively ineffective carcinogen, a significant proportion of cancer incidence has been attributed to

**Address correspondence to:*
Dr. Masatake Yamauchi, Laboratory of Precancerous Mutation, Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage, Chiba 263-8555, Japan.
e-mail: m_yam@nirs.go.jp

the recent extensive use of X-ray diagnostics in medical procedures (*14*). Similar speculation has also been argued elsewhere (*15,16*).

Through our investigation of genomic instability induced by X-ray irradiation in mouse cells, we have noted the existence of mutant cell clones that exhibit plasticity in their gene regulation. We previously abandoned such mutants because their plasticity seemed to make them unsuitable for further analyses. However, such unstable mutants were repeatedly isolated in our cell mutation experiments, suggesting that the phenomenon was reproducible. Such unstable mutants sometimes comprised ~ 10% of the mutant population, leading us to hypothesize that these mutants may play an important role in the initiation of radiation carcinogenesis, a process that has not been clearly elucidated.

The genomic instability examined in this study was manifested as reversible drug-resistant phenotypes with drastically elevated mutation frequencies in hypoxanthine phosphoribosyl transferase (Hprt, E.C.2.4.2.8) activity, as we reported previously (*17*). DNA methylation of the promoter region has been reported to suppress Hprt activity (*18-20*); however, the genomic instability identified in this study seemed to involve different mechanisms. Here, we report the molecular characterization of the *Hprt* promoter region in phenotypically plastic mutant clones.

2. Materials and Methods

2.1. *Plastic mutant cells*

Mutant cells exhibiting phenotypic plasticity were isolated from mouse FM3A cells and maintained in ES medium (Nissui, Tokyo, Japan) containing 2% fetal bovine serum (FBS, Nichirei, Tokyo, Japan) using a model 3161 CO_2 incubator (Forma Scientific, Marietta, OH, USA) with 5% CO_2 and 100% humidity, as previously described (*17,21*).

2.2. *DNA methylation analysis*

Genomic DNA was extracted from the cells using proteinase K-sodium dodecyl sulfate (SDS) treatment and purified by phenol-chloroform extraction, as described previously (*22*). Purified genomic DNA was amplified with oligonucleotide primers F71 5'-CAAA TGTATGTGTGCAATCC-3' and R81 5'-GTGTTCC CTGGCCGCCAAAC-3'. For nucleotide sequencing analysis of the PCR product, oligonucleotide primers F71-2 5'-TGTTGTATAAGATTGAACCCAG-3', F71-3 5'-ACCAAAAAAAAAAAAAAAAAGAT-3', R81-2 5'-GGCAAAAAGCGGTCTGAGG-3', and R81-3 5'-A TGGTTTAAAAAAAAAAAAAGG-3' were used.

For methylation analysis, genomic DNA was denatured and treated with sodium bisulfite, as described previously (*23*). The treated DNA was used as a template for PCR amplification using a set of oligonucleotide primers Meth-1064F 5'-ATGAGGAGG GAGAAAAATG-3' and Meth-R4-RV 5'-AAAACTCT ACTAAAATCCCCTTAAC-3'. Nucleotide sequences of the PCR products were determined by direct sequencing using the same oligonucleotide primers (Meth-1064F and Meth-R4-RV) or by TA cloning (Takara Bio Inc., Shiga, Japan) using universal primers M4 and RV.

3. Results and Discussion

3.1. *Identification of $6TG^R/HAT^R$ mutants exhibiting phenotypic plasticity*

As previously described, $6TG^R/HAT^R$ mutant cells exhibiting phenotypic plasticity were originally identified in a cell population irradiated with 5 Gy X-rays (*17*). Mutant cells exhibiting the same phenotypic plasticity were eventually isolated from un-irradiated cells. As summarized in Figure 1, X-ray exposure induced 192 $6TG^R$ mutants from 3.1×10^6 cells at a mutation frequency of 6.2×10^{-5}, 187 spontaneous $6TG^R$ mutants were obtained from the un-irradiated cell population at a frequency of 1.2×10^{-5}. Five Gy X-ray exposure enhanced the frequency of *Hprt*-deficient mutations 5-fold. Loss-of-heterozygosity at the *Hprt* locus in $6TG^R$ cells was examined by PCR using the UniSTS 178186 primers, as previously described (*17*). The *Hprt* locus was not detected in 94 clones of the 187 spontaneous mutants and in 138 clones of the 192 irradiated mutants, as shown in Figure 1. Cells that did not yield PCR products were regarded as having a deletion mutation in the *Hprt* allele and were not employed in further experiments. Using the $6TG^R$ mutant cells without LOH, namely, 93 spontaneous mutants and 54 irradiated mutants, we isolated revertants by culturing $6TG^R$ cells in HAT medium, as previously described (*17*).

As summarized in Figure 1, we isolated 4 revertant clones from 93 spontaneous $6TG^R$ mutants that did not show LOH, and 19 from 54 irradiated mutants. The reversion frequency was 4.3% among spontaneous mutants and 35.2% among irradiated mutants. As a result, the irradiated $6TG^R$ mutants contained approximately 10-fold more reversible mutant cell clones than the spontaneous mutants. The frequency of the plastic mutant was approximately 2.5×10^{-7} in the normal cell population and approximately 6.1×10^{-6} in the irradiated population, indicating that IR induced approximately 24 times more phenotypic plasticity in mouse FM3A cells. The plastic mutants changed their phenotype at a frequency of approximately 10^{-2}. The remaining $6TG^R$ mutants exhibited a stable phenotype and did not grow in HAT medium.

5-Aza-cytidine is a chemical compound that is incorporated into DNA molecules through cell

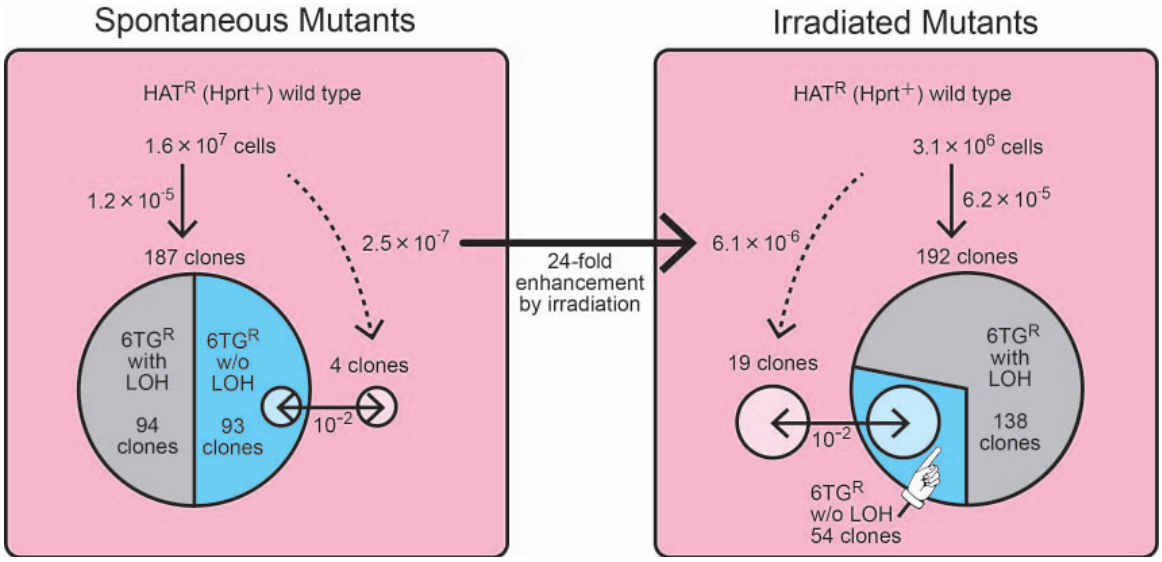

Figure 1. Isolation of the plastic mutants. The box on the left shows the un-irradiated cell population including the HAT^R wild-type phenotype. The box on the right shows the irradiated cell population. Total number of cells used for the drug selection experiments, number of $6TG^R$ clones and plastic mutants, and their frequencies are provided. Within the box, $6TG^R$ clones isolated from each wild-type population are enclosed in a circle. The grey shaded portion represents the $6TG^R$ clones with LOH, and the blue portion represents the $6TG^R$ clones without LOH. Small circles represent the plastic mutants identified in the $6TG^R$ clones; pale-red, HAT^R and pale-blue, $6TG^R$. The bi-directional arrows show the phenotypic plasticity, and the frequency at which the plastic mutants change their phenotypes is approximately 10^{-2}.

metabolism. The presence of 5-aza-cytidine in DNA strands inhibits methylation at the 5th position of the pyrimidine ring of cytidine molecules. To examine involvement of DNA methylation in phenotype plasticity, the effect of 5-aza-cytidine was investigated. As previously reported, inclusion of 5-aza-cytidine did not affect plasticity of the mutant phenotypes (*17*). Plastic mutants isolated in our experiments changed from HAT^R wild-type to a $6TG^R$ mutant phenotype, from a $6TG^R$ mutant to HAT^R wild-type in the presence of 5-aza-cytidine at an average frequency of approximately 10^{-2}.

3.2. *DNA methylation analysis of the Hprt promoter region*

In addition to the incorporation of 5-aza-cytidine in cell culture experiments, we further examined the methylation status of the *Hprt* promoter region utilizing bisulfite-induced modification of genomic DNA, whereby cytosine was converted to uracil, but 5-methyl-cytosine remained non-reactive. Positions and directions of the oligonucleotide primers are shown in Figure 2.

Prior to bisulfite treatment, the nucleotide sequence of the *Hprt* promoter region was amplified from genomic DNA with primers 71 and 81, and confirmed by sequencing with primers 71-2, 71-3, 81-2, and 81-3. The original nucleotide sequence of the *Hprt* promoter region is provided as clone number 0 in Figure 3.

The genomic DNA samples were prepared from both $6TG^R$ and HAT^R clones, and treated with sodium bisulfite. The promoter region of *Hprt* was then amplified by PCR with primers Meth-1064F and Meth-R4-RV. Meth-1064F was designed to bind at a position where bisulfite treatment did not affect the nucleotide sequence. On the other hand, Meth-R4-RV was designed by converting all cytosine residues to thymines at the 3'-downstream end of exon 1 (*24*), as shown in Figure 3.

PCR products were sequenced directly to provide strand-specific average sequences, and compared between $6TG^R$ and HAT^R cells, as well as between spontaneous and X-ray induced mutants. Surprisingly, all cytidine nucleotides in the *Hprt* promoter region were un-methylated in both active and inactive genes. In other words, there were no methyl-cytosine residues even in the $6TG^R$ clones, in which *Hprt* expression was expected to be suppressed.

PCR products were cloned into TA-plasmid vectors and sequenced to provide methylation maps of single DNA molecules. Sequence analysis of the TA-clones revealed the absence of consensus methylated cytosines between 10 clones as shown in Figure 3, which represents typical results obtained from DNA methylation analysis of a $6TG^R$ clone. Sample 0 represents the nucleotide sequence of the un-treated *Hprt* promoter region confirmed in this study. Of the 443 nucleotides in the region, the number of cytidine nucleotides was 125. The results of DNA sequencing obtained from 10 TA-clones of the PCR products amplified from the irradiated $6TG^R$ clone with Meth-1064F and Meth-R4-RV are presented as sample numbers 1-10.

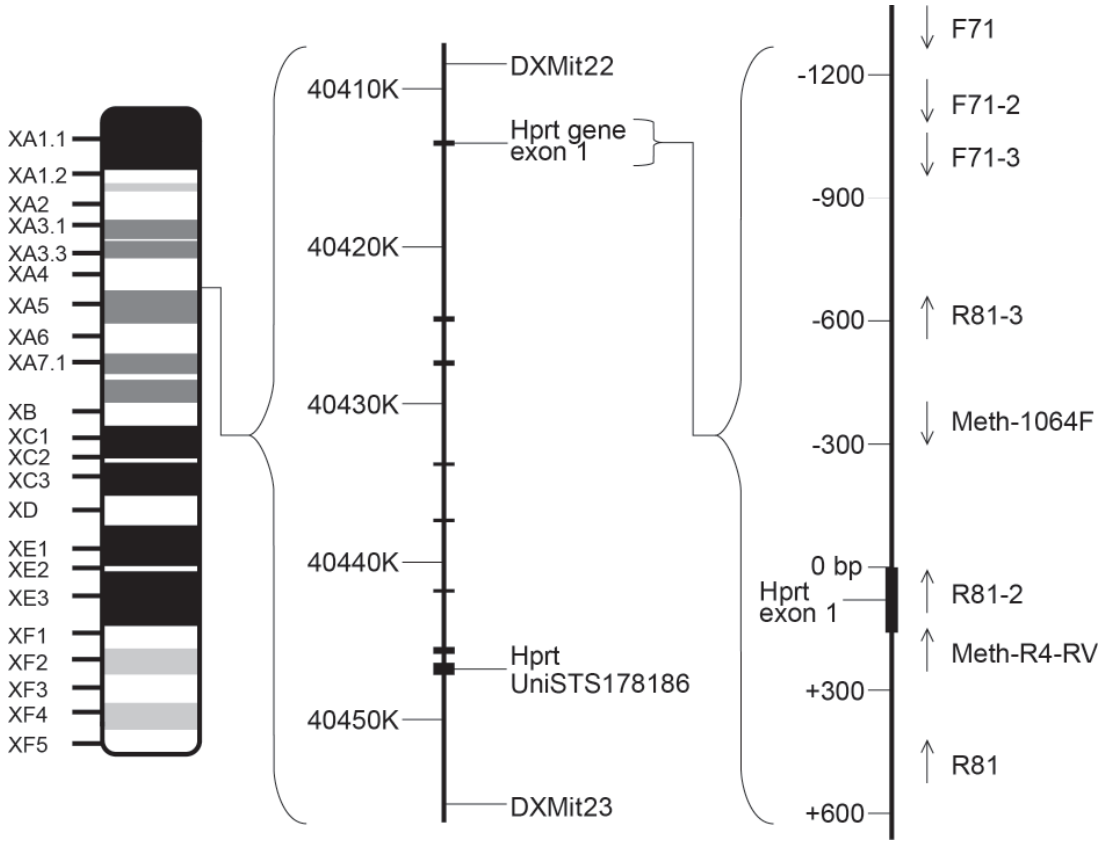

Figure 2. Genomic organization of the mouse *Hprt* allele and oligonucleotide primer design. Chromosomal location of the mouse *Hprt* gene is shown on the left. The center figure describes the genomic structure of the gene and the locations of the UniSTS primers and *Hprt* exon 1. The right figure describes the location of oligonucleotide primers used for the determination of nucleotide sequences of the normal and bisulfite-treated *Hprt* promoter regions.

Almost all of the cytidine residues were converted to thymines, suggesting that these cytidine residues were not methylated in the $6TG^R$ clone. Of 125 cytidine nucleotides in the region, all were converted to thymines in 7 TA-clones, 1 cytidine residue remained un-converted in 1 clone (#8), 2 cytidines remained in 1 clone (#1), and 5 remained in 1 clone (#9). There seemed to be no consensus in the position of methylated cytidine nucleotides. These results showed that DNA methylation was not involved in silencing of *Hprt* transcription.

In this report, we described the identification and DNA methylation analysis of a new class of genomic instability in cultured mouse FM3A cells, which exhibit mutant phenotypic plasticity. The frequency of plastic $6TG^R$ clones was enhanced approximately 24-fold by X-ray irradiation, as shown in Figure 1. In other words, IR increased the plasticity in *Hprt* gene regulation approximately 24-fold in mouse FM3A cells. Mutation frequency was drastically increased to approximately 10^{-2} in plastic mutants.

Interestingly, additional radiation exposure of the plastic mutants derived from both spontaneous and irradiated cells did not affect the frequency of phenotypic changes in either direction, as reported previously (*17*). The plastic mutation phenotype identified here appeared to be stable. Once the genomic instability was acquired by the cells, it was transmitted stably to the daughter cells for at least three months, implying that the genomic instability induced by IR and manifested as phenotypic plasticity can be transmitted stably to daughter cells and may contribute to carcinogenesis.

We speculated that these phenotypic changes could be attributable to the change in *Hprt* expression mediated by DNA methylation. DNA methylation is one of the most common mechanisms in the regulation of transcription, especially in gene suppression often observed in X chromosome inactivation (*18-20*). As observed in our previous study, 5-aza-cytidine treatment did not affect the frequency of plastic mutations in either direction, implying that DNA methylation was not involved in the plasticity of the mutant phenotypes we examined. In this study, the speculation was confirmed by detailed DNA methylation analysis of the promoter region of *Hprt* in both $6TG^R$ and HAT^R clones derived from the same parental cells. Cytidine nucleotides in the promoter region of *Hprt* genes were totally un-methylated even in $6TG^R$ clones, in which transcription of the *Hprt* gene must be down regulated. We therefore concluded that DNA methylation was not involved in plastic mutations and that Hprt activity was regulated by some different mechanism(s).

Figure 3. DNA methylation analysis of the *Hprt* promoter region in a 6TG^R clone. As mentioned in the text, sample 0 represents the nucleotide sequence of the untreated *Hprt* promoter region. Samples 1-10 represent the nucleotide sequences of TA-clones derived from a 6TG^R clone that showed plasticity in its mutant phenotype. Positions where unconverted cytidines were identified are marked with asterisks. Positions of oligonucleotide primers for sequencing of the converted DNA are shown. Exon 1 of *Hprt* is shown in the open black box. The green box represents the first ATG codon of *Hprt*. The number of cytidine nucleotides found in each sequence is also given.

Acknowledgements

The authors thank Mrs. Reiko Ishikawa and Mrs. Takako Hatada for their excellent technical assistance. This study was supported by a grant-in-aid for scientific research provided by the Japan Society for the Promotion of Science (JSPS).

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(Received October 19, 2010; Revised November 7, 2010; Accepted November 8, 2010)

Original Article

Availability and use of emergency obstetric care services in public hospitals in Laos PDR: A systems analysis

Xaysomphou Douangphachanh[1], Moazzam Ali[2,*], Phathammavong Outavong[3], Phengsavanh Alongkon[1], Menorath Sing[1], Kuroiwa Chushi[4]

[1] *Department of Postgraduate Studies and Research, University of Health Sciences, Ministry of Health, Vientiane, Lao PDR;*
[2] *Reproductive Health & Research, World Health Organization (WHO), Switzerland;*
[3] *Health System Development, WHO Laos, Vientiane, Lao PDR;*
[4] *Division of International Health, Yotsukaido Tokushukai Medical Center, Chiba, Japan.*

Summary **The maternal mortality ratio in Laos in 2005 was 660 per 100,000 lives birth which was the third highest in Asia-Pacific Region. The objective was to determine the availability and use of emergency obstetric care (EmOC) in provincial and district hospitals in Borikhamxay, Khammouane, and Savannakhet provinces using UN guidelines. A hospital-based cross sectional survey was conducted from January to March 2008. All district (30) and provincial hospitals (3) from three provinces were included. Analysis was based on hospital records reflecting 12 months of facility data. Data indicates that only 14 hospitals (42.4%) were providing EmOC services, *i.e.*, 9 basic, 5 comprehensive services. The proportion of births in EmOC facilities was only 11.2%, the met need was a very low 14.5%, and the cesarean section rate was only 0.9%. The case fatality rate in Borikhanxay province was 2.8%; in Khammouane and in Savannakhet provinces it was less than 1%. Record keeping at hospitals was poor. Signal functions provided in the last three months showed only 48.5% of the facilities performed assisted vaginal delivery. This is the first study in Lao PDR to assess EmOC services. Almost all the indicators were below the UN recommendations. Health planners must take evidence-based decisions to rectify and improve the situation in the hospitals regarding EmOC services. These data can therefore help government to assign and allocate budgets appropriately, and help policymakers and planners to identify systemic bottlenecks and prioritize solutions and will help in improving maternal health.**

Keywords: Emergency obstetric care, maternal health, process indicators, public hospitals, Lao PDR

1. Introduction

Maternal mortality remains a major public health problem worldwide; ninety-eight percent of cases occur in the developing countries (*1*,*2*). According to the United Nations Population Fund, half a million women die annually from treatable or preventable complications of pregnancy and childbirth (*3*,*4*). The majority of the deaths were due to hemorrhage, prolonged/obstructed labor, and post-partum sepsis, complications of abortion, pre-eclampsia/eclampsia, ectopic pregnancy, and ruptured uterus (*5*).

Numerous studies demonstrated that woman's lives could be saved, if the emergency obstetric care (EmOC) was available (*6*-*14*). To address this issue, a set of process indicators was formally issued by UNICEF, WHO, and UNFPA in 1997 (*14*). They have been used in research surveys and to assess services available at selected hospitals. They are useful in determining the availability, use, and to some extent, the quality of EmOC. They are also useful for monitoring changes in availability, utilization, and quality (*14*).

According to the UN guidelines, EmOC services are classified into basic and comprehensive based on the number of signal functions which are provided. Basic EmOC comprises of six signal functions *i.e.*,

**Address correspondence to:*
Dr. Moazzam Ali, Reproductive Health & Research, World Health Organization (WHO), Switzerland.
e-mail: denube5@yahoo.com

provision and administration of intravenous (IV) and intramuscular (IM) antibiotics, oxytocin, and anticonvulsants, manual removal of placenta, assisted vaginal delivery, and removal of retained products of placenta. Comprehensive emergency obstetric care (EmOC) comprises all basic EmOC signal functions and plus cesarean section and blood transfusion (*14,15*). The guideline also recommended at least five basic EmOC and one comprehensive should be available for every 500,000 populations (*14*).

Maternal health poses a serious problem in the Lao PDR. According to the WHO, maternal mortality ratio (MMR) in Laos in 2005 was 660 per 100,000 live births, which was the highest in the Southeast Asia Region (*16-18*), owing to the lack of quality obstetric care services, the dearth of trained birth assistants and non-functional referral systems (*19*). It is uncertain whether Laos can achieve the Millennium Development Goal 5 of reducing MMR to 185 per 100,000 live births by the year 2015 (*20*).

To combat the high MMR, the government of Laos initiated strategies. The Ministry of Health launched the Safe motherhood, safe deliveries and neonatal care (SMICD) project in 2005 in collaboration with United Nations for Children Fund (UNICEF). To reduce maternal deaths it has also focused on improving access to quality reproductive health services, antenatal, perinatal and postnatal care, EmOC services, and better referral system (*21*). According to the Lao PDR's national health policy, the EmOC should be available in provincial and district hospitals nationwide (*22*). However, there is no baseline study on the existence of EmOC availability and quality of services in Laos. This study tried to assess the availability and utilization of EmOC services in selected central rural provinces of Laos using the UN guidelines.

2. Methods

2.1. *Study design*

A hospital-based, cross-sectional study was carried in January to March 2008 in three central rural provinces to collect information from district and provincial hospitals using the UN guidelines. Besides interviewing key persons for example the director and deputy director to acquire the information on the availability of EmOC services, we also obtained information from hospital records in past 12-month period to assess its quality and the utilization of EmOC services.

2.2. *Study site and sampling*

Laos has seventeen provinces spread across three regions of North, Center and South. From these three regions we selected central region for data collection keeping in view the ease of access, time and monetary constraints. From the central region, out of seven provinces, we randomly selected three rural provinces – Borikhamxay, Khammouane, and Savannakhet. The total population of these three provinces in 2005 was 1.4 million that accounted for 25% of the national population of Laos. There are three provincial (one from each province) and 30 district hospitals (six hospitals in Bolikhamxay, nine in Khuammuane and fifteen in Savannakhet) in these provinces. All these thirty-three hospitals were recruited in this study.

2.3. *Field procedure, study tool and analysis*

Three teams, one in each province, who were trained by the chief investigator on research purpose, design and questionnaire, undertook the study. Data sources were the health facility's records, including the labor and birth registry, the operating theater registry, the antenatal registry, and the gynecological ward registry. To ensure consistency of responses and to minimize errors in data entry, 10% of the total sample was randomly checked before final data entry.

The need assessment was conducted mainly using pre-established tools (*14*), by the UN guidelines along with a few additional questions to interview hospital directors and senior health care workers available at the time of the study (*23*). The English questionnaire stated-above was translated into Lao language and was later back translated into English to ensure consistency and clarity by independent translators.

The questionnaire focused on obtaining information on availability of EmOC services *i.e.*, according to UN recommendations, there should be at least one comprehensive and four basic EmOC facilities per 500,000 populations. It also looked at proportion of births taking place in hospitals, *i.e.*, according to UN minimum recommendations and based on the assumption that at least 15% of pregnant women in a given population will develop complications and require access to EmOC, at least 15% of all births should occur in health facilities. It also assessed the met needs; *i.e.*, all pregnant women with complications should have access to and be treated at health facilities providing EmOC. The minimum recommendation is 100%. It also looked at caesarean section rate; UN process indicators recommend that at least 5% of births be undertaken by caesarean section and, keeping in mind the overuse of this technique, no more than 15%.

Finally the quality aspect was also explored by estimating case fatality rate; a measure of the quality of services provided by health facilities, estimates the number of women who come to the facility with complications and die there.

A pilot study was conducted before the actual survey and adjustment were made in questionnaire based on the feedback. Data collection took place from January to March 2008. The records reflect 12 months of

facility data. The crude birth rate used to calculate the expected number of births was 31.6/1,000 population. Incomplete forms were returned immediately for collection of information. Data sources from district and provincial hospitals included delivery, operating theater, antenatal, and gynecology ward registers. Study permission was obtained from the ethics review committees of the University of Tokyo, Japan, and the University of Health Sciences, Ministry of Health, Lao PDR.

To assess EmOC services, data were coded and analysis was carried out using Microsoft Excel; it was used to produce frequencies and percentages.

3. Results

3.1. *Brief profile of the study areas*

In three provinces the per capital income were US$ 491. Access to TV per 1,000 was 10% and radio per 1,000 was 2.7 (*24*).

Borikhumxay: All 6 districts were recruited in our study from Borikhamxay province. The combined population of these districts was 231,544, with a population density of 15 persons/km^2. The average literacy rate was 77.2% (female literacy 68.1%), and the average village per district was 55 villages. The urban population was 26.3%, with an unemployment rate of 1.2%. Only 4.3% had access to piped water. Electricity was 51.6% (*24*).

Khammouane: All 9 districts were recruited in our study from Khammouane province. The combined population of these districts was 349,542, with a population density of 21 persons/km^2. The average literacy rate was 69.9% (female literacy 59.6%), and the average village per district was 89 villages. The urban population was 21.3%, with an unemployment rate of 0.9%. Only 4.8% had access to piped water. Electricity was 57% (*24*).

Savannakhet: All 15 districts were recruited in my study from Savannakhet province. The combined population of these districts was 857,581, with a population density of 38 persons/km^2. The average literacy rate was 68.5% (female literacy 59.2%), and the average village per district was 103 villages. The urban population was 22.4%, with an unemployment rate of 1%. Only 4.7% had access to piped water. Electricity was 40.2% (*24*).

3.2. *Availability of EmOC and signal functions*

Of 33 hospitals in the study sites, only 14 hospitals were providing either basic or comprehensive EmOC service. Of 14 hospitals providing EmOC, nine offered basic EmOC hospitals, and five comprehensive EmOC hospitals. According to the UN recommendation, however, 11.5 basic hospitals and 2.9 comprehensive hospitals were needed to cover 1,442,233 populations.

We also noted the signal functions for basic EmOC specifically in the three months preceding data collection in the selected hospitals (Table 1). Of 33 hospitals, 32 (97.0%) were providing parental antibiotics and oxytocin; 18 (54.5%) parental sedatives; 31 (93.9%) manual removal of placenta; 25 (75.8%) removal of retained product of placenta; and 16 (48.5%) provided assisted vaginal delivery.

In addition, among 33 hospitals, we also found that only 11 hospitals had functioning ambulances to transfer patients with emergency obstetric complications to a higher level of care.

3.3. *Proportion of all births and met needs in basic and comprehensive EmOC facilities*

We found that only 11.2% of all births in the three provinces studied occurred in health facilities, the coverage by province from the highest to the lowest were 15.5% in Borikhamxay, 14.7% in Khammouane and 8.6% in Savannakhet (Table 2).

According to UN guidelines, all pregnant women with complications (100%) should have access to and be treated at health facilities providing EmOC. The estimated proportion of women, who suffered from delivery complications and received EmOC services, was 14.5% as the whole. The proportions by province of complications treated in hospitals varied; from the highest to the lowest were 21.0% in Khammouane, 18.0% in Borikhamxay and 11.0% in Savannakhet (Table 3).

3.4. *Cesarean deliveries as a proportion of all births*

In our study, cesarean section accounted for 0.9%, with all estimated births at health care facilities in three provinces combined. No health facilities performed cesarean section at a rate near recommended 5% (Table 4). The rates varied by province from the highest to the lowest were 1.5% in Khuammouane, 0.9% in Borikhamxay, and 0.7% in Savannakhet, respectively.

3.5. *Case fatality rate*

The case fatality rate among women with obstetric complications in an EmOC facility should not exceed one percent. This indicator, the combined case fatality rate, a measure of the quality of services provided by health facilities in three provinces, results showed the average case fatality rate of three provinces was 0.9%, which was highest in Borikhamxay (2.8%) while 0.5% in Khammouane and 0.0% in Savannakhet (Table 5).

4. Discussion

This is the first study in Lao PDR that utilized the

Table 1. Signal function in three provinces

Signal functions of EmOC in selected districts in three provinces (n = 33)	Present (services provided in the last three months) (%)	Absent (services not provided in the last three months) (%)
Parental antibiotics	97.0	3.0
Parental oxytocics	97.0	3.0
Parental sedatives	54.5	45.5
Manual removal of placenta	93.9	6.1
Removal of retained products of placenta	75.8	24.2
Assisted vaginal delivery	48.5	51.5

Table 2. Proportion of births in EmOC facilities, Lao PDR

	Total population	Number of deliveries	Expected number of births	Proportion (%)	Recommended by UN (%)
Combined data from three provinces	1,442,233	5,089	45,462	11.2	> 15
Borikhamxay	231,544	1,133	7,317	15.5	
Khammouane	349,542	1,619	11,046	14.7	
Savannakhet	857,581	2,337	27,099	8.6	

Table 3. Met needs in EmOC facilities, Lao PDR

	Total population	Number of women with complications treated	Expected number of complications in population	Met needs (%)	Recommended by UN (%)
Combined data from three provinces	1,442,233	989	6,819.3	14.5	100
Borikhamxay	231,544	198	1,097.5	18.0	
Khammouane	349,542	347	1,656.9	20.9	
Savannakhet	857,581	444	4,064.9	10.9	

Table 4. Cesarean deliveries as a proportion of population

	Total population	Number of cesarean sections	Expected number of births	Proportion (%)	Recommended range by UN (%)
Combined data from three provinces	1,442,233	412	45,462	0.9	5-15
Borikhamxay	231,544	64	7,317	0.9	
Khammouane	349,542	170	11,046	1.5	
Savannakhet	857,581	178	27,099	0.7	

Table 5. Case fatality rate, Lao PDR

	Total population	Number of maternal deaths/complications	Case fatality rate (%)	Recommended maximum by UN (%)
Combined data from three provinces	1,442,233	6/641	0.9	1
Borikhamxay	231,544	5/176	2.8	
Khammouane	349,542	1/189	0.5	
Savannakhet	857,581	0/276	0.0	

standardized UN guidelines (*14*) in assessment of availability and quality of the emergency obstetric care services. The study collected hospital data from three central rural provinces in Lao PDR. Our study findings showed that the number of hospitals providing basic EmOC was less than desired, however as in other studies hospitals providing comprehensive EmOC services were adequate. Our study substantiated findings from other studies in Gambia, Kenya and Pakistan (*25-27*). That showed that the availability and quality of the EmOC services – coverage of institutional birth, proportion of women with complications received EmOC services, caesarean section rate and case fatality rate – were also generally lower than the recommended levels.

According to UN recommendations, there should be at least one comprehensive and four basic EmOC facilities per 500,000 populations. Based on the UN estimates, approximately 11.5 basic and 3 comprehensive EmOC facilities are needed in the three provinces. Our study showed that only 9 hospitals offered basic EmOC and 5 provided comprehensive EmOC services. The comprehensive EmOC were higher than UN recommendations while basic EmOC were lower, similar tendency was reported in previous papers (*5*,*28*). For basic EmOC services, compared to Khammouane province, Borikhamxay, and Savannakhet province could not meet the UN recommendations. Thus highlighting the need to upgrade the existing and where necessary, to build hospitals especially for basic EmOC to provide services for treating common obstetric complications. It has to be noted that out of nine basic EmOC, six were in Khammouane province that was mainly attributed to

Nam Theun 2 hydroelectric project, which is obliged by the government to use income generated by the project to fund the Lao national growth and poverty eradication strategy. It specially emphasizes the health sector, education, and basic infrastructure targeting the poor and has focused on increasing road access and developing new health facilities as the Nhommalath district hospital and health centers in this province (*29*).

According to UN minimum recommendations and based on the assumption that at least 15% of pregnant women in a given population will develop complications and require access to EmOC, thus at least 15% of all births should occur in health facilities. If fewer than 15% of all births take place in health facilities providing EmOC facilities that means that women in need of services are not receiving them. We found that only 11.2% of all births in the three provinces studied occurred in health facilities, which means that some women who need life-saving assistance from EmOC services did not receive it. The use of EmOC services in Savannakhet province was particularly low, and the proportion of complications treated at health facilities was also only 8.6%; we conjecture that it could be attributed to the fact that many women in Laos, like in many other developing countries, could not attend the facilities due to lack of knowledge, low education, poverty, distance from health facilities, and self-efficacy beliefs (*30-32*).

Cesarean section is a life saving procedure, and its rate is an important indicator in monitoring facilities providing comprehensive EmOC services. This study found that the number of cesarean sections was also very low (0.9%), indicating that many women in need may not be getting it. Its importance to save lives necessitates its provision through skilled personnel in hospitals (*33*). In addition, as found also in other studies, it was noted that only 11 in 33 health facilities had functioning ambulances, and what ambulances were available were used mainly to transfer and refer patients to a higher level of care in emergencies (*23,34*).

The combined percentage at all health facilities in the three provinces studied showed the case fatality rate to be 0.9%, which seems acceptable and in line with the UN guidelines. However, in light of other facts, a closer assessment is warranted. During the study we found that in hospitals the records of the complications and deaths are not responsibly kept and maintained. In Borikhamxay province, the case fatality rate was highest, unacceptably high, at 2.8%. The alleged reason is that the transportation system here is much worse than that of other provinces due to poor road conditions, especially in the rainy season, making the terrain inaccessible, leaving people with little choice regarding hospital selection, thus the high case fatality rate reported there. In Savannakhet province where the case fatality rate was 0%, we found poor record-keeping in hospitals, and this presumably accounts for the better case fatality rate results (*i.e.*, < 1%). Thus one has to be careful in interpretation of the results from this study due to general poor quality of record keeping, there is possibility that many deaths reports were not recorded and could have affected the results.

Monitoring the performance of key functions informs us of the capacity of the health system to provide crucial interventions when obstetric emergencies occur (*14,35*). Data from all health facilities in the three provinces showed that although most of the facilities were performing signal functions like administration of oxytocics, antibiotics, manual removal of placenta, followed by removal of retained products of placenta, but only 48.5% of the facilities performed assisted vaginal delivery. It was noted that many studied health facilities did not have the infrastructure to perform surgery and provide blood transfusions. These data can therefore help government to assign and allocate budgets appropriately, and help policymakers and planners to identify systemic bottlenecks and prioritize solutions.

The situation is challenging to policymakers who must redirect monetary and skilled human resources to fill the deficit in EmOC service provision and do everything possible to achieve MDG5 and reduce the maternal mortality ratio to 185 deaths per 100,000 live births by the year 2015.

5. Conclusions and Recommendation

To reduce maternal mortality among these three provinces, we recommend strengthening and upgrading the existing basis EmOC facilities, and required establishing more basic EmOC facilities because many obstetric complications can be resolved by provision of quality EmOC services at these levels. Better record keeping, an efficient referral system, and better access to emergency care, especially by providing ambulances, are also needed in these provinces to effectively present maternal deaths and complications during pregnancy.

The application, regular and proper monitoring of process indicators can provide timely and accurate information about the situation of health systems, enabling immediate action to be taken to rectify deficits and ultimately to reduce maternal deaths.

To improve utilization, policymakers need to emphasize the importance and the quality of hospital services to promote community's confidence in them. The starting point can be improvements in human resources and in management and monitoring to ensure the availability of supplies and maintain accurate medical records (*36,37*). It is important to strengthen institutional/hospital capacity through regular staff training and self-confidence building to allow medical staff in skillfully performing EmOC services, including assisted vaginal delivery, cesarean section, and other life-saving procedures. In addition, a large scale,

national level survey is required to clearly estimate the health systems needs at hospital, district and at national level in order to realistically divert more resources based on evidence.

Finally, measures must be taken to increase the people's confidence in the quality of available services from public sector. Through education, there is also a need to create awareness of obstetric complications and maternal and prenatal morbidity and mortality issues in the communities to understand the risks involved in pregnancy that can lead to timely and appropriate decisions in saving women lives.

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(Received October 12, 2010; Revised November 5, 2010; Accepted November 14, 2010)

(**Declaration:** *When the study was conducted the corresponding author was employed at The University of Tokyo, Tokyo, Japan. This report contains the collective views of an international group of experts, and does not necessarily represent the decisions or the stated policy of the World Health Organization.*)

Original Article

Association between circulating leptin and insulin resistance, the lipid profile, and metabolic risk factors in North Indian adult women

Abhishek Gupta[1], Vani Gupta[1,*], Suraksha Agrawal[2], Shankar M. Natu[3], Chandra G. Agrawal[4], Mahendra P. S. Negi[5], Sunita Tiwari[1]

[1] *Department of Physiology, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India;*
[2] *Department of Medical Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India;*
[3] *Department of Chemical Pathology, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India;*
[4] *Department of Medicine, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India;*
[5] *Division of Biometry and Statistics, Central Drug Research Institute, Lucknow, India.*

Summary **Leptin plays an important role in the regulation of body weight and operates by inhibiting food intake and stimulating energy expenditure. The purpose of the present study was to ascertain the relationship between serum leptin levels and the lipid profile, insulin resistance, and metabolic risk factors in North Indian adult women. In a transactional case-control study of 390 women, subjects were 186 women with metabolic syndrome according to National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) guidelines and 204 healthy control women without metabolic syndrome, all of whom were between 20-40 years of age. Circulating leptin levels were determined by sandwich enzyme-linked immunosorbent assay, insulin resistance was determined by homeostasis model assessment for insulin resistance (HOMA-IR), and the lipid profile was determined using an enzymatic method. Results indicated that circulating leptin (13.38 ± 9.00 *vs.* 8.16 ± 6.31 ng/mL, $p < 0.001$), HOMA-IR (2.68 ± 2.05 *vs.* 1.72 ± 1.20, $p < 0.001$), the lipid profile, and other metabolic risk factors (waist circumference, waist-to-hip ratio, body mass index, and fasting plasma insulin) were significantly higher in women with metabolic syndrome than in women without the syndrome ($p < 0.001$). Further, in women with metabolic syndrome serum leptin was significantly ($p < 0.05$ or $p < 0.001$) and positively correlated with HOMA-IR ($p = 0.000$) and other metabolic risk factors but negatively correlated with fasting plasma glucose, triglycerides, and high-density lipoprotein cholesterol. Circulating leptin was found to be significantly associated with hyperlipidemia, insulin resistance, and other metabolic risk factors in North Indian adult women.**

Keywords: Leptin, insulin resistance, lipid profile, metabolic risk factors

1. Introduction

Metabolic syndrome describes the clustering of abdominal obesity, lipid abnormalities, hypertension, and hyperglycemia and is a strong, independent contributor to the onset of coronary heart disease and type 2 diabetes (*1*). Although the mechanisms underlying metabolic syndrome are not well understood, current evidence suggests that obesity plays a central role (*2*,*3*). As an endocrine organ, adipose tissue produces a number of adipokines, such as leptin, that are mainly associated with cylindrical obesity (*4*), whereas others such as interleukin-6, tumor necrosis factor-α, resistin, and adiponectin may be more closely linked to abdominal adiposity (*5*).

**Address correspondence to:*
Dr. Vani Gupta, Department of Physiology, Chhatrapati Shahuji Maharaj Medical University (formerly King George's Medical University), Lucknow-226003 (UP), India.
e-mail: vaniphysiology@gmail.com;
vaniadhya@yahoo.com

However, there is evidence that leptin is associated with metabolic syndrome and/or with the factors related to it. "Adipokines" are hypothesized to be a possible link between obesity and other components of metabolic syndrome (*6*). Although the prevalence of metabolic syndrome is greater in obese people, not all obese persons suffer from metabolic syndrome, and nonobese individuals may also be affected.

Leptin is a 16 kDa metabolic hormone produced by the leptin gene, whose name is derived from the Greek word "leptos", which means "thin". It was discovered at the end of the year 1994 (*7*). It is produced and released mainly by adipocytes (*8*) and it circulates in serum in both free and bound form. Sinha *et al.* (*9*) noted the presence of leptin-binding proteins and reported that in lean subjects the majority of leptin circulated in the bound form, whereas in obese subjects the majority of leptin circulated in free form.

Leptin is produced at many sites but the amount of body fat is the main determinant of the circulating levels of this hormone. Leptin plays a central role in maintaining the energy balance in humans (*10*). It also regulates energy homeostasis and body weight by adipose tissue mass regulation (*8,11*). The leptin serum concentration has been found to be proportional to body mass but may be lowered rapidly by fasting or inflammatory reaction. It has a central role in energy storage regulation and fertility (*12*). It has several systemic effects such as body mass control, reproduction, angiogenesis, immunity, and cardiovascular function (*13,14*).

In humans, the circulating leptin level increases with obesity (*13*) and has been shown to be directly proportional to the amount of body fat mass, suggesting that a hallmark of obesity is not leptin deficiency but leptin resistance. Hyperleptinemia and/or leptin resistance may play an important role in insulin resistance by causing insulin resistance. Compared to males, females have higher leptin levels if leptin levels are expressed as a percentage of body adiposity (*15*). In recent years, the presence of leptin has been found to be associated with diabetes, glucose metabolism, and insulin metabolism (*16,17*). The association between plasma leptin and insulin in adults has also been examined (*18*).

The present study measured serum leptin levels in North Indian women with metabolic syndrome. These women were classified on the basis of National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) III criteria, and the relationship between metabolic syndrome and homeostasis model assessment for insulin resistance (HOMA-IR), the lipid profile, and other metabolic risk factors was investigated in these women and age-matched healthy controls. The relationship between leptin levels and insulin resistance was also investigated, as were clinical and metabolic parameters in healthy controls.

2. Materials and Methods

2.1. *Subject characteristics*

A case-control study was conducted with North Indian adult women ages 20-40 years. A total of 390 adult women were enrolled in this study and consisted of 186 women with metabolic syndrome (wMetS) according to NCEP-ATP III criteria and a control group of 204 age-matched healthy women without metabolic syndrome (woMetS) who were non-alcoholic, non-diabetic, and who had no cardiac, respiratory, inflammatory, endocrine, or metabolic disease. Pregnant and lactating women with any gynecological or obstetrical problems and women receiving medication such as hormone replacement therapy were excluded from this study.

A structured form was completed to collect information regarding subjects' medical, personal, family, dietary, and menstrual history. This study was approved by the Ethics Committee of this Institute and the Department of Biotechnology (DBT), New Delhi, India and "all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research". Written informed consent was obtained from all participants.

2.2. *Criteria for metabolic syndrome*

The NCEP-ATP III criteria for metabolic syndrome (*19*) are based on simple clinical and biochemical parameters. The current subjects were classified as having metabolic syndrome if they had three or more risk factors, which included any 3 of the following: 1) waist circumference (WC) > 88 cm (35 in); 2) triglycerides (TG) ≥ 150 mg/dL (1.69 mM); 3) high-density lipoprotein cholesterol (HDL-C) < 50 mg/dL (1.29 mM); 4) systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 85 mmHg; and 5) fasting plasma glucose (FPG) ≥ 110 mg/dL (6.1 mM). For adult Asian Indian women (*20*), the cut-off point for waist circumference was > 80 cm and that for body mass index (BMI) was > 23 kg/m^2 (modified with various components of NCEP-ATP III).

2.3. *Anthropometric measurements*

BMI, height, weight, WC, and hip circumference (HC) were evaluated in all subjects. The waist-to-hip ratio (WHR) was calculated from the WC and HC. The WHR is an indicator for measuring central/visceral obesity (WC was measured at the narrowest point superior to the hip and was divided by the circumference of the hips measured at their greatest gluteal protuberance). BMI was calculated as the ratio of body weight to body height squared and was expressed in kg/m^2. Using

an appropriate cuff size, a physician measured blood pressure on the right arm in a sitting position after 5 min of rest. The first and fourth Korotkoff sounds were recorded as systolic and diastolic BP. Blood pressure was measured again after 5 min of rest and the average was used in analysis.

2.4. *Biochemical measurements*

Blood samples for measuring serum biochemical parameters were obtained from all women in the morning after 12 h of fasting on the 10th day of menstruation. Serum and plasma were separated out from a total sample of 4.0 mL blood. Blood plasma glucose and the serum lipid profile were estimated using a glucose oxidase-peroxidase and enzymatic method, respectively (Randox Laboratories Ltd., Antrim, UK). Fasting plasma insulin (FPI) was estimated using an immuno-radiometric assay (Immunotech Radiova, Prague, Czech Republic). Insulin resistance, which indicates proneness to developing metabolic syndrome, was evaluated using the HOMA-IR method (*21*) was determined using the formula: [FPG (mM) × fasting insulin (μU/mL)]/22.5.

2.5. *Detection of serum leptin levels*

In a total of 541 women, leptin levels were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) (Version 6.0, Cat No. CAN-L-4260, Diagnostics Biochem Canada Inc., London, UK). A monoclonal antibody specific for leptin was immobilized onto a microtiter plate and another specific for a different epitope of leptin was conjugated to biotin. Biotin-conjugated anti-leptin antibody, streptavidin-horseradish peroxidase conjugate, and corresponding substrate were used for yellow color development. A colored product formed in proportion to the amount of leptin present in the serum sample. The absorbance was measured on a microtiter plate reader at a wavelength of 450 nm. The intra-assay and inter-assay coefficients of variation were 4.3% and 5.8%, respectively.

2.6. *Statistical analysis*

Quantitative variables are expressed as mean ± standard deviation (S.D.). An unpaired *t*-test was performed to assess differences between the two groups in terms of biochemical parameters. Groups were also compared by one-way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test. Pearson's correlation coefficient (*r*) was calculated to determine the association between the serum levels of circulating leptin level and insulin resistance, the lipid profile, and metabolic risk factors. As most of the parameters were not normally distributed, all parameters were log-transformed before analysis. A two-tailed ($\alpha = 2$) probability value of $p < 0.05$ was considered statistically significant. All statistical analysis was performed with STATISTICA (version 6.0) software.

3. Results

Out of a total of 390 North Indian adult women (age 20-40 yrs), 204 did not have metabolic syndrome (control group: woMetS) and 186 had metabolic syndrome (study group: wMetS).

3.1. *Clinical and biochemical characteristics in women wMetS and woMetS*

Differences between women wMetS and woMetS (Table 1) in terms of biochemical and anthropometric parameters, *i.e.*, weight (64.65 ± 12.02 *vs.* 52.33 ± 9.54), BMI (27.22 ± 5.07 *vs.* 22.06 ± 4.05), HC (99.32 ± 12.18 *vs.* 88.17 ± 7.45), WHR (0.87 ± 0.05 *vs.* 0.82 ± 0.06), FPI (10.31 ± 6.95 *vs.* 7.56 ± 4.86), and HOMA-IR (2.68 ± 2.05 *vs.* 1.72 ± 1.20), were highly significant ($p < 0.001$) but differences were not significant in terms of age (28.32 ± 5.94 *vs.* 27.18 ± 6.87), height (154.23 ± 6.28 *vs.* 154.15 ± 5.78) and pulse rate (78.90 ± 6.50 *vs.* 77.89 ± 6.48). Further comparison of women with and without metabolic syndrome revealed that serum leptin concentrations increased significantly with metabolic syndrome (13.38 ± 9.00 *vs.* 8.16 ± 6.31, $p < 0.001$) (Table 1).

3.2. *Metabolic risk factors in women wMetS and woMetS*

Comparison of metabolic risk factors revealed significant differences between women wMetS and woMetS (Table 2) in terms of WC (86.35 ± 13.98 *vs.* 72.14 ± 9.48, $p < 0.001$), SBP (132.40 ± 11.36 *vs.* 118.46 ± 9.01, $p < 0.001$), DBP (88.46 ± 7.81 *vs.* 80.46 ± 6.54, $p < 0.001$), high TG (142.59 ± 35.98 *vs.* 105.28 ± 23.27, $p < 0.001$), low

Table 1. Comparison of demographic and biochemical parameters for groups woMetS and wMetS

Variables	Women woMetS ($n = 204$)	Women wMetS ($n = 186$)
Age (yrs)	27.18 ± 6.87	28.32 ± 5.94
Height (cm)	154.15 ± 5.78	154.23 ± 6.28
Weight (kg)	52.33 ± 9.54	64.65 ± 12.02*
BMI (kg/m^2)	22.06 ± 4.05	27.22 ± 5.07*
HC (cm)	88.17 ± 7.45	99.32 ± 12.18*
WHR	0.82 ± 0.06	0.87 ± 0.05*
PR (count/min)	77.89 ± 6.48	78.90 ± 6.50
FPI (μU/mL)	7.56 ± 4.86	10.31 ± 6.95*
HOMA-IR	1.72 ± 1.20	2.68 ± 2.05*
Serum leptin (ng/mL)	8.16 ± 6.31	13.38 ± 9.00*

Data are shown as mean ± S.D. * $p < 0.01$. Abbreviations: BMI, body mass index; HC, hip circumference; WHR, waist-to-hip ratio; PR, pulse rate; FPI, fasting plasma insulin.

HDL-C (41.98 ± 4.66 *vs.* 43.92 ± 6.18, $p < 0.001$), and FPG (101.09 ± 17.25 *vs.* 90.54 ± 10.42, $p < 0.001$).

3.3. *Serum lipid parameters in women wMetS and woMetS*

Lipid profiles including serum total cholesterol (172.13 ± 34.64 *vs.* 145.14 ± 29.51), low-density lipoprotein-cholesterol (LDL-C; 101.88 ± 31.80 *vs.* 80.16 ± 28.48), very low density lipoprotein (28.52 ± 7.20 *vs.* 21.06 ± 4.65), the TC/HDL-C ratio (4.15 ± 0.97 *vs.* 3.36 ± 0.80), the HDL-C/LDL-C ratio (2.47 ± 0.87 *vs.* 1.87 ± 0.75), and a low LDL-C/HDL-C ratio (0.46 ± 0.19 *vs.* 0.64 ± 0.35) differed significantly ($p < 0.001$) between women wMetS and woMetS (Table 3).

3.4. *Metabolic risk factors in women woMetS and wMetS sub-grouped according to BMI*

When the women were divided into groups woMetS and wMetS according to BMI and sub-grouped into BMI < 23 kg/m^2 and > 23 kg/m^2, there was a significant difference ($p < 0.001$) between sub-groups woMetS and wMetS (Table 4) in terms of WC, WHR, and serum leptin levels, and there was no significant difference ($p > 0.05$) in terms of HOMA-IR and serum HDL-C. In contrast, serum TG was not significant in the group wMetS but was significant in the group woMetS (Table 4).

3.5. *Comparative summary of WC, BMI, and leptin levels on the basis of the number of metabolic syndrome determinants according to NCEP-ATP III guidelines*

The circulating level of leptin, WC, and BMI were compared among individuals with different numbers of metabolic syndrome determinants (according to the NCEP-ATP III guidelines) (Figure 1). Individuals with 0 (Control), 1, 2, 3, and 4-5 metabolic risk components numbered 22 (5.64%), 139 (35.64%), 43 (11.02%), 153 (39.23%) and 33 (8.46%), respectively. *p* values were significant and a linear trend in the serum level of leptin and WC was observed with an increasing number of metabolic syndrome determinants (Table 5).

3.6. *Correlation between circulating leptin levels and HOMA-IR, the lipid profile, and metabolic risk factors in women with metabolic syndrome (study group)*

Pearson's correlation coefficient (*r*) showed that the leptin level had a highly significant inverse relationship with FPG ($r = -0.09$, $p = 0.251$), TG ($r = -0.01$, $p = 0.913$), HDL-C ($r = -0.08$, $p = 0.259$), and the HDL-C/LDL-C ratio ($r = -0.05$, $p = 0.481$) but a positive correlation with WC ($r = 0.74$, $p < 0.001$), WHR ($r = 0.53$, $p < 0.001$), BMI ($r = 0.63$, $p < 0.001$), SBP ($r = 0.06$, $p = 0.428$), DBP ($r = 0.10$, $p = 0.159$), TC ($r = 0.09$, $p = 0.215$), LDL-C ($r = 0.11$, $p = 0.131$), the TC/HDL-C ratio ($r = 0.12$, $p = 0.120$), the LDL-C/HDL-C

Table 2. Comparison of metabolic risk factors for groups woMetS and wMetS

Variables	Women woMetS (*n* = 204)	Women wMetS (*n* = 186)
Age (yrs)	27.18 ± 6.87	28.32 ± 5.94
WC (cm)	72.14 ± 9.48	86.35 ± 13.98*
TG (mg/dL)	105.28 ± 23.27	142.59 ± 35.98*
HDL-C (mg/dL)	43.92 ± 6.18	41.98 ± 4.66*
SBP (mmHg)	118.46 ± 9.01	132.40 ± 11.36*
DBP (mmHg)	80.46 ± 6.54	88.46 ± 7.81*
FPG (mg/dL)	90.54 ± 10.42	101.09 ± 17.25*

Data are shown as mean ± S.D. * $p < 0.01$. Abbreviations: WC, waist circumference; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose.

Table 3. Comparison of serum lipid parameters for groups woMetS and wMetS

Variables	Women woMetS (*n* = 204)	Women wMetS (*n* = 186)
TC (mg/dL)	145.14 ± 29.51	172.13 ± 34.64*
LDL-C (mg/dL)	80.16 ± 28.48	101.88 ± 31.80*
VLDL (mg/dL)	21.06 ± 4.65	28.52 ± 7.20*
TC/HDL-C	3.36 ± 0.80	4.15 ± 0.97*
HDL-C/LDL-C	1.87 ± 0.75	2.47 ± 0.87*
LDL-C/HDL-C	0.64 ± 0.35	0.46 ± 0.19*

Data are shown as mean ± S.D. * $p < 0.01$. Abbreviations: TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; VLDL, very low density lipoprotein; HDL-C, high-density lipoprotein cholesterol.

Table 4. Comparison of metabolic risk factors in women woMetS and wMetS sub-grouped according to BMI

Metabolic risk factors	Women woMetS (*n* = 204)		Women wMetS (*n* = 186)	
	BMI < 23 kg/m^2 (*n* = 119)	BMI > 23 kg/m^2 (*n* = 85)	BMI < 23 kg/m^2 (*n* = 36)	BMI > 23 kg/m^2 (*n* = 150)
WC	67.89 ± 7.01	78.08 ± 9.33*	69.78 ± 6.12	90.33 ± 12.31*
WHR	0.79 ± 0.04	0.85 ± 0.06*	0.80 ± 0.04	0.88 ± 0.04*
Serum leptin	5.92 ± 2.83	10.06 ± 5.36*	6.74 ± 3.46	16.29 ± 8.99*
HOMA-IR	1.63 ± 1.12	1.86 ± 1.30	2.59 ± 1.54	2.70 ± 2.16
Serum TG	100.59 ± 21.57	111.85 ± 24.08*	146.61 ± 32.08	141.63 ± 36.89
Serum HDL-C	44.38 ± 6.19	43.28 ± 6.14	41.99 ± 4.37	41.97 ± 4.74

Data are shown as mean ± S.D. * $p < 0.01$. Abbreviations: WC, waist circumference; WHR, waist-to-hip ratio; HOMA-IR, homeostasis model assessment for insulin resistance; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol.

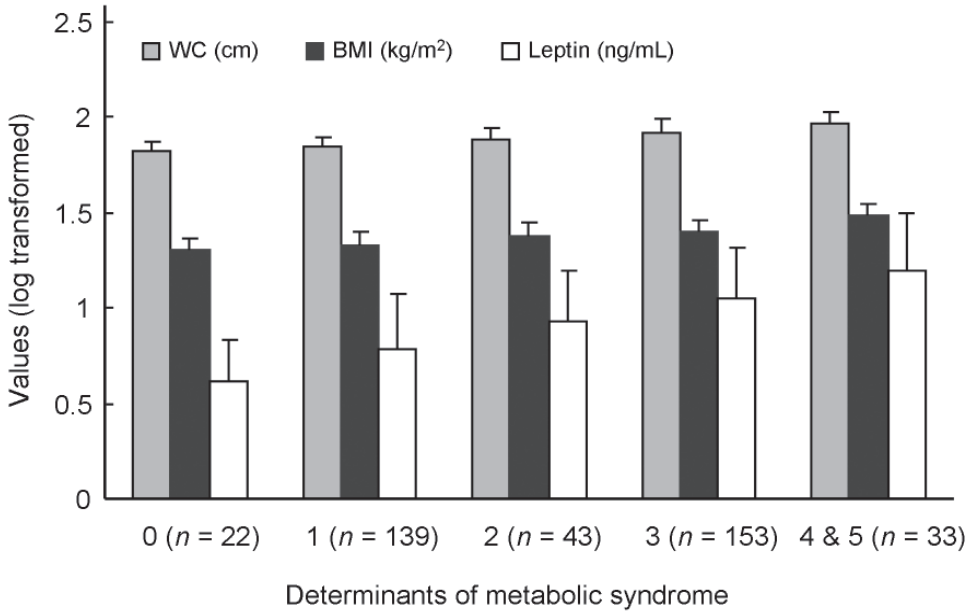

Figure 1. Comparison of serum leptin levels, WC, and BMI with the number of determinants of metabolic syndrome (NCEP-ATP III guidelines). Log-transformed data are expressed as mean ± S.D. In comparison to women with different numbers of determinants of metabolic syndrome, there was a significant increase in the serum leptin level with an increase in number of determinants of metabolic syndrome; this was attributed to an increase in waist circumference independent of BMI. However, women with 2 and 3 determinants had no significant change in BMI but a significant increase in the serum leptin level and WC.

Table 5. Comparison of WC, BMI, and leptin levels on the basis of number of metabolic syndrome determinants according to NCEP-ATP III guidelines

Parameters	Control (n = 22)	MRF 1 (n = 139)	MRF 2 (n = 43)	MRF 3 (n = 153)	MRF 4 and 5 (n = 33)
WC (cm)	66.5 ± 7.6	71.33 ± 7.42	77.63 ± 13.22[ab]	84.67 ± 13.6[abc]	94.15 ± 13.18[abcd]
BMI (kg/m^2)	20.39 ± 3.04	21.55 ± 3.27	24.04 ± 4.07[ab]	25.13 ± 3.47[ab]	30.57 ± 5.21[abcd]
Leptin (ng/mL)	4.53 ± 1.67	7.28 ± 3.73	10.26 ± 6.36[ab]	13.49 ± 8.09[abc]	19.07 ± 11.5[abcd]

Data are shown as mean ± S.D. [a] $p < 0.01$ compared to control. [b] $p < 0.01$ compared to MRF 1. [c] $p < 0.01$ compared to MRF 2. [d] $p < 0.01$ compared to MRF 3. Abbreviations: WC, waist circumference; BMI, body mass index; MRF, metabolic risk factors.

ratio (r = 0.12, p = 0.101), FPI (r = 0.17, p = 0.024), and HOMA-IR (r = 0.11, p = 0.032) (Figures 2 and 3).

4. Discussion

After the discovery of leptin secretion from adipose tissue, leptin's role has been further elucidated in the field of endocrinology. Even though leptin limits food ingestion and increases energy expenditure, it has been found at high levels in obese individuals. The present study noted a relationship between serum leptin levels and insulin resistance, the lipid profile, and metabolic risk factors in adult women, and these three indices indicate a greater risk of developing metabolic syndrome. Using a North Indian population of 390 adult women, this study found that leptin was strongly associated with metabolic syndrome in women wMetS. Women wMetS have higher leptin levels compared to subjects without metabolic syndrome. Furthermore, serum leptin level increased with the number of determinants of metabolic syndrome. Leptin levels were also found to be positively correlated with WHR, BMI, WC, SBP, DBP, TC, TG, FPG, FPI, and HOMA-IR but inversely correlated with HDL-C.

Leptin is an adipocyte secretory product that is not only involved in food intake and energy metabolism but also clearly has a role in glucose metabolism. Leptin may also act as a positive modulator of insulin. In skeletal muscle and beta cells, leptin may promote lipid oxidation and inhibit lipid synthesis, thus improving insulin sensitivity.

The present study found significant differences between women wMetS and woMetS in terms of WHR, BMI, fasting glucose, insulin, insulin resistance, and higher serum leptin levels ($p < 0.001$) (Table 1). However, there is only limited evidence regarding the association between leptin and metabolic syndrome as defined by conventional criteria because a high serum leptin level is associated with central obesity, which accompanies other components of metabolic syndrome like insulin resistance and dyslipidemia, after adjustment for body composition (*22,23*). The increased serum leptin level in obesity may be secondary to 'leptin resistance'. Resistance to the action

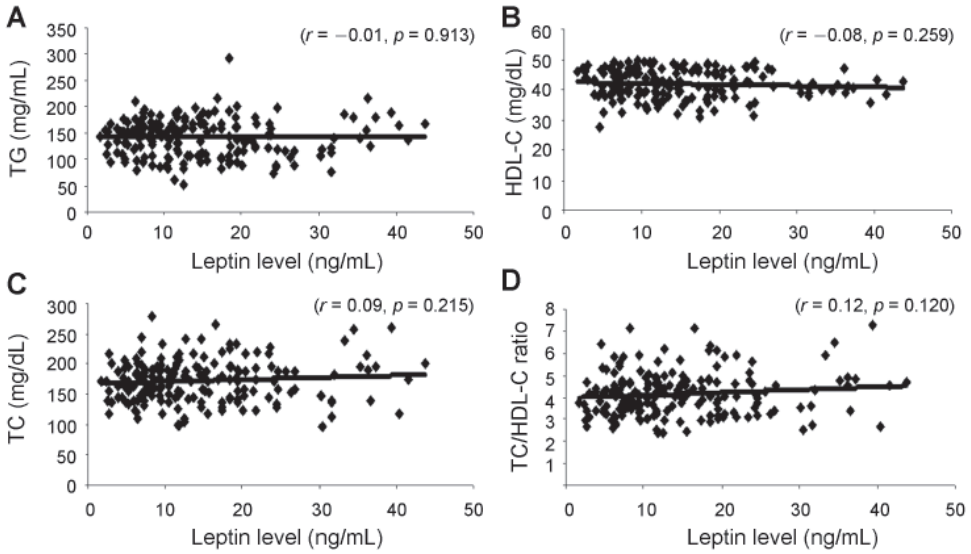

Figure 2. Relationship between serum leptin levels and TG (A), HDL-C (B), TC (C), and TC/HDL-C ratio (D) in North Indian women with metabolic syndrome.

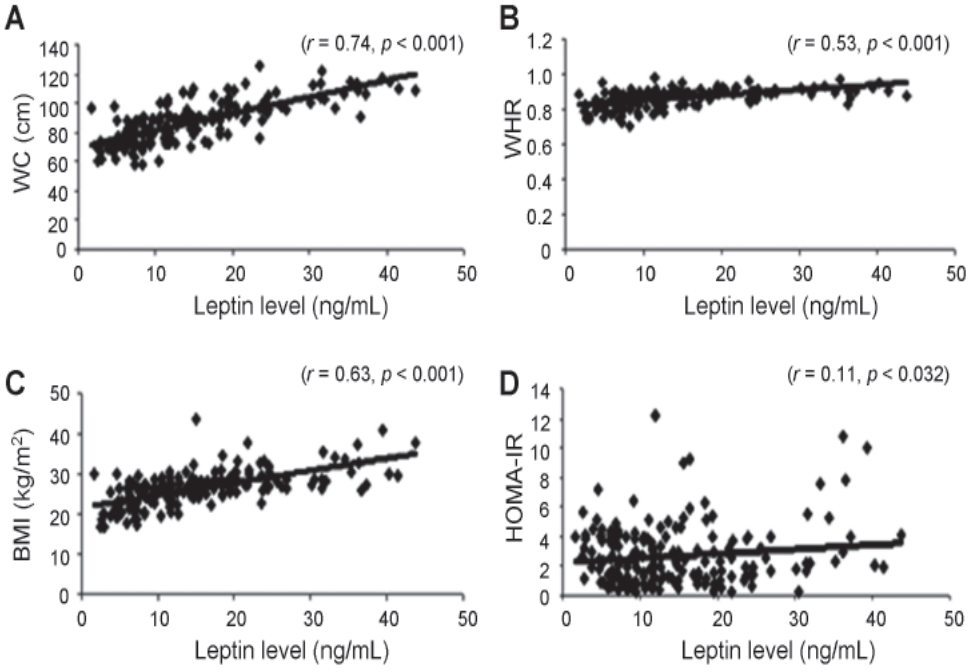

Figure 3. Relationship between serum leptin levels and various metabolic risk factors in North Indian women with metabolic syndrome. (A) WC; **(B)** WHR; **(C)** BMI; **(D)** HOMA-IR. Leptin was positively correlated with these metabolic risk factors.

of leptin could promote obesity *via* decreased energy expenditure and a failure to decrease food intake (*24*). Furthermore, leptin acts *in vivo* to lower glucose and insulin throughout the body, so resistance to this action could induce insulin resistance. One explanation for the insulin resistance seen in obesity might be that the high leptin levels interfere with insulin signaling. Another possibility is that there is diminished activation of AMPK in myocytes due to impaired leptin signalling. The resulting decrease in fatty acid oxidation will lead to an increase in intra-myocellular lipids and thus to insulin resistance.

That said, comparing the metabolic risk factors according to the NCEP-ATP III guidelines in women wMetS and woMetS revealed a significant difference in all risk factors for the development of metabolic syndrome. The current results also indicated that circulating leptin increases with an increase in the determinants of metabolic syndrome ($p < 0.001$). When women were stratified according to the presence of an increasing number of determinants of metabolic syndrome, a significant increase in the

serum leptin level was observed with an increasing number of determinants of metabolic syndrome. This was attributed to an increased waist circumference independent of BMI. There was no significant change in BMI for subjects with 2 and 3 determinants. However, the serum leptin level and WC increased significantly in these subjects (Figure 1 and Table 5). This shows that the presence and number of determinants of metabolic syndrome are closely associated with high levels of circulating leptin and a large WC but are independent of BMI in well-functioning adult women. The association between the presence of metabolic syndrome and leptin is partially attenuated by fat mass and visceral fat area.

The present study and other previous studies have shown that increased serum leptin levels are associated with metabolic abnormalities including abdominal obesity, glucose intolerance, IR, and high blood pressure. The present study found that serum leptin levels were negatively correlated with HDL-C and positively correlated with anthropometric variables, blood pressure, total cholesterol, triglycerides and insulin, and insulin resistance. Strong correlations between leptin and BMI and body fat distribution (waist and WHR) were observed. Similar associations were seen with leptin and body compositions in Asian Indian immigrants compared to Caucasians (*25*). Esteghamati *et al.* (*26*) found that high leptin levels are associated with insulin resistance and metabolic syndrome independent of BMI but that these associations are significantly mediated by the effects of central obesity. Similar to a study by Baratta *et al.* (*27*), the present study found a weak relationship between leptin and lipid parameters but a strong one between BMI and HOMA-IR. In humans, leptin has been found to be associated with factors for cholesterol metabolism (*28*). Leptin specifically represses the gene coding for stearoyl-CoA desaturase-1, an enzyme involved in the synthesis of triglycerides and very low density lipoprotein in the liver (*29*). The causal relationship between leptin and lipid levels has yet to be explained. Further studies are needed to define the factors that affect how leptin and insulin resistance interact, but some authors have posited a strong correlation between leptin and insulin sensitivity (*30*).

Previous studies and this study also suggest that control of metabolic risk factors warrants attention and caution to prevent metabolic syndrome in healthy individuals. Therefore, future studies need to consider how these risk factors influence the relationship between metabolic syndrome and leptin and between the syndrome and other adipokines as well.

As shown here, circulating leptin was significantly associated with obesity, the lipid profile, insulin resistance, and metabolic risk factors in adults of North India. Future studies are needed to identify other genetic or lifestyle risk factors that may contribute to this association and to determine the best strategies for lowering inflammation and the prevalence of metabolic syndrome in adults.

Acknowledgements

The authors would like to thank the participants who participated in this study and the participating physicians and residents of the Department of Medicine and Physiology, Chhatrapati Shahuji Maharaj Medical University, Uttar Pradesh Lucknow, India for their generous support. This work was supported by a grant from the Department of Biotechnology, Ministry of Science & Technology, New Delhi (Grant No. BT/PR9780/GBD/27/68/2007).

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(Received May 23, 2010; Revised June 22, 2010; Re-revised July 27, 2010; Accepted August 7, 2010)

Original Article

Frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 genetic variants in the Northern Indian population

Saurabh S. Rathore[1], Surendra K. Agarwal[2], Shantanu Pande[2], Tulika Mittal[1], Balraj Mittal[1,*]

[1] *Department of Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India;*
[2] *Cardio-Vascular & Thoracic Surgery, Sanjay Gandhi Post Graduate Institute of Medical Sciences Lucknow, India.*

Summary **Dose requirements for oral anticoagulants in thromboembolic events are influenced by polymorphisms in *VKORC1* and *CYP2C9* genes. The Indian population comprises multiple ethnic groups but no data is available on allele frequencies of these genes for North Indians. The present study aimed at establishing the allele and genotype frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 alleles in the North Indian population. One hundred and two healthy subjects from the Northern Indian region were genotyped for *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 by polymerase chain reaction and restriction fragment length polymorphism. Allele frequencies were compared with that of the HapMap populations. The allele frequencies for *VKORC1* -1639 A, *CYP2C9**2 and *CYP2C9**3 were found to be 14.22%, 4.90% and 3.92% respectively. This report also describes the inter-ethnic differences in the Northern Indian frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 alleles with that of other populations and HapMap project data. *VKORC1* -1639 G>A allele is present at moderately high frequency in the Northern Indian population. The frequencies of *CYP2C9**2 and *CYP2C9**3 alleles are also found to be different from other populations.**

Keywords: *VKORC1*, *CYP2C9*, polymorphism, genotype, allele frequency, North Indians

1. Introduction

Oral anticoagulants, warfarin/acitrom and related coumarins (acenocoumarol and phenprocoumon), are widely used in thromboembolic prophylaxis. These drugs have a narrow therapeutic index, and their use is associated with increased risk of clot formation when treatment is subtherapeutic or bleeding when supratherapeutic. In recent years, common genetic variations in vitamin K epoxide reductase (VKOR) and certain cytochrome P450 genes have been discovered that significantly influence oral anticoagulant maintenance dose requirements.

VKOR converts vitamin K epoxide to vitamin K and vitamin K hydroquinone in the vitamin K cycle. Acitrom (or warfarin) inhibits VKOR (*1*), specifically the VKORC1 subunit (*2,3*), thereby diminishing available vitamin K and vitamin K hydroquinone in the tissues. Single nucleotide polymorphisms (SNPs) in the *VKORC1* gene have been linked to reduced efficacy of vitamin K recycling as a result of lower VKOR activity (*3*). Therefore, common polymorphisms in *VKORC1* have been shown to greatly influence dose requirements for oral anticoagulants. The *VKORC1* -1639 G>A variant has been genotyped in a number of different populations. This polymorphism has pronounced differences in its frequency by ethnic group as it is actually the majority allele (around 90%) in Japanese populations and appears to explain the lower warfarin dose requirement for individuals of Japanese descent (*4*). This variant is also quite common in Caucasians, with an allele frequency typically around 40% in predominantly Caucasian populations (*5*). However, there is limited information for Indian populations as only a single study on a small number of Indians living

**Address correspondence to:*
Dr. Balraj Mittal, Department of Genetics, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow-226014, India.
e-mail: bml_pgi@yahoo.com;
balraj@sgpgi.ac.in

in Taiwan has been reported so far (*6*).

CYP2C9 is primarily responsible for oxidative metabolism of narrow therapeutic index compounds like oral anticoagulants (*7*). Multiple single base pair substitution polymorphisms have been identified in the gene coding for CYP2C9 protein. Variability in the metabolism and the therapeutic effect of CYP2C9 substrates can be attributed to the genetic polymorphisms of *CYP2C9* gene. About 34 variant alleles have been reported (*8*). The two most important variants shown to have clinical implications for warfarin dosing and prevention of adverse events are *CYP2C9**2 (Chromosome 10, Exon 3, 430 C>T, rs1799853, Arg144Cys) and *CYP2C9**3 (Chromosome 10, Exon 7, 1075 A>C, rs1057910, Ile359Leu). Individuals with the *2 and *3 variants, who are more likely to need lower doses of warfarin, take a longer time to reach the target International Normalized Ratio (INR) on starting warfarin therapy and have an increased risk of bleeding complications (*9*,*10*). In the Indian context, there are two reports of *CYP2C9**2 and *3 allelic frequency distribution only on subjects from South India (*11*,*12*).

Studies across different populations have shown wide differences in the distribution of the above three alleles in different ethnic groups. The Indian population comprises multiple ethnic groups and no data is available for North Indians. Hence, the present study was aimed at establishing the allele and genotype frequencies of *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 in the North Indian population. This report also compares North Indian *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 frequencies with those of other populations in the HapMap project data.

2. Materials and Methods

2.1. *Subjects and DNA Extraction*

Unrelated healthy volunteers of North Indian origin (n = 102), of either sex (males = 51, females = 51) between the age of 18-60 years with a mean age (S.D.) 28.5 (± 7.8) years were included in the study. North Indian ethnicity was based on place of residence in the last three generations, food habits and mother tongue (Hindi or related languages). Blood samples were collected in ethylenediaminetetraacetic acid and genomic DNA was extracted from peripheral blood leukocyte pellets using the standard salting-out method (*13*). The quality and quantity of DNA was checked by gel electrophoresis and spectrophotometry using the NanoDrop Analyzer (ND-1000) spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The ratio of absorbance at 260 and 280 nm for DNA was between 1.7 and 1.9. The isolated DNA was stored at −70°C.

2.2. *Genotyping of VKORC1 (-1639 G>A, rs9923231) allele*

Genotyping of *VKORC1* (-1639 G>A) was performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) as described by Sconce *et al.* (*14*). Sequences for the forward and reverse primers were 5'-GCCAGCAGGAGAGGGA AATA-3' and 5'-AGTTTGGACTACAGGTGCCT-3', respectively. The PCR conditions consisted of 25 cycles of 1 min at each of the following: 94°C, 59°C, and 72° C. The 5'-untranslated region (UTR) polymerase chain reaction products (10 µL) were digested with 2 units of restriction enzyme Msp1 (Fermentas, MA, USA) in a final volume of 30 µL in the appropriate digestion buffer at 37°C for at least 16 h. The digested products were visualized on 10% polyacrylamide gels stained with ethidium bromide.

2.3. *Genotyping of CYP2C9 430 C>T (CYP2C9*2, rs1799853) and CYP2C9 1075 A>C (CYP2C9*3, rs1057910) allele*

*CYP2C9**2 which is responsible for amino acid change Arg144Cys was detected by PCR-RFLP as described (*15*) using primers (5'-CACTGGCTGA AAGAGCTAACAGAG-3') and (5'-GTGATATGG AGTAGGGTCACCCAC-3') to amplify a 372-bp amplicon in a 50 µL PCR mix comprising 10 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, 50 mM KCl, 200 mM dNTPs, 0.2 mM of each of the primers, 2.5 units of Taq DNA polymerase (Bangalore Genei, Bangalore, India), and 1 µL of genomic DNA. PCR was performed with an initial denaturation for 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 10 sec at 60°C, 1 min at 72°C, and a terminal extension for 7 min at 72°C. Twenty µL of PCR product were digested overnight with restriction endonuclease Sau96I (New England Biolabs, Ipswich, MA, USA), and analysis was done by 3% agarose gel electrophoresis. Wild type alleles (Arg) were cut into fragments of 179, 119, and 74 bp, whereas mutant alleles (Cys) showed fragments of 253 and 119 bp because of loss of one restriction site.

*CYP2C9**3 which codes for the amino acid change Ile359Leu was detected by a PCR-RFLP assay (*13*) using primers (forward) 5'-AGGA AGAGATTGAACGTGTGA-3' and (reverse) 5'-GGCAGGCTGGTGGGG AGAAGGCCAA-3'. PCR conditions were the same as described above. A 130-bp amplicon was digested with StyI (New England Biolabs); wild type alleles (Ile) remained uncut, but mutant alleles (Leu) were cleaved into two fragments of 104 and 26 bp.

For quality control, 10% of the samples were genotyped by other laboratory personal and no discrepancy was observed.

2.4. *Statistical analysis*

Genotype frequencies in the study population were checked for Hardy-Weinberg equilibrium. 95% confidence intervals (CI) were calculated using Confidence Interval Analysis software version 1.0. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. *Allele and genotype frequencies of VKORC1 alleles in the North Indian population*

The *VKORC1* -1639 G>A minor allele frequency in the study population ($n = 102$) was found to be 14.22% (95% CI: 10.08-19.67) (Table 1). Out of 102 subjects, 2 subjects were homozygous for *VKORC1* (-1639 AA) (1.96%, 95% CI: 0.54-6.87) and 25 subjects were heterozygous having both the alleles (-1639 GA) (24.51%, 95% CI: 17.19-33.68) (Table 1). The genotype frequencies of *VKORC1* -1639 G>A in the study population were found to be in Hardy-Weinberg equilibrium.

3.2. *Frequencies of CYP2C9*2 and *3 in the North Indian population*

None of the patients were homozygous for *2C9**2 or *2C9**3. The *CYP2C9**2 mutant allele frequency in our study population ($n = 102$) was found to be 4.9% (95% CI: 2.68-8.79) (Table 2). Out of 102 subjects, 2 subjects were homozygous (TT) for *CYP2C9**2 (1.96%, 95% CI: 0.54-6.87) and 6 subjects were heterozygous (CT) (5.88%, 95% CI: 2.72-12.24). The *CYP2C9**3 allele frequency in the study population ($n = 102$) was found to be 3.92% (95% CI: 2.00-7.55) (Table 3). Out of 102 subjects, 8 subjects were heterozygous (AC) (7.8%, 95% CI: 4.03-14.72) but no homozygous (CC) was detected in 102 subjects (Table 3).

Table 1. *VKORC1* -1639 G>A genotype and allele frequencies in the North Indian population

Genotypes	Number of subjects*		Frequency	95% Confidence interval (CI)
	Males	Females		
GG	35	40	73.53	64.23-81.12
GA	14	11	24.51	17.19-33.68
AA	2	0	1.96	0.54-6.87
Alleles	Number of alleles**		Frequency	95% Confidence interval (CI)
G	175		85.78	80.33-89.92
A	29		14.22	10.08-19.67

* Total number of subjects genotyped for *VKORC1* -1639 G>A allele was 102 (males = 51 and females = 51); ** Total number of alleles = 204.

Table 2. *CYP2C9* 430 C>T (*CYP2C92) frequencies in the North Indian population**

Genotypes	Number of subjects*		Frequency	95% Confidence interval (CI)
	Males	Females		
CC	47	47	92.16	85.28-95.97
CT	4	2	5.88	2.72-12.24
TT	2	0	1.96	0.54-6.87
Alleles	Number of alleles**		Frequency	95% Confidence interval (CI)
C	194		95.10	91.2-97.32
T	10		4.90	2.68-8.79

* Total number of subjects genotyped for *CYP2C9**2 allele was 102 (males = 51 and females = 51); ** Total number of alleles = 204.

Table 3. *CYP2C9* 1075 A>C (*CYP2C93) genotype and allele frequencies in the North Indian population**

Genotypes	Number of subjects*		Frequency	95% Confidence interval (CI)
	Males	Females		
AA	46	48	92.16	85.28-95.97
AC	5	3	7.84	4.03-14.72
CC	0	0	0	0.00-3.63
Alleles	Number of alleles**		Frequency	95% Confidence interval (CI)
A	196		96.08	92.45-98.00
C	8		3.92	2.00-7.55

* Total number of subjects genotyped for *CYP2C9**3 allele was 102 (males = 51 and females = 51), ** Total number of alleles = 204.

4. Discussion

The present study performed genotyping on 204 chromosomes (102 individuals) for polymorphisms in *VKORC1* and *CYP2C9* genes. This group is considered to represent individuals from all the states of Northern India. All the blood samples were randomly collected from healthy relatives of patients coming to our institute from a large capture area of North India. The number of subjects was enough to establish frequencies of minor alleles as this number was higher than that available in the HapMap data.

The *VKORC1* -1639 G>A minor allele frequency in the study population was 14.22% which was significantly different from East Asians as well as Caucasian populations (*16*). The frequency of *VKORC1* -1639 A was significantly higher in Chinese (CHB, 94.6%), Japanese (JPT, 90.1%) and European (CEU, 39.8%) populations (*16*). Our frequency in North Indians was similar to Indians in Taiwan (mostly South Indians) (*6*), but moderately lower than the Gujarati Indian population of USA (GIH, 19.3%), the migrants from a state in the Western part of India (*16*).

The frequencies of *CYP2C9* (*CYP2C9**2 and *CYP2C9**3) in the study population were also compared to previously reported studies on Indians and five major population data groups from the HapMap project (*16*). The *CYP2C9**2 frequency was higher in European population (CEU, 10.4%) but 0% in Chinese, Japanese and African populations (*16*). The *CYP2C9**2 allele frequency in North Indians is comparable to that of South Indians but there are no reports about frequency in Gujarati Indians. Regarding *CYP2C9**3, the frequencies in North Indians were lower than Indians in South and West India. The frequency was slightly higher in European and Chinese populations (CEU, 5.8% and CHB, 5.4%) than the North Indian population of our study (16). The *CYP2C9**3 frequency was lower in the Japanese population (JPT, 2.3%) and there are no reports about the African population (*16*) (Table 4).

A clinically important observation that the oral anticoagulant daily dose requirement is highest in the black population, with the white population generally requiring an intermediate dose and the Asian population the lowest dose, led to investigation of the role of genetics in oral anticoagulant dose requirements. There are many reports associating oral anticoagulant dose requirement with patient genotypes. The G allele frequency of *VKORC1* A>G has been found in concordance with the clinical observation that Chinese patients require smaller doses of warfarin than their Caucasian counterparts to achieve the same degree of anticoagulation (*5*). Lee *et al*. (*6*) reported that the mean weight-normalized warfarin dose was lower for Chinese and Malays than for Indians.

The *CYP2C9**2 genotype is absent and *3 has a greatly decreased frequency in East Asians, whereas African Americans carry a lower frequency of *2 than whites (*17*). In individuals with African heritage the frequency of *CYP2C9**2 and *3 is much lower in blacks than in whites. About 20% of the Caucasian population is heterozygous (*1*2) and 2% is homozygous (*2*2) for *CYP2C9**2 genotype. A smaller proportion of the population is homozygous (*3*3) or heterozygous (*1*3 or *2*3) for the *3 genotype. For *CYP2C9**2, enzyme activity decreases by 30%, and for *3 it decreases by 80% (*18*). The lowest enzyme activity is seen in individuals carrying two *3 alleles. The decreased enzyme activity is associated with lower drug dose requirements. Patients with the *CYP2C9**1*2 genotype required, on average, a 20% lower warfarin dose to maintain a target INR between 2 and 4 compared to the anticoagulated patient population studied (*19*). Japanese patients have been shown to require a lower warfarin dose, and those who are of wild-type *CYP2C9* genotype can clear warfarin more efficiently than their white counterparts (*20*). Based on these facts, it appears that warfarin maintenance dose in Indians should be higher than other Asian populations but lower than Caucasians. However, there is also significant variation in frequency of *2C9**3 in different Indian populations and that may result in variation of dosage requirements for anticoagulation therapy also.

In conclusion, we report frequencies of three important genetic variants, namely *VKORC1* -1639 G>A, *CYP2C9**2 and *CYP2C9**3 in the North Indian population which are different from other populations. These factors may be taken into consideration for genome-based dosing regimens for oral anticoagulant therapies in the future.

Table 4. Minor allele frequencies of *VKORC1* -1639 G>A, *CYP2C92 and *3 in the North Indian and HapMap selected populations**

SNP ID	Major/Minor allele	Number of subjects					
		NI	CEU	CHB	GIH	JPT	AFR
rs9923231 (*VKORC1*)	G/A	14.2	39.8	94.6	19.3	90.1	2.2
rs1799853 (*CYP2C9**2)	C/T	4.9	10.4	0	n.r.	0	0
rs1057910 (*CYP2C9**3)	A/C	3.9	5.8	5.4	13.1	2.3	n.r.

Data of the minor allele frequencies of various populations were obtained from the HapMap project (*16*). Abbreviations: NI, North Indian (the present data); CEU, CEPH (Utah residents with ancestry from Northern and Western Europe); GIH, Gujarati Indians in USA; JPT, Japanese in Tokyo, Japan; CHB, Han Chinese in Beijing, China; AFR, African; n.r., not reported.

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(Received August 13, 2010; Revised November 16, 2010; Re-revised November 17, 2010; Accepted November 17, 2010)

Original Article

Loss-of-heterozygosity analysis of 6-thioguanine-resistant mutants induced by radon exposure in mouse FM3A cells

Masatake Yamauchi[1,*], Kumiko Fukutsu[2], Mari Sakagami[1], Yuji Yamada[2]

[1] *Laboratory of Precancerous Mutation, Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, Inage, Chiba, Japan;*
[2] *Department of Radiation Dosimetry, Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences, Inage, Chiba, Japan.*

Summary **Radon is an inert gas that can migrate from soils and rocks and accumulate in enclosed areas such as buildings and underground mines. The ubiquitous occurrence of radon in the environment is the primary cause of harmful radiation exposure to the public. To investigate the mutagenic effect of radon, mouse FM3A cells growing on soft agarose plates were exposed to alpha particles disintegrated from radon-222 and daughter elements. Mutation induction at the *hypoxanthine phosphoribosyl transferase* (*Hprt*) allele was examined at radon concentrations of 10, 230, 1,100, 6,500, 200,000, 1,000,000, and 10,000,000 Bq/m^3 for an exposure period of 1 week. A typical inverse dose-rate effect was observed in the frequencies of 6-thioguanine-resistant (6TG^R) mutations, and lower mutation frequencies were exhibited at 230, 1,100, 6,500, and 200,000 Bq/m^3 than at 10, 1,000,000, and 10,000,000 Bq/m^3. Loss-of-heterozygosity (LOH) analysis at the *Hprt* locus revealed that deletion mutations were dominant at radon concentrations of 230, 1,100, 6,500, and 10,000,000 Bq/m^3, but not at 10, 200,000, and 1,000,000 Bq/m^3. These results suggested that alpha particles released from radon in the normal atmosphere did not exhibit the measured mutagenic effect in mouse FM3A cells, but that increased concentrations of radon led to a significant increase in the mutagenic effect of radon. At 6,500 Bq/m^3, radon exposure induced the least number of 6TG^R mutants but all had LOH deletion mutations, which is the typically observed type of mutation in radiation carcinogenesis. Our results suggest that certain concentrations of environmental radon may have specific carcinogenic potential, and it should be avoided by proper ventilation wherever possible.**

Keywords: Radon, ionizing radiation, mouse FM3A cells, mutation, hypoxanthine phosphoribosyl transferase (Hprt)

1. Introduction

Radon is a ubiquitous noble gas generated by the disintegration of uranium and radium in the earth's crust. Radon itself disintegrates and generates daughter elements (*1*). For example, radon-222 disintegrates and generates the following cascade of daughter elements: polonium-218, lead-214, polonium-214, lead-210, bismuth-210, polonium-210, and lead-206. Alpha particles are released at relatively high energy levels throughout the disintegration of radon and its daughter polonium elements and this process is known as alpha decay. Alpha particles are identical to the helium nucleus, which is composed of 2 protons and 2 neutrons. The large mass of typical alpha particles allows them to travel only about a few centimeters in the atmosphere and 40-80 micrometers in human tissue. Because alpha particles cannot penetrate skin, external exposure to radon is not problematic for the general public. However, inhalation of radon releasing

*Address correspondence to:
Dr. Masatake Yamauchi, Laboratory of Precancerous Mutation, Experimental Radiobiology for Children's Health Research Group, Research Center for Radiation Protection, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage, Chiba 263-8555, Japan.
e-mail: m_yam@nirs.go.jp

alpha particles may cause serious health problems. The frequent occurrence of lung cancer in underground miners (*2-4*), as well as in the general public (*5-7*) has been reported. Additionally, radon exposure is suspected to be one of the major causes of lung cancer in non-smokers (*8-10*). Radon exposure also has a suspected role in the initiation of leukemia (*11-13*). Thus, the omnipresence of radon is a primary source of harmful radiation exposure for the general public.

Radon is an established carcinogen, but the molecular basis of carcinogenesis caused by radon has not been properly elucidated. High-energy alpha particles are estimated to kill at least 3 cells along their path, and the by-stander effect could cause mutations in surrounding cells (*14-16*). The by-stander effect caused by the cytoplasmic crossing of alpha particles and the subsequent transmission of chemical signals were suggested to play major roles in mutagenesis and carcinogenesis in cell populations hit by alpha particles (*17-19*). However, other reports voice the opposite position and claim that the proposed by-stander effect of alpha particles could be within experimental error (*20-22*).

We established a radon exposure apparatus and examined the mutagenic activity of alpha particles released during the alpha decay process of radon-222 using mouse FM3A cells. Mouse FM3A cells were derived from a mammary tumor in the C3H He/N mouse and grown on soft agar plates in an atmosphere containing 100% humidity. The ability of mouse FM3A cells to grow on these plates allowed us to investigate the mutagenic potential of radon gas by directly exposing cells growing on soft-agar plates to the atmosphere. Here we report the isolation of 6-thioguanine ($6TG^R$) resistant mutants induced by various concentrations of radon and the results of loss-of-heterozygosity (LOH**)** analysis of these mutants. Our results imply that there is a specific radon concentration range that has a greater carcinogenic potential.

2. Materials and Methods

2.1. *Cell culture*

Mouse FM3A cells were maintained in ES liquid medium (Nissui, Tokyo, Japan) containing 2% fetal bovine serum (FBS) (Nichirei, Tokyo, Japan) in a CO_2 incubator with an atmosphere containing 5% CO_2 and 100% humidity, as described previously (*23*,*24*). Prior to radon exposure, cells were cultured for 48 h in HAT medium containing 10^{-4} M hypoxanthine, 10^{-6} M aminopterin [also known as methotrexate (MTX)], and 10^{-5} M thymidine, and for 24 h in HT medium containing 10^{-4} M hypoxanthine and 10^{-5} M thymidine, as described previously (*24*). For radon exposure, cells were cultured on ES agarose plates containing 5% FBS and 0.5% agarose.

2.2. *Radon exposure*

Radon gas was generated from ceramic plates coated with radium-226. Various amounts of a ceramic radon source were stored in individually sealed lead chambers that were connected to the CO_2 incubator. Different radon concentrations were achieved using an appropriate radon chamber containing different amounts of ceramic plates coated with radium. Radon gas was injected into the CO_2 incubator through a sterilizing filter at a flow rate of approximately 0.1 liter per minute. For a natural background control, the room atmosphere was injected into the incubator through the filter at the same flow rate. Radon concentration was monitored using the AlphaGUARD radon monitor (Genitron, Frankfurt, Germany), and an average concentration was calculated.

Cells grown on ES agarose plates were exposed to an atmosphere containing radon gas at a concentration of 10, 230, 1,100, 6,500, 200,000, 1,000,000, and 10,000,000 Becquerel per cubic meter (Bq/m^3) for 1 week. After radon exposure, cells were allowed to recover for 48 h prior to further treatment.

2.3. *Isolation of $6TG^R$ mutants*

For selection of $6TG^R$ clones, cells were plated onto ES plates containing 5% FBS, 0.5% agarose, and 10^{-5} M 6TG, as described previously (*24*). The number of cells used in the drug selection experiment was estimated from the number of colonies formed on ES plates without 6TG using an appropriate dilution of the cell suspension. Colonies that formed on the selection plates containing 6TG were independently isolated and cultured for further analysis. All chemicals were obtained from Wako Chemical (Osaka, Japan), unless otherwise specified.

2.4. *LOH analysis*

Genomic DNA was extracted from cells by proteinase K-sodium dodecyl sulfate (SDS) treatment and purified using the phenol-chloroform extraction method, as described previously (*23*,*24*). LOH at the *hypoxanthine phosphoribosyl transferase* (*Hprt*) locus coding for the *Hprt* gene was examined using the polymerase chain reaction (PCR) with the following oligonucleotide primers UniSTS178186 *Hprt*-F 5'-GAAATGTCA GTTGCTGCGTC-3' and UniSTS178186 *Hprt*-R 5'-GCCAACACTGCTGAAACATG-3' (*25*). The reaction mixture was prepared as recommended by the manufacturer (Takara, Shiga, Japan). The reaction was initiated for 5 min at 94°C, and followed by 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C using the GeneAmp PCR System 9700 (Applied Biosystems Inc., Carlsbad, CA, USA). PCR products were analyzed using 3% agarose gel electrophoresis (*24*).

3. Results

3.1. *Induction of mutation by radon exposure*

The apparatus for radon gas exposure was installed as described in Figure 1. Before cell culture, the CO_2 incubator was equilibrated for 24 h with a sterile atmosphere containing radon using a specific radon chamber.

Prior to radon exposure, mouse FM3A cells were cultured in HAT medium for 48 h to eliminate naturally occurring *Hprt*-deficient cells that were resistant to 6TG, as described previously (*24*). HAT medium was used because Hprt activity is not an absolute requirement for survival, and cells that lack Hprt activity will grow in normal growth medium, which can affect the results of experiments that examine mutation frequency.

Cells were allowed to recover from the toxic effects of MTX in HT medium for 24 h. Approximately 1.0×10^4 cells were plated onto ES agarose plates and cultured for 7 days without lids in a sterile atmosphere containing various concentrations of radon, 5% CO_2, and 100% humidity. The radon concentrations used in our experiments were as follows: 10 (ordinary atmosphere natural background control), 230, 1,100, 6,500, 200,000, 1,000,000, and 10,000,000 Bq/m^3.

Following radon exposure, cells were suspended in ES medium by rinsing the surface of agarose plates with the medium, recovered in normal growth medium (ES + 2% FBS), and used for selection experiments to isolate drug-resistant mutants.

3.2. *Selection and isolation of 6TG^R mutants*

Cells were plated on ES agarose plates containing 5% FBS and 10^{-5} M 6TG. 6TG^R colonies appeared after 1 week. Each colony was isolated independently and grown for further analysis. The number of colonies isolated and their mutation frequencies are summarized in Table 1. The control experiment with natural background (10 Bq/m^3) showed 43 6TG^R colonies at a mutation frequency of 5.0×10^{-6}. The mutation frequency was similar to the background level at 230 Bq/m^3 (34 colonies at a mutation frequency of 4.0×10^{-6}), increased at 1,100 and 6,500 Bq/m^3 (22 at 2.6×10^{-6} and 10 at 1.2×10^{-6}, respectively), dropped to the background level at 200,000 Bq/m^3 (37 at 4.3×10^{-6}), and became enhanced again at 1,000,000 and 10,000,000 Bq/m^3 (138 at 1.6×10^{-5} and 91 at 1.0×10^{-5}, respectively). These mutation results exhibited a typical inverse dose-rate effect (Figure 2).

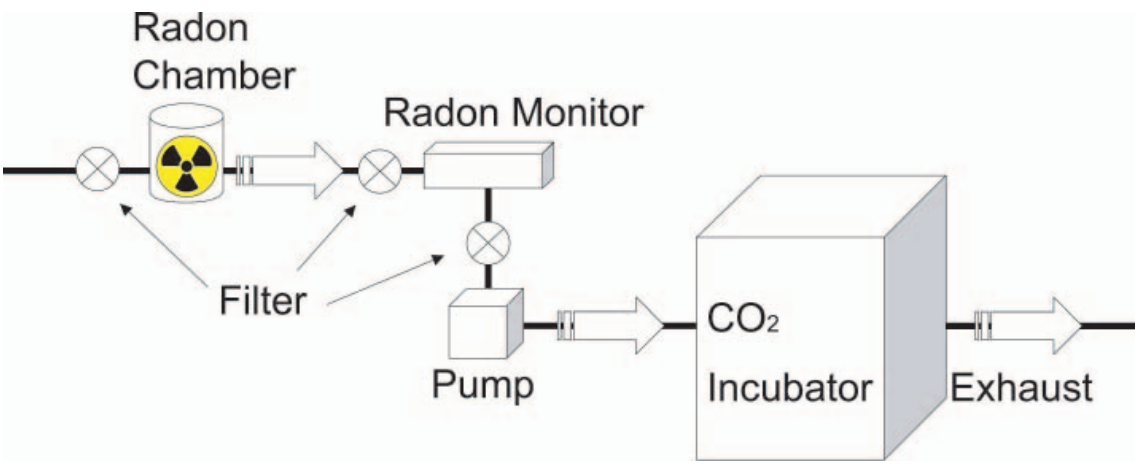

Figure 1. Radon exposure apparatus. Radon gas was generated from ceramic plates coated with radium-226 and stored in a sealed lead chamber that was connected to the CO_2 incubator. Different radon concentrations were achieved by using an appropriate radon chamber containing appropriate amounts of ceramic plates coated with radium. The sealed lead chamber was prepared for radon gas, which was injected into the CO_2 incubator through a sterilizing filter at a flow rate of 0.1 liter per minute. For the background control, room atmosphere was injected into the incubator through the filter at the same flow rate. Radon concentration was measured using the AlphaGUARD radon monitor (Genitron), and an average concentration was calculated.

Table 1. 6TG^R mutation frequencies and the ratio of LOH mutants induced by radon

Radon concentration (Bq/m^3)	Number of 6TG^R colonies	Mutation frequency	Number of LOH mutants (%)
10	43	5.0×10^{-6}	13 (30.2)
230	34	4.0×10^{-6}	23 (67.6)
1,100	22	2.6×10^{-6}	21 (95.5)
6,500	10	1.2×10^{-6}	10 (100)
200,000	37	4.3×10^{-6}	8 (21.6)
1,000,000	138	1.6×10^{-5}	51 (37.0)
10,000,000	91	1.0×10^{-5}	69 (75.8)

3.3. *LOH analysis at the Hprt locus*

The genomic structure of the *Hprt* locus in $6TG^R$ cells was examined with PCR using a set of UniSTS primers (*24*,*25*). The results of the LOH analysis are summarized in Table 1.

LOH at the *Hprt* locus was detected in 13 out of 43 $6TG^R$ colonies isolated from the control group. LOH increased at 230, 1,100, and 6,500 Bq/m^3 (23 out of 34, 21 out of 22, and 10 out of 10, respectively), returned to the background level at 200,000 and 1,000,000 Bq/m^3 (8 out of 37, and 51 out of 138, respectively), and again increased at 10,000,000 Bq/m^3 (69 out of 91) (Figure 3). DNA sequence analysis of the genomic *Hprt* gene was not performed.

4. Discussion

In this report, we established a radon exposure experimental system using mouse FM3A cells. A CO_2 incubator was equilibrated with an atmosphere containing various concentrations of radon gas.

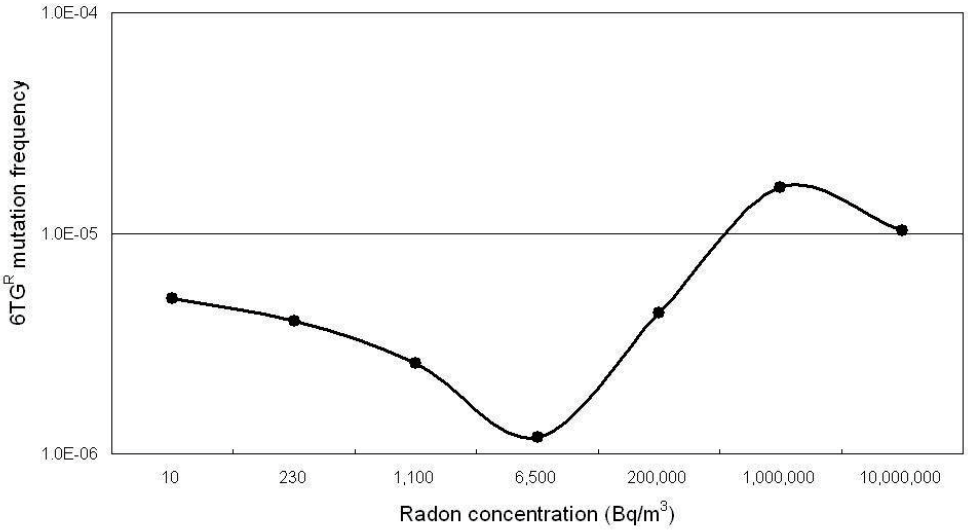

Figure 2. Inverse dose-rate effect on mutation frequencies induced by radon exposure. An inverse dose-rate effect was observed in the mutation frequencies of mouse FM3A cells induced by radon exposure at various radon concentrations for 1 week. The mutation frequencies are represented on a logarithmic scale. The radon concentration in the background control atmosphere was 10 Bq/m^3.

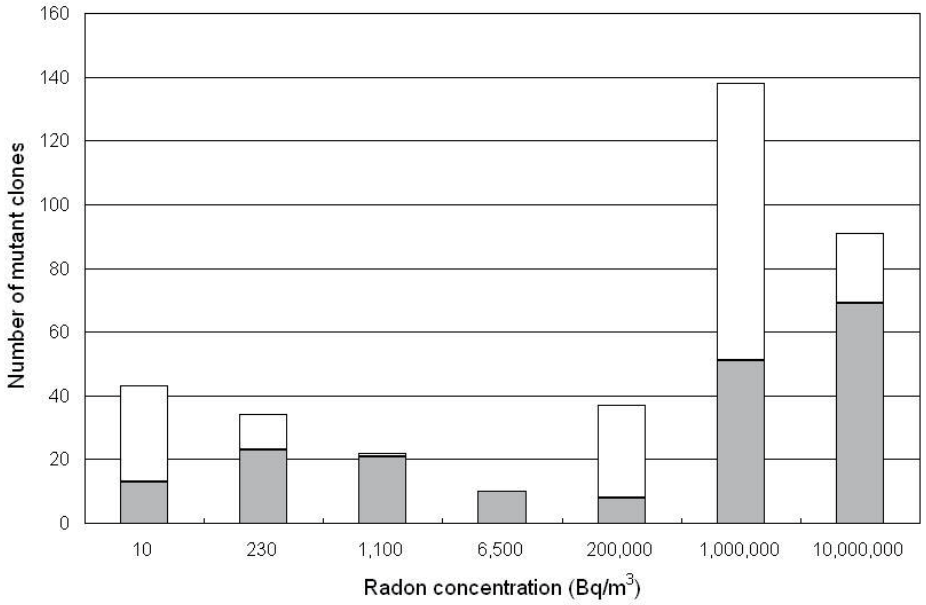

Figure 3. Ratio of LOH mutants induced by radon exposure. LOH analysis was performed on $6TG^R$ mutants induced by various concentrations of radon exposure for 1 week. Each bar represents the number of mutant cells and the shaded bottom area represents the number of LOH mutants at the specified radon concentration. The radon concentration in the background control atmosphere was 10 Bq/m^3.

Since the $6TG^R$ mutant phenotype can only be manifested by a lack of Hprt activity in mammalian cells, a mutation detection system using a combination of $6TG^R$ and Hprt-deficiency has been widely used in experiments examining mutagenic activity of various mutagens, including chemicals (*26*,*27*) and radiation (*28*,*29*), on mammalian cells.

$6TG^R$ mutants were isolated at a frequency of 5.0×10^{-6} in our control experiments with a natural background radon concentration of 10 Bq/m^3, consistent with our previous observations (*24*). The mutation frequency was 4.0×10^{-6} at 230 Bq/m^3, showing that radon exposure did not significantly enhance mutation induction at the *Hprt* locus.

At 1,100 Bq/m^3 and 6,500 Bq/m^3, mutation induction was enhanced 2- and 4-fold, respectively. At 200,000 Bq/m^3, mutation induction returned to the control level, exhibiting a typical inverse dose-rate effect (Figure 2). Mutation induction was again enhanced at the higher concentrations of 1,000,000 Bq/m^3 and 10,000,000 Bq/m^3, resulting in 3- and 5-fold enhancements, respectively.

Molecular examination of the *Hprt* locus revealed that the ratio of LOH mutants in all mutant clones, which presumably occurred by deletion mutations, did not correlate with mutation frequency. The ratio of LOH mutants was 13/43 (30.2%) in the mutant cells induced in ordinary atmosphere, consistent with our previous observations (*24*). However, the ratio of LOH mutants drastically increased as radon concentration increased, as shown in Table 1. The ratio of LOH mutants was 23/34 (67.6%) at 230 Bq/m^3, 21/22 (95.5%) at 1,100 Bq/m^3, and reached a maximum of 10/10 (100%) at 6,500 Bq/m^3.

Surprisingly, the ratio of LOH mutants was reduced to 8/37 (21.6%) at 200,000 Bq/m^3, which was lower than the background level, and 51/138 (37.0%) at 1,000,000 Bq/m^3, which was close to the control level. We cannot explain these observations because the ratio of deletion mutants generally increases as the dose increases in radiation mutagenesis.

The ratio of LOH mutation was again increased at 10^7 Bq/m^3 to 69/91(75.8%). Although the ratio was lower at 10^7 Bq/m^3 than the induction level at 1,100 and 6,500 Bq/m^3, 7 times more mutants were isolated at 10^7 Bq/m^3. The increases in both the number of mutants and the ratio of LOH mutants at the highest exposure concentration used in our experiments may be explained by the dose-effect of ionizing irradiation. However, this result sheds light on the unusually high proportion of LOH mutants at 1,100 and 6,500 Bq/m^3. Our observations may have resulted from a combination of different mechanisms such as direct hit by alpha particles and by-stander effects.

Recently, there has been considerable concern about the potential carcinogenic effects of very low doses of alpha particle radiation. The ubiquitous existence of radon contributes to approximately half of the radiation exposure of the general public, suggesting that the deleterious mutagenic effects of radon are one of the greatest threats to the health of the general public. It has been estimated that as many as 15% of all lung cancer cases may be caused by exposure to residential radon (*1*,*7*). Our examination of the mutagenic effect of radon using mouse FM3A cells provides a molecular basis for these statistics. Detailed molecular analysis of the *Hprt* locus in the mutant cells obtained in our experiments may provide useful information that will reveal the underlying mechanisms that induce mutations. Further experiments with various exposure periods should be performed to understand the molecular basis of the health effects of radon.

Acknowledgements

The authors thank Mrs. Reiko Ishikawa and Mrs. Takako Hatada for their excellent technical assistance. This study was supported by the grant-in-aid for scientific research provided by the Japan Society for the Promotion of Science (JSPS).

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(Received July 14, 2010; Revised October 5, 2010; Accepted October 12, 2010)

Original Article

No relation between folate and homocysteine levels and depression in early pregnant women

Hiroko Watanabe[1,*], Nobuhiko Suganuma[2], Ayako Hayashi[3], Yumiko Hirowatari[4], Tsuneharu Hirowatari[4], Masami Ohsawa[5]

[1] *Department of Clinical Nursing, Division of Maternity Nursing, Shiga University of Medical Science, Otsu, Shiga, Japan;*
[2] *Human Health Sciences, Graduate School of Medicine, Kyoto University, Sakyou-ku, Kyoto, Japan;*
[3] *Department of Nursing, St. Luke's International Hospital, Chuo-ku, Tokyo, Japan;*
[4] *Hirowatari Ladies' Clinic, Akubicho, Chita-gun, Aichi, Japan;*
[5] *Narita Hospital, Naka-ku, Nagoya, Aichi, Japan.*

Summary **The objective in this study was to evaluate the association between folate and homocysteine (Hcy) levels and depressive symptoms in early pregnancy. A cross-sectional study was conducted with 86 pregnant women in the first trimester. A Japanese version of the Center for Epidemiologic Studies Depression (CES-D) scale was used to screen for depression. Non-fasting blood samples were collected from the women to measure folate and Hcy levels. Fifty-three (61.6%) women scored at or above a clinical cut-off of 16, and were classified with depression. In logistic regression analyses, no significant associations were observed between the incidence of depression in the first trimester and elevated Hcy and deficiencies of serum folate, folate intake, vitamin B6 intake and vitamin B12 intake. Folate and Hcy concentrations, and folate consumption, may not be protective against depression in early pregnancy.**

Keywords: Folate, homocysteine (Hcy), pregnancy, depression, nutrition

1. Introduction

Proper nutrition during pregnancy is vital to the health of the woman and her fetus (*1,2*). Women are particularly vulnerable to the adverse effects of poor nutrition on mood because pregnancy and lactation increase nutrient requirements. It has been proposed that depletion of nutrient reserves throughout pregnancy and a lack of recovery postpartum may increase a women's risk for maternal depression (*3*). An association between depressive symptoms and low levels of several dietary B vitamins has been suggested, including folate, vitamin B2 (VB2), vitamin B2 (VB6), vitamin B12 (VB12), all possibly mediated by homocysteine (Hcy) (*4-6*).

Folate deficiency appears to be the most closely linked not only to neural tube defects (*7,8*), but also to depressive disorders. Evidence has been steadily mounting over the past several decades implicating folate in processes thought to underlie the regulation of mood (*9*). Folate and VB12 are essential for normal central nervous system function and may modulate mood through several mechanisms. Severe deficiencies of these vitamins cause loss of memory, mental dysfunction, and depression. Similarly, fatigue, confusion, dementia, and irritability are common clinical signs of folate deficiency. Zuckerman *et al.* (*10*) reported that depressed women were more likely to have poor nutrition. The active metabolite of folate is required for remethylation of Hcy in the production of methionine, which is involved in a number of biochemical processes involving the three aforementioned neurotransmitters (*11*). Thus, a deficiency in folate would impact the production and function of these neurotransmitters.

A deficiency of either folate or VB12 causes elevated Hcy concentrations, which may contribute to the pathogenesis of major depressive disorder (MDD) by mediating a vascular response (*12*). Patients

**Address correspondence to:*
Dr. Hiroko Watanabe, Department of Clinical Nursing, Division of Maternity Nursing, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan.
e-mail: watana@belle.shiga-med.ac.jp

diagnosed with MDD tend to have lower concentrations of serum or red-cell folate than healthy control subjects (*13*,*14*). Poor folate status has been associated with severity of depression and prolonged episodes of MDD. Women have roughly twice the risk of MDD as men. In addition, women of childbearing age are at high risk for MDD. The lifetime risk for MDD in community samples has varied from 10 to 25% for women, with peak prevalence between 25-44 years of age (*15*). A similar proportion of women are affected by MDD in pregnancy and the postpartum period (*16*).

Numerous researches have explored the relationship between dietary deficiency and depression among women in middle pregnancy and postpartum, but not in early pregnancy. Heron *et al.* (*5*) reported that women with antenatal depression have a 6.5-fold increased risk of the more widely known postpartum depression. Of the 10 to 15% of women who develop postpartum depression, up to 0.2% will develop postpartum psychosis, a serious illness associated with suicide, infanticide and homicide (*17*). Depression is a significant disease that has potentially deleterious effects on the woman and her infant, such as uterine irritability, pregnancy-induced hypertension, antepartum bleeding, decreased uterine artery blood flow and preterm delivery (*18*-*21*). However, the association between folate deficiency and prevalence of depression in the early pregnancy is not well known. The objective in this study was to evaluate the association between folate and Hcy levels and depressive symptoms in early pregnancy.

2. Materials and Methods

2.1. *Subjects*

A cross-sectional study on healthy women in early pregnancy who had antenatal care at the obstetrics and gynecology clinic of Narita Hospital or Hirowatari Ladies' Clinic, both of which are located in Aichi prefecture, Japan, was conducted from February to May 2009. Women were at 6-11 weeks of gestation, which was estimated from the first day of the last menstrual period, and fetal growth was confirmed by ultrasound examination. Women with pregestational diabetes of multiple deliveries were excluded from the study. The study was approved by the Ethics Committees of Kyoto University. Written consent was obtained from all participants, after obstetricians explained the purpose, significance, and protocol of the study.

2.2. *Sociodemographic and anthropometric information*

Maternal body mass index (BMI) was calculated using prepregnancy weight and height (kg/m^2). A self-administered questionnaire was used to gather information on age, parity, medical history, number of episodes of nausea/d, number of vomits/d, and current smoking status. Smokers were defined as women who had smoked any number of cigarettes, regardless of smoking status before conception.

2.3. *Dietary assessment*

Dietary habits during the last month of gestation were assessed with a brief self-administered diet-history questionnaire (BDHQ), which was completed by each woman while waiting for the medical examination. The BDHQ took approximately 10 min to complete. The BDHQ is a four-page structured questionnaire, consisting of the following seven sections: general dietary behaviors, major cooking methods, consumption frequency and portion size of six alcoholic beverages, semiquantitative frequency of intake of 56 selected food and nonalcoholic beverage items, dietary supplements, amount and consumption frequency of 19 staple foods and open-ended items for foods consumed regularly (≥ 1 time/week). The food and beverage items and portion sizes in the diet-history questionnaire (DHQ) were derived primarily from the National Nutrition Survey of Japan data and several recipe books for Japanese dishes (*22*). Measures of dietary intake for 147 food and beverage items, energy, fat, total carbohydrates, alcohol, and dietary fiber were calculated by using an ad hoc computer algorithm developed for the DHQ, which was based on the Standard Tables of Food Composition in Japan (*23*). Values of dietary intake were energy-adjusted.

2.4. *Depressive symptom*

A Japanese version (*24*) of the Center for Epidemiologic Studies Depression (CES-D) scale was used to screen for depression. The CES-D is a widely used instrument for assessing general depressive symptom in Western (*24*) and Japanese (*25*) subjects. It is a short self-report scale designed to measure depressive symptoms associated with depression and has been validated against longer scales (*25*). Participants were asked to score the prevalence of salient symptoms of depression (*e.g.* sadness, hopelessness, fatigue, crying, low self-esteem, and changes in sleep or appetite) within the past seven days on a four-point response scales (0: 'less than 1 day', 1: '1-2 days', 2: '3-4 days', and 3: '5-7 days'). The total CES-D scores range from 0 to 60. Participants scoring more than 16 were categorized as having CES-D depression.

2.5. *Blood collection and analysis*

Blood samples for nutritional analyses and hematology were obtained at 4-11 weeks of gestation. Non-fasting blood samples were collected from pregnant women for measurement of folate, Hcy, hemoglobin, hematocrit,

serum iron level, albumin, and total protein. Blood samples were drawn from the antecubital vein into potassium ethylenediaminetetra-acetic acid and serum separator tubes. Serum folate was measured by chemiluminescent immunoassay method (Kemirumi ACS folate II: Siemens Healthcare Diagnostics, Deerfield, IL, USA). Plasma Hcy was measured by high performance liquid chromatography technique (YMC-Rack Pro C18: YMC Co., Ltd., Kyoto, Japan). The intra-assay coefficient of variation (CV) was 7.6% for folate and 10.6% for Hcy, respectively. Serum albumin was measured by Bromcresol green method (Albumin HR II: Wako Pure Chemical Industries, Osaka, Japan) and CV was less than 2.0%. Serum total protein was measured by Biuret method (Total protein HR II: Wako Pure Chemical Industries) and CV was less than 1.5%.

2.6. *Statistical analysis*

All statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). Nutritional intake and biomarkers values were compared between the two groups. Differences in continuous variables were determined by independent *t*-test or the Mann-Whitney-Wilcoxon test for non-normally distributed data. Significant differences between the proportion of the depression group and the non-depression group based on biomarkers and nutrition intake were evaluated by using Pearson's chi-square analyses. Crude and adjusted odds ratios for depression were calculated *via* median. The depression model was adjusted for parity, gestational weeks, and number of vomits/d. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. *Demographic data*

Of 94 pregnant women initially recruited, 86 were finally analyzed. Subjects' scores on the CES-D ranged from 1 to 38, with a mean of 17.0. Fifty-three (61.6%) women scored at or above a clinical cut-off of 16, and were classified with depression. The mean CES-D scores were 22.4 ± 5.6 for the depression group and 8.5 ± 3.7 for the non-depression group, respectively (Table 1). The gestational weeks of the subjects ranged from 6 to 11. No significant differences in age, height, prepregnancy weight, prepregnancy BMI, or gestational weeks were observed between the two groups. Around 20% of women reported using vitamin supplements of any kind. The proportion of primipara was significantly higher in the depression group than that in the non-depression group ($p = 0.04$). Episodes of nausea/d and number of vomits/d in the first trimester were not different in the two groups.

3.2. *Biological markers*

Logarithmically transformed serum folate concentrations were inversely correlated with plasma Hcy concentrations ($r = -0.227$, $p < 0.05$) (Table 2). We also assessed the degree of correlation between CES-D scores and blood biomarkers. A small, significant correlation was observed between CES-D scores and total protein ($r = 0.263$, $p < 0.05$) and hemoglobin concentrations ($r = 0.222$, $p < 0.05$), but CES-D scores were not significantly related to serum folate, plasma Hcy, albumin, iron, hematocrit, or hematocrit concentrations.

Table 1. Demographic characteristics

Items	All ($n = 86$)	Non-depression ($n = 33$, 38.4%)	Depression ($n = 53$, 61.6%)	p value*
Age (year)	30.9 ± 4.6 (20-41)	30.6 ± 4.5 (24-39)	30.8 ± 4.7 (20-41)	0.86
Height (cm)	159.4 ± 5.5 (148-172)	159.8 ± 5.9 (148-172)	159.1 ± 5.3 (150-170)	0.56
Prepregnancy weight (kg)	51.1 ± 5.9 (40-66)	52.0 ± 5.9 (42-66)	50.5 ± 5.5 (40-63)	0.24
Prepregnancy BMI (kg/m^2)	20.1 ± 1.8 (17.0-25.0)	20.3 ± 1.7 (17.0-23.4)	19.9 ± 1.9 (17.0-25.0)	0.34
Gestational week	8.2 ± 1.2 (6-11)	8.6 ± 1.0 (6-10)	8.1 ± 1.3 (6-11)	0.07
CES-D scores**	17.0 ± 8.4 (1-38)	8.5 ± 3.7 (1-15)	22.4 ± 5.6 (16-38)	< 0.001
Primipara (%)	54.4	39.4	62.3	0.04
Smoking (%)	21.1	18.2	23.1	0.59
Anemia (%)***	7.8	15.2	3.8	0.54
Vitamin supplements	20.2	18.8	21.2	0.79
Episodes of nausea/d				0.54
none (%)	19.8	24.2	17.0	
1-2 times (%)	33.7	36.4	32.1	
≥ 3 times (%)	46.5	39.4	50.9	
Number of vomits/d				0.19
none (%)	77.9	75.8	77.4	
1-2 times (%)	15.1	9.1	18.9	
≥ 3 times (%)	7.0	12.1	3.8	

Values are presented as mean ± S.D. (Range) or %. * p values: depression *vs.* non-depression pregnant women (Mann-Whitney U test). ** CES-D: Center for Epidemiologic Studies Depression Scale. Depression: CES-D ≥ 16. *** Anemia: Hemoglobin less than 11.0 g/dL.

Median (interquartile range) serum folate concentrations were 8.0 (5.5) ng/mL in the non-depression group and 8.2 (4.5) ng/mL in the depression group, respectively. The median (interquartile range) plasma Hcy concentrations were 6.0 (2.4) nmol/mL in the non-depression group and 6.1 (1.4) nmol/mL in the depression group, respectively. The median of serum folate, plasma Hcy, total protein and albumin, and the mean of serum iron, hemoglobin, and hematocrit concentrations were not significantly different between the non-depression and depression groups (Table 3).

3.3. *Dietary intake*

The mean folate intake was 197.1 ± 59.4 μg/1,000 kcal in the non-depression group, and 179.9 ± 52.4 μg/1,000 kcal in the depression group, respectively. Total energy intake in the first trimester was 1,519.6 ± 395.9 kcal/d in the non-depression group and 1,413.9 ± 470.9 kcal/d in the depression group, respectively (Table 4). The mean of energy intakes, and all other nutrient intakes per 1,000 kcal, was not significantly different between two groups.

3.4. *Risk factors of depression in the first trimester*

The risks of depression in early pregnancy, according to the biomarker concentrations and nutrient intakes which were used by each median, are shown in Table 5. In logistic regression analyses, no significant associations were observed between the incidence of depression in the first trimester and elevated Hcy or lower serum folate, folate intake, VB6 intake, or VB12 intake, even adjusted for parity, number of vomits/d, and gestational weeks (Table 5).

4. Discussion

Nearly 62% of women were found to have depression symptoms in the first trimester, even though they were healthy and well-nourished women. The mean of the CES-D score was 17.0 ± 8.4 in these subjects, a little bit higher than the 16.0 threshold used to indicate a positive screen for depression, but lower than those of non-pregnant Japanese women aged 18-30 years old (*26*). Similar results were observed among 60 pregnant American women at an average of 15 weeks

Table 2. Correlation between biomarkers and CES-D scores in early pregnancy

Items	CES-D	Serum folate	Plasma Hcy	Total protein	Albumin	Serum iron level	Hemoglobin	Hematocrit
CES-D	1	0.098	0.036	0.263*	−0.091	0.001	0.222*	0.143
Serum folate			−0.227*	0.052	0.216	0.019	0.179	0.207
Plasma Hcy				0.144	−0.092	−0.113	−0.099	−0.187

CES-D: Center for Epidemiologic Studies Depression scale. * $p < 0.05$.

Table 3. Biomarkers in non-depression and depression groups in early pregnancy

Biomarkers	Non-Depression ($n = 33$)	Depression ($n = 53$)	p value
Serum folate (ng/mL)*	8.0 (5.5)	8.2 (4.5)	0.862[a]
Plasma Hcy (nmol/mL)*	6.0 (2.4)	6.1 (1.4)	0.964[a]
Total protein (g/dL)*	7.1 (0.5)	7.3 (0.6)	0.063[a]
Albumin (g/dL)*	4.3 (0.3)	4.3 (0.3)	0.074[a]
Serum iron (μg/dL)**	93.6 ± 41.8 (13.0-163.0)	92.6 ± 40.4 (19.0-201.0)	0.673[b]
Hemoglobin (g/dL)**	11.9 ± 1.2 (8.7-13.8)	12.4 ± 1.0 (9.3-15.7)	0.099[b]
Hematocrit (%)**	37.2 ± 2.9 (30.0-42.5)	38.1 ± 2.9 (28.7-47.2)	0.254[b]

Depression: Center for Epidemiologic Studies Depression (CES-D) scale ≥ 16. * Values are median (Interquartile range). ** Values are mean ± S.D. (Range). [a] Student's t-test [b] Mann-Whitney U test.

Table 4. Energy adjusted nutrient intake/d by BDHQ in non-depression and depression groups

Items	Non-depression ($n = 33$)	Depression ($n = 53$)	p value*
Energy (kcal)	1,519.6 ± 395.9 (814.1-2,258.6)	1,413.9 ± 470.9 (779.1-2,738.4)	0.159
% energy from protein (g)	14.2 ± 2.1 (8.8-19.4)	13.6 ± 2.2 (8.9-18.9)	0.119
% energy from total fat (%)	30.1 ± 4.2 (22.9-38.2)	28.8 ± 5.3 (16.1-39.4)	0.309
% energy from carbohydrate (%)	54.7 ± 5.4 (42.2-64.4)	56.4 ± 6.9 (42.5-70.5)	0.289
Calcium (mg/1,000 kcal)	304.1 ± 91.1 (169.6-613.2)	285.5 ± 84.9 (72.9-499.6)	0.549
Iron (mg/1,000 kcal)	4.3 ± 0.9 (2.7-6.8)	3.9 ± 0.8 (2.3-6.8)	0.063
Zinc (mg/1,000 kcal)	4.2 ± 0.5 (2.9-5.4)	4.0 ± 0.6 (2.3-5.1)	0.159
Folate (μg/1,000 kcal)	197.1 ± 59.4 (96.7-336.9)	179.9 ± 52.4 (63.9-362.4)	0.199
Vitamin B6 (mg/1,000 kcal)	0.7 ± 0.1 (0.3-1.0)	0.6 ± 0.1 (0.3-0.8)	0.289
Vitamin B12 (μg/1,000 kcal)	3.9 ± 1.5 (1.0-8.0)	3.7 ± 1.8 (0.5-10.1)	0.277
Vitamin C (mg/1,000 kcal)	78.1 ± 28.9 (30.5-132.2)	85.6 ± 43.3 (13.7-195.2)	0.746

Values are presented as mean ± S.D. (Range). BDHQ: brief self-administered diet-history questionnaire. Depression: Center for Epidemiologic Studies Depression (CES-D) scale ≥ 16. * p values: depression *vs.* non-depression pregnant women (Mann-Whitney U test).

Table 5. Incidence of depression (CES-D ≥ 16) and odds ratio by maternal biomarkers and nutrition

Variables	Crude OR			Adjusted OR*		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Serum folate (ng/mL)**						
< 8.1	0.74	0.31-1.78	0.51	0.63	0.24-1.61	0.34
≥ 8.1	1.0			1.0		
Plasma Hcy (nmol/mL)**						
< 6.1	1.0			1.0		
≥ 6.1	1.04	0.43-2.49	0.93	0.62	0.23-1.72	0.36
Intake folate (μg/1,000 kcal)**						
< 187.9	1.19	0.50-2.84	0.69	1.12	0.44-2.83	0.82
≥ 187.9	1.0			1.0		
Intake VB6 (mg/1,000 kcal)**						
< 0.66	1.25	0.52-2.98	0.62	1.36	0.54-3.42	0.52
≥ 0.66	1.0			1.0		
Intake VB12 (μg/1,000 kcal)**						
< 3.3	1.96	0.80-4.78	0.14	1.79	0.70-4.56	0.22
≥ 3.3	1.0			1.0		

* Adjusted odds ratio from a logistic regression model controlling for parity, number of vomits/d and gestational weeks. ** Values are medians. OR, odds ratio; CI, confidence interval. Depression: Center for Epidemiologic Studies Depression (CES-D) scale ≥ 16.

(*27*). Several studies have linked nausea and vomiting of pregnancy with depression (*28,29*). Kelly *et al.* (*30*) reported that pregnant depressive women have significantly more somatic complaints, such as nausea, stomach ache, shortness of breath, and headache, than non-depressive women. Inconsistent with their findings, the prevalence of episodes of nausea and number of vomits in this study was not different between the depressive and non-depressive women. This may mean that the incidence of nausea/d and vomits/d in early pregnancy does not appear to affect the CES-D score.

Women in early pregnancy did not consume adequate amounts to meet the nutrient requirements for energy intake, calcium, iron, folate, and vitamin D, which are similar to the findings of Mito *et al.* (*31*). Nearly 90% of subjects had a folate intake below the recommended 400 μg/d (data not shown). Furthermore, only 20% of pregnant women took vitamin supplements, which is a little higher than the 12% reported by Kondo *et al.* (*32*). Thus, we strongly feel that awareness of the importance of adequate folate intake has not improved in Japanese women since 1998, the year the guidelines on folate intakes (*33*) were issued.

Considerable research has reported that folate deficiency, low folate status, or high Hcy levels have been linked in clinic studies to depression and persistent depressive symptoms (*34-36*), especially in elderly people. The Rotterdam study of older men and women found that hyperhomocysteinemia, VB12 deficiency, and folate deficiency were related to depressive disorders (*37*). However, Penninx *et al.* (*38*) found no association between folate/Hcy and depression. In addition, Morris *et al.* (*14*) observed that serum Hcy was not found to be associated with depression diagnoses even in young populations. Consistent with their findings, our study found that all biomarkers including folate, Hcy, total protein, and albumin were no difference between non-depressive and depressive women in early pregnancy. We also found that the intake of folate and other vitamins such as B6 and B12 showed no significant differences between them. CES-D score was significantly correlated only with total protein and hemoglobin, but not with serum folate and plasma Hcy concentrations. The association of their deficiency with depression is still unclear.

A few researchers have examined their association with depression throughout pregnancy. In a study among 865 Japanese postpartum women by Miyake *et al.* (*39*), 14% of women had postpartum depression at two to nine months postpartum. They reported that no significant association was observed between the intake of folate and the risk of postpartum depression by using the Edinburgh Postnatal Depression Scale. Cho and colleagues (*40*) evaluated the effect of prenatal multivitamins containing folic acid on reducing the incidence of depression among 1,277 pregnant Korean women using Goldberg's depression scale. They noted that intake of multivitamins containing folic acid was not associated with lower rates of depression at either less than or greater than 20 weeks' gestation. Regarding the antenatal and postpartum periods, their findings do not suggest an association between low folate and vitamin intakes and depressive disorders.

The major limitation of our study was that we were not able to examine the risk factors for depressive episodes including history of depression, lack of partner, lack of social support, poverty, family violence, and increased life stress (*18,41*). In addition, the study subjects were few and not randomly selected. The study was conducted in only two clinics. Therefore, our findings may not be generalizable to other pregnant Japanese women. Further research is needed.

In conclusion, nearly 62% of women were found to have depression symptoms in the first trimester. CES-D score was significantly correlated only with total protein

and hemoglobin, but not with serum folate, plasma Hcy concentrations, or intake of folate, VB6, or VB12. Our results suggest that consumption of these may not be protective against early pregnancy depression. Almost none of the pregnant women in this study were meeting recommended folate intakes. Pregnancy can be an opportune time to improve nutrition, and presents an ideal time for health promotion activities. Health care providers should encourage all women of reproductive age to have a well-balanced diet behavior which leads to improved birth outcomes.

Acknowledgements

The study was funded by the JEX Co., Ltd. (Osaka, Japan). The authors appreciate the great help of the research nurses for the recruitment of study participants at Hirowatari Ladies' Clinic and Narita Hospital.

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(Received September 21, 2010; Accepted October 13, 2010)

Original Article

Second trimester pregnancy termination with 400 μg vaginal misoprostol: Efficacy and safety

Shipra Kunwar[1,*], Pradip K. Saha[2], Poonam Goel[2], Anju Huria[2], Rimpi Tandon[2], Alka Sehgal[2]

[1] *Department of Obstetrics & Gynaecology, Era's Lucknow Medical College, Lucknow, India;*
[2] *Department of Obstetrics and Gynaecology, Government Medical College & Hospital, Chandigarh, India.*

Summary **The present study was designed to evaluate the efficacy and safety of misoprostol (400 μg) given intravaginally repeated at 6 hourly intervals for a maximum of 6 doses for second-trimester pregnancy terminations. The study was conducted on women who had to undergo pregnancy termination between 13 and 26 weeks of gestation for various indications but mainly intrauterine death over a period of 2 years. A standard regime of 400 μg of misoprostol 6 hourly intravaginally was given until a maximum of 6 doses. Sixty women underwent second trimester terminations. The mean induction abortion interval was 11.8 h. The success rate at the end of 48 h was 96.6%. Side-effects were in the form of incomplete abortion, excessive blood loss, and fever. No patient had a uterus rupture. Intravaginal misoprostol 400 μg given 6 hourly seems to be an effective, safe, and acceptable method for second trimester pregnancy terminations.**

Keywords: Second trimester pregnancy termination, vaginal misoprostol, prostaglandin

1. Introduction

Second trimester pregnancy terminations, though a common problem in obstetrics practices, can be tricky situations. Previously, the most commonly used drug oxytocin had high failure rates because it was ineffective in stimulating a preterm uterus. Prostaglandins have been the most commonly used agents for many years up to now.

Misoprostol (prostaglandin E1 analogue) originally designed for treatment of peptic ulcer has now gained wide popularity in obstetrics. This agent is effective for cervical ripening and labor induction at term, and treatment of post-partum hemorrhage as well as first and second trimester abortions (*1*). The agent has been also used in scarred uterus (*2*). The main advantages of misoprostol are ease of administration, cost-effectiveness, and stability at room temperature (*3,4*).

More than thirty different dosages of misoprostol regimes have been described in the literature for use in obstetrics and gynacology (*5*). A review article published in contraception stressed the fact that, although misoprostol is effective for second trimester preganancy terminations especially for those with intrauterine deaths, further studies are required to establish optimal dose and requirements of second trimester terminations (*6*).

The present study was undertaken to establish efficacy and safety of misoprostol (400 μg) given vaginally and repeated at 6 hourly intervals for a maximum of 6 doses, for second trimester abortions.

2. Materials and Methods

2.1. *Study design*

The study was conducted in the Department of Obstetrics and Gynaecology at Government Medical College and Hospital, Chandigarh, India, between December 2005 and December 2007. It was a prospective study. A total number of 60 patients were included in the study.

2.2. *Subject*

The study was conducted on 60 healthy women admitted for termination of pregnancy between 13-26

**Address correspondence to:*
Dr. Shipra Kunwar, House No. 3, Gulistan Colony, Lucknow 226001, India.
e-mail: shipra.kunwar@gmail.com

weeks of pregnancy for any indication but most commonly for intrauterine death (Table 1).

2.3. *Method*

A detailed history including age, parity, and period of gestation were noted and details of clinical examination including cervical findings were also recorded. Ultrasound (USG) was done to confirm gestational age, congenital malformation, and placental localization. Written informed consent of the patient was taken and a standard regime of moistened misoprostol (400 µg) 6 hourly intravaginally was used for a maximum of 6 dosages. Any complications including: fever, diarrhea, shivering, and excessive blood loss were recorded. Failure was noted when after 6 doses of misoprostol the patient did not go into labor. In case of failure a repeat dose of misoprostol 400 µg for 6 doses was used after giving a rest of 24 h. Before the next dose, the patient was examined and if the patient was having strong and frequent contractions, *i.e.*, more than 3 contractions in 10 min and if the cervix was more than 4 cm dilated, the next dose was deferred. All incomplete abortions were surgically evacuated. Patients with previous caesarean or any scarred uterus, multiple pregnancies, or severe anemia were excluded. After abortion the amount of blood loss was noted and products of abortion checked for completeness. All patients received prophylactic antibiotics, which included a combination of ciprofloxacin and tinidazole for 5 days. All patients were followed up for a period of 6 weeks but 5 patients did not come for follow up. No patient reported any complications in the follow up period.

3. Results

All patients were between the age group of 19 to 33 years with mean parity of 2.3. Principal indication for termination of pregnancy was intrauterine death of fetus (Table 1). Majority of the patients had a period of gestation between 16 to 18 weeks (Table 2). Mean induction abortion interval (IAI) was 11.8 ± 9.9 h at the end of 48 h (Table 3). Mean number of dosages required was 2.64 (Table 4). Success rates at the end of 24 h and 48 h were 88.3% and 96.6%, respectively (Table 5). Two patients did not abort even after 6 dosages. These patients were given a repeat dose of misoprostol after rest of 24 h. The main complications were incomplete abortion requiring surgical evacuation in 6 patients; fever in one patient, but none of the patients had rupture of the uterus. Three patients had excessive blood loss and only one required blood transfusion. No patient had sepsis.

Table 1. General characteristics of patients and indications

Characteristics of patients	
Total (*n*)	60
Median age (years)	24.5 (19-33)
Median gestation (weeks)	21.1
Indication (*n*)	
IUD	34
CMF	3
PTPROM	23

Abbreviations: IUD, intrauterine device; CMF, congenital malformations; PTPROM, preterm labor or preterm premature rupture of membranes.

4. Discussion

Second trimester abortions are painful and stressful procedures. Various agents have been used and compared with misoprostol in second trimester pregnancy terminations. Misoprostol has proved to be better than extraamniotic instillation of PGF2-α (*7*), extraamniotic instillation of ethacridine lactate (*8*), and prostaglandin E2 (*9*).

Misoprostol is now widely used for second trimester terminations. However, there is still a need to find out the best route and dose with minimum IAI along with minimal side effects and complications. A number of routes of misoprostol administration have been studied and it has been shown that misoprostol

Table 2. Gestational age distribution

Gestational age (weeks)	Number of patients
13 to 15	3
16 to 18	17
19 to 21	14
22 to 24	11
25 to 26	15

Table 3. Results of misoprostol administration

Characteristics	Results
IAI (h)	11.8
Partial failure requiring surgical evacuation (*n*)	6
Excessive blood loss (*n*)	3
Blood transfusion (*n*)	1
Failure (*n*)	2

Table 4. Number of doses of 400 µg of misoprostol required

Number of doses	Number of patients	Percent (%)
1 dose	19	31.6
2 doses	17	28.3
3 doses	12	20.0
4 doses	6	10.0
5 doses	4	6.7
6 doses	2	3.3

Table 5. Number of abortions during treatment period

IAI	Number of patients	Percent (%)
Up to 6 h	10	16.6
Up to 12 h	31	51.6
Up to 18 h	38	63.3
Up to 24 h	53	88.3
Up to 48 h	58	96.6

demonstrates a route-dependent pharmacokinetic profile and that the best absorption is through vaginal administration (*5*). Although the oral route is more acceptable (*10*), vaginal administration of misoprostol is more effective and reliable with minimal side effects (*11*). Direct vagina-to-uterus transport as described for progesterone may explain the better clinical efficacy of the vaginally administered drug (*12*). Sublingual misoprostol has a better pharmacokinetic profile, *i.e.*, peak concentrations achieved in shortest time and higher peak concentrations (*13*), but clinical efficacy is poorer as compared to vaginal misoprostol for second trimester abortions (*14*,*15*). As stated earlier, more than 30 regimes of misoprostol administration have been suggested adding to the confusion.

Further, various adjuncts to misprostol administration have been suggested including moistening of misoprostol tablets with saline (*16*) or acetic acid (*17*). A saline-moistened tablet is better than a dry one and acetic acid-moistened tablet is better (although not statistically different) than a saline-moistened one (*17*). Vaginal misoprostol along with a laminaria tent has been tried (*18*). Mifepristone given orally 36-48 h decreases the mean IAI (*19*), but increases the cost of treatment (*20*).

Mean IAI in our study was 11.8 ± 9.9 h, which was comparable to other studies using 400 μg (*14*,*21*) and was 5 h less than a study published by Dickinson *et al.* (*3*). Mean dose requirement was less than the study by Dickinson *et al.* (2.64 and 3, respectively), with a total dose requirement being 1,054 μg and 1,200 μg, respectively. This higher dose requirement and longer IAI in the study of Dickinson *et al.* could be due to an inclusion of previous caesarian sections in their study. On the other hand, complete abortion rate was similar to the previous study (*14*).

Side effects such as fever and diarrhea were minor in nature, which could be due to less frequent dosages. It has been shown in the previous study that serum levels of misoprostol could accumulate when vaginal misoprostol was repeated at an interval shorter than 6 h (*14*), therefore a 6 hourly dosage schedule seems to be more practical and associated with less chance of hypertonicity besides being more convenient with a better side effect profile. This has been supported by a previous comparative study showing that vaginal administration of 400 μg was more effective than a 200 μg regime and that side effects were lower than a 600 μg dose (*22*).

In conclusion, vaginal misoprostol for second trimester abortion seems to be a cheap, convenient, and effective choice.

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(Received July 15, 2010; Accepted October 31, 2010)

Case Report

Ruptured hepatic subcapsular hematoma following laparoscopic cholecystectomy: Report of a case

Kentaro Shibuya, Yutaka Midorikawa*, Hiroyuki Mushiake, Masato Watanabe, Tatsuo Yamakawa, Yasuyuki Sugiyama

Department of Surgery, Teikyo University School of Medicine University Hospital, Kawasaki, Japan.

Summary **Laparoscopic cholecystectomy is now a standard procedure for cholecystolithiasis because of its minimally invasive nature compared to the conventional method. However, severe complications that have never been seen for open surgery have also been reported. Here, we report the case of a 28-year-old woman who underwent laparoscopic cholecystectomy and then developed a ruptured subcapsular hematoma. On postoperative day 1, she developed shock, and postoperative bleeding was suspected. During re-operation, a ruptured subcapsular hematoma of the whole right lobe of the liver with active bleeding was found, and hemostasis was achieved. In this case, it was assumed that the rupture of the subcapsular hematoma was due to compression of the liver by the clamp for retrieving the spilled gallstones during the first operation and perioperative administration of non-steroidal anti-inflammatory drugs.**

Keywords: Laparoscopic cholecystectomy, complication, ruptured subcapsular hematoma

1. Introduction

Laparoscopic cholecystectomy (LC) for symptomatic cholecystolithiasis is now a routine procedure worldwide. Due to its minimally invasive nature, including a smaller incision and shortened duration of admission, patients suffering from cholecystolithiasis have benefited greatly (*1*). However, despite these advantages, several kinds of severe complications occur with LC, some of which have not been observed following open cholecystectomy (*2*). The most frequently reported complication during LC is common bile duct injury, of which the incidence is 0.125%-0.25% (*2*), followed by bleeding from trocar sites and the liver bed, vascular injury (*3*), bile leakage, pneumoperitoneum, abscess formation due to peritoneal spilled gallstones (*4*), and bowel injury. On the other hand, intestinal volvulus (*5*) or ischemia (*6*), pseudoaneurysm of the cystic or hepatic arteries (*7*), trocar site hernia (*8*), gas embolism (*9*), portal vein thrombosis (*10*), and migration of endoclips (*11*) are relatively rare but serious complications following LC.

In this paper, the case of a patient who developed a ruptured subcapsular hematoma of the liver after LC is presented, and particular attention is given to the incidence, differential diagnosis, and treatment of this complication.

2. Case report

A 28-year-old woman was admitted to our hospital complaining of right upper quadrant abdominal pain. She was diagnosed as having cholecystolithiasis, and LC was performed. During surgery, the gallbladder was injured, and the spilled gallstones were retrieved, during which some tiny subcapsular hematomas developed because the aspiration clamp compressed the liver (Figure 1A). Although the subcapsular hematomas gradually became larger during the operation, they were left untreated (Figures 1B and 1C). For treatment of the abdominal pain after the operation, the patient took flurbiprofen axetil, a non-steroidal anti-inflammatory drug (NSAID), 3 times (150 mg in total). On postoperative day 1, she developed hypotension, tachycardia, and severe anemia, and laboratory tests

**Address correspondence to:*
Dr. Yutaka Midorikawa, Department of Surgery, Teikyo University School of Medicine University Hospital, Mizonockuhi 3-8-3 Mizonokuchi, Takatsu-ku, Kawasaki 213-8507, Japan.
e-mail: mido-tky@umin.ac.jp

showed a low hemoglobin (4.4 g/dL) level (postoperative, 9.8 g/dL). Postoperative X-ray examination which was performed routinely showed displacement of the drainage tube that had been placed in the right subphrenic region (Figure 2). Ultrasonography demonstrated intraperitoneal fluid collection, suggesting hemorrhage after LC. The patient's hemodynamic condition worsened, and an emergency re-operation was performed. Laparoscopic exploration showed a ruptured subcapsular hematoma of the phrenic side of the entire right lobe of the liver with active bleeding (Figure 3A). Laparotomy was then performed (Figure 3B), the hematoma was removed, and hemostasis was achieved using an argon beam coagulator (Figure 3C). Further exploration within the abdomen showed no liver parenchymal or gallbladder bed injury, and no hemorrhage from the cystic artery (the amount of intraabdominal bleeding was 1,466 mL). The patient's postoperative course after re-operation was uneventful, and she was discharged without further complaints.

3. Discussion

Postoperative hemorrhage after LC is rare but one of the most life-threatening complications, and the sources of bleeding are diverse, *i.e.*, stump of the cystic artery, gallbladder bed, vessels of the greater omentum, and rupture of a subcapsular hematoma of the liver (*2*).

Seven cases of subcapsular hematoma of the liver after LC have been reported, two cases of which were accompanied with rupture, as in our case (*12*,*13*). Most patients with subcapsular hematoma of the liver complain of right upper quadrant pain or discomfort, and physical examination shows tachycardia and hypotension (*12-18*); they also develop severe anemia with shock in cases with rupture, as in our case (*12*,*13*). Subcapsular hematoma after LC usually occurs within a few days, and within 24 h in cases of rupture, and, therefore, careful monitoring is necessary for patients with such complaints for a few postoperative days, even after LC is performed as a day surgery procedure, if possible (*19*).

If subcapsular hematoma is not accompanied with rupture, interventional procedures such as CT- or US-guided aspiration of the hematoma is effective, or it may be treated conservatively (*14-18*), whereas laparoscopic or laparotomized exploration of the abdomen and hemostasis are necessary for ruptured subcapsular hematomas (*12*,*13*). In the present case, we confirmed the intraperitoneal fluid collection, which led us to diagnose severe postoperative hemorrhage and therefore, the patient was not considered to be a

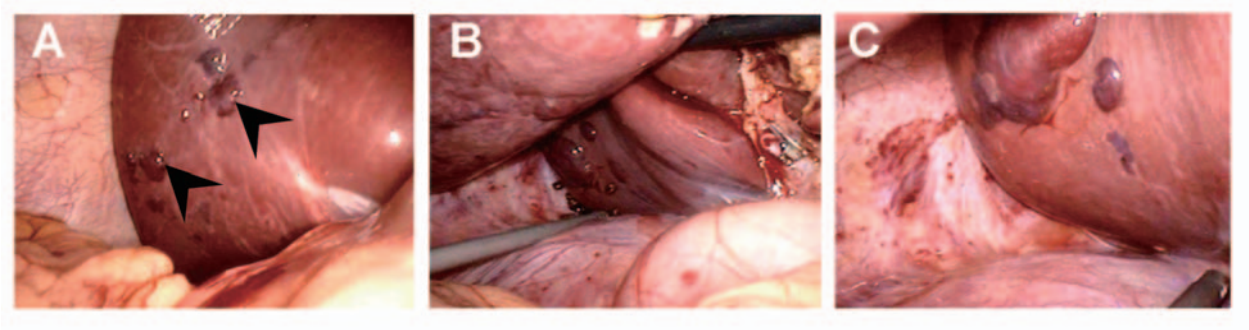

Figure 1. Laparoscopic and intraoperative view of the first surgery. (A) Small subcapsular hematoma found during laparoscopic cholecystectomy (arrow heads). **(B)** and **(C)** Subcapsular hematoma which grew gradually during operation.

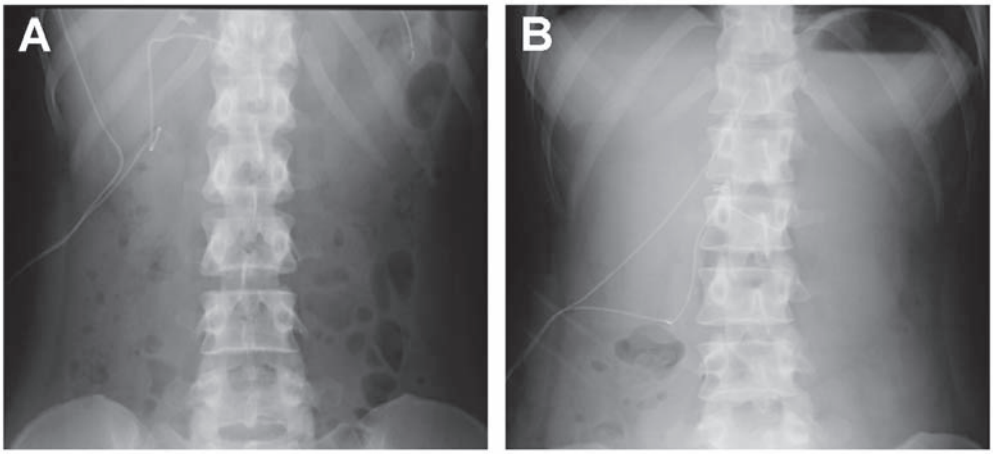

Figure 2. X-ray obtained after surgery. (A) A drainage tube was placed in the right subphrenic space (arrow head) postoperatively. **(B)** The tube is displaced to the left side by the huge hematoma on postoperative day 1.

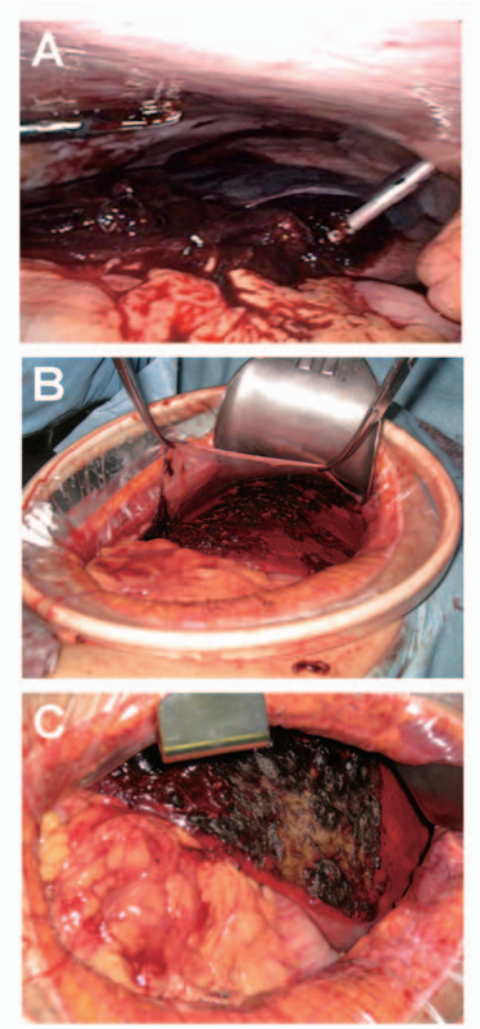

Figure 3. Laparoscopic and intraoperative view of the second surgery. (A) Laparoscopic view of the ruptured subcapsular hematoma of the right lobe of the liver. **(B)** Ruptured subcapsular hematoma of the right lobe of the liver after laparotomy for hemostasis. The ablated liver capsule is lifted. **(C)** The right lobe of the liver after hemostasis using an argon beam coagulator.

candidate for interventional therapy, but for surgery.

The causes of subcapsular hematoma of the liver after LC are not known. Some researchers have assumed that ketorolac, which is a NSAID that is used to relieve severe pain after surgery, may cause subcapsular hematoma of the liver, because ketorolac induces substantial gastrointestinal bleeding and their patients had no evidence of parenchymal injury during LC (*13*,*16*,*18*). In the present case, some minor subcapsular hematomas of the liver were recognized because the aspiration clamp had compressed the liver surface in order to retrieve the spilled gallstones during the initial procedure (*4*). During the procedure, you can see that the small subcapsular hematomas grow gradually by compressing the liver with the aspiration clamp. The present patient had also received frequent NSAID injections postoperatively, which may have exacerbated the small subcapsular hematomas and led to rupture.

In summary, the case of a patient with a ruptured subcapsular hematoma of the liver after LC was presented. In order to avoid such a rare but severe postoperative complication, surgeons should keep in mind that the liver is not to be handled roughly during the procedure, and that NSAIDs may result in lethal hemorrhages and therefore use them with caution perioperatively.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) 19591601 (Y.M.).

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(Received August 26, 2010; Revised September 16, 2010; Accepted October 12, 2010)

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Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: 03-5840-8764, Fax: 03-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

Acknowledgements

Acknowledgements to reviewers

BioScience Trends is a peer-reviewed international journal published bimonthly by *International Research and Cooperation Association for Bio & Socio-Sciences Advancement* (IRCA-BSSA). During the editing time, many scientists kindly provided lots of support to BioScience Trends. To ensure the quality of articles, they have contributed great expertise and time to the peer-review. Here, we greatly appreciate the efforts of the following scientists as reviewers:

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(*December 25, 2010*)

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Original Articles should be reports on new, significant, innovative, and original findings. An Article should contain the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure legends, and Tables. There are no specific length restrictions for the overall manuscript or individual sections. However, we expect authors to present and discuss their findings concisely.

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Abstract. A one-paragraph abstract consisting of no more than 250 words (200 words in Policy Forum essays) must be included. It should state the purpose of the study, basic procedures used, main findings, and conclusions.

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Introduction. The introduction should be a concise statement of the basis for the study and its scientific context.

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